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Enzymatic Hydrolysis of an Organic Sulfur Compound

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Abstract

Sulfatases which cleave sulfate esters in biological systems are key enzymes that deserve special attention due to their significant roles in organic sulfur (OS) mineralization and inorganic sulfur (SO_4^{2-}) release. In this study, *in-vitro* experiments were conducted to evaluate S bonded substrate hydrolysis by a commercially available arylsulfatase (EC 3.1.6.1) from *Aerobacter aerogenes*. The enzyme-substrate interactions were assessed to determine: 1) rate of hydrolysis, 2) catalytic efficiency, 3) thermal stability, and 4) optimal pH of this enzyme. Arylsulfatase exhibited substrate hydrolysis with a high affinity for *p*-nitrophenyl sulfate (potassium 4-nitrophenyl sulfate (*p*NPS)). The optimum activity for the enzyme was observed to occur at a pH of 7.1. The optimal temperature was 37˚C but ranged from 35˚C - 45˚C. The apparent K_m and K_{cat} of the enzyme for *p*NPS hydrolysis at the optimal pH, and temperature were determined to be 1.03 mM and 75.73 μM/min, respectively. This work defines the catalytic and kinetic properties of arylsulfatase (EC 3.1.6.1) and confirms the optimal conditions for sulfatase activity testing. The resulting information is useful in elucidating the contributions that individual enzymes have for specific reactions rather than relying on traditional total enzyme activity measurements.

Keywords

Enzymes, Sulfatases, Organic Sulfur Mineralization

1. Introduction

Sulfatases are a heterogeneous family of enzymes that constitute a biologically and industrially important group of proteins and play critical roles in the hydrolysis of sulfate groups from sulfated biomolecules [1]. The International Union

of Biochemistry and Molecular Biology (IUBMB) classified the family into 17 classes from EC 3.1.6.1 - EC 3.1.6.18, as deduced from the nucleotide sequence similarities [2], substrate specificity and sensitivity to inhibitors [3]. Although sinigrin sulfohydrolase; myrosulfatase (EC 3.1.6.5) was removed from the group in 1964, the major classes recognized include arylsulfatases (EC 3.1.6.1), steroid sulfatases (EC 3.1.6.2), glucosulfatases (EC 3.1.6.3), chondrosulfatases (EC 3.1.6.4), and alkylsulfatases (EC 3.1.6.19) [4] [5].

Sulfur is documented as being a critical component for plant and animal growth and development, and in several reactions that occur in living cells [6]. However, reductions in S emissions from industrial sources, decreased use of S containing fungicides and pesticides, increased use of low S containing fertilizers, and high yielding crops over the past twenty years have resulted in low S availability [7] [8]. The deficiencies in plant available S in soils have long been recognized as a cause of delayed maturity, stunting of plants, and interveinal chlorosis in crop productions worldwide [9] [10].

Inorganic S is generally much less abundant than organically bound S in most agricultural soils [11]. As a result, organic S compounds are typically unavailable to plants. Ester sulfates constitute the most important organic S reserve in aerobic soils accounting for up to about 70% of the total S in such soils [12]. Organic S compounds must, therefore, be converted by biochemical hydrolysis of sulfate-esters or by microbiological mineralization of C-bonded S to release inorganic SO_4^{2-} before plant uptake [13]. The hydrolysis of the aromatic ester-sulfate molecule is catalyzed by periplasmically located sulfatases that cleave sulfate from the organic S molecules moiety.

Enzymes of the sulfatase group are major components in the release of SO_4^{2-} from organic sulfur compounds. Sulfatases are found to be intracellular or bound to cell components and abiotic or as extracellular secretions from intact cells or released from dead or lysed cells that originate from the cell membranes [14]. The enzyme focus in this study, for example, arylsulfatase is a class of sulfatase in which both extracellular and intracellular forms have been detected in a wide range of soils, and its activity has been used as a potential indicator for biochemical mineralization of organic ester-sulfates in soils [15] [16]. It has been shown that arylsulfatase in soils is of both plant and microbial origins, and their alterations in soil rhizospheres can cause shifts in the ecology and functionality of the soil microbial communities. The changes in soil microbial communities, therefore, influence the activities of the enzyme and may significantly impact the biochemical mineralization of plant S [15] [17]. Significant positive correlations between arylsulfatase activity and total SOM have been reported [18]. Deng and Tabatabai [18] stated that the actions of arylsulfatase were highly correlated with soil organic C content and suggested that organic matter plays a vital role in protecting soil enzymes. In many instances, the mineralization of S from soil organic matter increases the supply of S for plant nutrition [6].

Arylsulfatase (aryl-sulfate sulfohydrolase, EC 3.1.6.1) is a class of glycosulfo-

hydrolase involved in the desulfation of sulfated polysaccharides. It catalyzes the hydrolysis of aryl sulfate-ester bonds, producing aryl compounds and inorganic sulfate (SO_4^{2-}). Arylsulfatases are found in a wide range of organisms including mammals, bacteria, fungi, and higher plants, and their primary structures are similar to each other although they originate from different species [19] [20]. There have been several reports of arylsulfatases isolated from such bacteria as *Pseudomonas* [21] [22], *Enterobacter* [23], *Salmonella typhimurium* [24], *Klebsiella* [25], and *Serratia* [26].

Arylsulfatase enzymes (ARS) are one of the sulfate starvation-induced (SSI) proteins produced by microorganisms during S starvation. Recent studies suggest that in bacteria, these proteins are potentially critical enzymes in the cellular responses against S limitation [14] [27] [28]. Aryl-sulfohydrolase enzymes are implicated in the desulfation of aromatic sulfate esters. In *Pseudomonas aeruginosa*, the repressive effects in vivo were traced to two independent effectors, sulfites and either sulfides or cysteines whereas, in *Klebsiella pneumoniae*, sulfate and cysteine repress arylsulfatase synthesis independently of each other [26]. Arylsulfatases have been classified as type I and II according to their substrate specificity and sensitivity to inhibitors. Type I enzymes are specific for *p*-nitrophenyl sulfate (*p*NPS) and *p*-acetylphenyl phosphate substrates and are inhibited by cyanide [29] [30]. Type II enzymes are more catalytic on *p*-nitrocatechol sulfate (*p*NCS) (2-hydroxy-5-nitrophenyl sulfate) and are inhibited by phosphate and sulfate [14]. However, to survive under sulfate-limiting conditions, microorganisms will have to synthesize SSI proteins to fulfill their S requirements [14].

Specific for the catalyzed reaction, the proficiency of an enzyme as a catalyst and its similar affinity for an altered substrate in the transition state can be assessed by comparing the K_{cat}/K_m, with the rate constant of the corresponding reaction under similar conditions and in the absence of a catalyst [31]. Some enzymes catalyze slow reactions while others are involved in fast reactions. Those enzymes that catalyze slow reactions are of interest because they offer sensitive targets for inhibition by transition-state analogs [32]. The stereochemical and stereoisomeric structures of the substrate or the types of elements attached to it will also influence hydrolysis rates [33]. Enzymes have specific atomic configurations on their active sites and, any modifications to the substrates that are specific for a particular enzyme will likely denature the enzyme rendering the protein ineffective. Such parameters as time, pH, temperature, and enzyme concentration influence enzymatic activity co-operatively.

Our understanding of the precise roles and functions of sulfatase enzymes in mineralizing organic S especially in soils is constrained to some extent by limitations of the methods used since there is no standard method to determine soil enzyme reactions or hydrolysis. Para-nitrophenyl sulfate (*p*NPS) is a widely used substrate for assays of soil arylsulfatase activities; however, it may not accurately reflect the relative hydrolysis of various soil sulfatases. Thus, understanding the

behavior of enzymes in pure systems with different substrate concentrations and identifying the contributions the individual proteins have for specific reactions rather than relying on total enzyme activities, as has been the case with traditional assay methods, would improve our understanding of enzyme hydrolysis in terrestrial and aquatic environments.

With this study, we focused on a commercially available sulfatase from *Aerobacter aerogenes*. The enzyme was selected because most early work on the regulation of arylsulfatase synthesis was performed with *Aerobacter aerogenes* [34]. This organism synthesizes arylsulfatase when grown in medium containing methionine, taurine or choline sulfate as the sulfur source (non-repressed condition). The synthesis is; however, repressed using inorganic sulfate, or any of the sulfur compounds which are thought to be direct intermediates in the conversion of sulfate to cysteine as the sulfur source [34]. The goals are to determine the catalytic and kinetic properties of arylsulfatase (EC 3.1.6.1), using organic S compounds as substrates. This paper reports the features of the enzyme from *Aerobacter aerogenes.* Information obtained will also aid in the scientific predictions in rate-limiting steps during the decomposition or degradation of organic matter and transformation of soil elements [35].

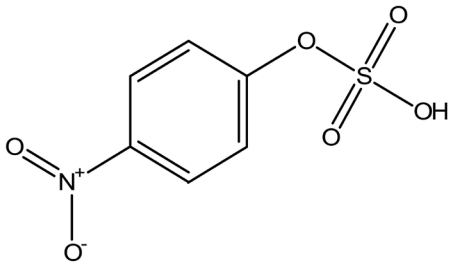
2. Materials and Methods

2.1. Organic Sulfate Compound and Enzyme

P-nitrophenyl sulfate (potassium 4-nitrophenyl sulfate) (*p*NPS) substrate (**Figure 1**), and arylsulfatase from *Aerobacter aerogenes* were used without further purification in this study. Both, the organic sulfate compound and the enzyme, were purchased from Sigma-Aldrich, St. Louis, Missouri, USA.

2.2. Assay Buffer and Conditions

The optimal pH for the arylsulfatase as reported by the manufacturer was 5.0, and the optimal temperature is 37˚C. One unit (U) of the enzyme was reported to liberate 1.0 μmol *p*-nitrocatechol sulfate per hour at the proper pH and temperature. Two concentrations of the (0.05 $U \cdot mL^{-1}$ and 0.033 $U \cdot mL^{-1}$) of the arylsulfatase enzyme was used to hydrolyze the *p*-nitrophenyl sulfate substrate (*p*NPS). The effects of pH, temperature, and time on the enzyme-substrate



p-nitrophenyl sulfate

Figure 1. Substrate structure.

reaction was determined by incubation using a wide range of temperatures ranging from 10˚C to 80˚C; pH ranging from 2 to 9, and time ranging from 1 to 10 hours, using *p*NPS substrate concentration of 5 times the K_m [36].

2.3. Kinetic Determination

The substrate concentrations used to establish the kinetic parameters in the assays ranged from 0 to 12 mM. The enzyme activity was estimated by measuring the rate of inorganic SO_4^{2-} (product) released and determined using methods described by Tabatabai and Bremner [4] and Dodgson and Spencer [37]. The sulfate compound (substrate) was dissolved in a 0.5 M acetate buffer solution, pH 5.8, with final enzyme concentrations of 0.05 $U{\cdot}mL^{-1}$ and 0.033 $U{\cdot}mL^{-1}$. All reaction mixtures were carried out at a total volume of 3 mL. At the end of incubation, the reaction was stopped by adding 0.5 *N* NaOH, and 0.5 M $CaCl_2$ to the product. The intensity of yellow color produced (due to the liberation of SO_4^{2-} from *p*NPS substrate) was measured at 400 nm with a spectrophotometer (Thermo Electron Corp. Model: Genesys 10 UV). Standard curves derived from spectrophotometric readings of known concentrations of SO_4^{2-} were used to calculate the sulfate concentrations in test solutions. The controls were established by incubating the substrate without enzyme to correct for the inorganic S released due to chemical hydrolysis. The amount of inorganic SO_4^{2-} issued was determined colorimetrically. The resulting data was plotted against substrate concentration. The initial rates of inorganic S released versus substrate concentrations were fitted to the Michaelis-Menten equation:

$$V = \frac{V_{\max}[\mathrm{S}]}{K_m + [\mathrm{S}]} \tag{1.2}$$

To study the effects of incubation time, temperature and pH on enzyme activity, these conditions were varied while using an excess of substrate (1.0 mM). The Michaelis-Menten enzyme model was used to determine the kinetic parameters. The constant (K_m), and maximum rate ($V_{\max}$) were calculated by way of non-linear regression analysis using GraphPad Prism version 4.00 statistical software (GraphPad Prism Software, Inc. Ca, USA).

The measurements to determine the temperature coefficients (Q_{10}) were at intervals of 10˚C (between 20˚C and 80˚C); while the activation energy (E_a), was assessed using the Arrhenius equation. Except otherwise stated, experiments were conducted in triplicate.

3. Results and Discussion

3.1. Activation Energies and Kinetic Parameters

The data obtained for arylsulfatase activity from the bacterial source (*Aerobacter aerogenes*), was fitted to the Michaelis-Menten enzyme kinetics to determine the K_m and $V_{\max}$ values for the substrate used. The $V_{\max}$ is dependent on enzyme concentration and is defined as the velocity obtained when the enzyme is satu-

rated. The K_m values are considered to be a measure of enzyme affinity for the substrate. The lower K_m value, the higher the enzyme affinity for the substrate [38]. In this study, the K_m value for the purified arylsulfatase from *Aerobacter aerogenes* resulted in a concentration of 1.03 mM. Comparably, the K_m of arylsulfatase purified from *Aerobacter aerogenes* in a separate study was valued at 0.187 mM [39]. *Pseudomonas aeruginosa* activity was measured as sulfate released from 4-nitrocatechol sulfate and was assessed at 0.10^5 mM [40]. Okamura *et al.*, [34] reported a much higher K_m value of arylsulfatase purified from the bacteria, *Klebsiella aerogenes* (9.0 mM). Although the enzymes were able to hydrolyze various aromatic sulfate esters, lower values were obtained in our study, indicating high enzyme affinity for *p*NPS. The variations in K_m appear to reflect the differences in substrate concentrations and the types and sources of the enzymes.

The maximum velocity (V_{max}) of the enzyme, reveals the number of substrate molecules converted into products by an enzyme molecule in a unit time when the catalyst is fully saturated with substrate [41]. Our results indicate the V_{max}, of the enzyme from *Aerobacter aerogenes,* was 75.7 uM/min.

The enzyme turnover number K_{cat} represents the number of substrate molecules each enzyme site converts to product per unit time. The enzymatic activity in this study was valued at 1.5×10^3 s^{-1} which; is equal to the kinetic constant. High K_{cat} values indicate greater enzyme specificity for catalyzing the substrate reaction. Tazisong *et al.* [42] and Berg *et al.* [41] reported that the K_{cat} of most enzymes associated with their physiological substrates is typically found in the range of 1 to 10^4 s^{-1}. The specificity constant, K_{cat}/K_m, incorporates the rate constants for all the reaction steps used to measure the catalytic efficiency of the enzyme-substrate reaction. The K_{cat}/K_m in this assessment was valued at 8.7×10^5 $M^{-1} \cdot s^{-1}$. Typically high K_{cat}/K_m values indicate higher enzyme affinity for the substrate.

3.2. Effects of Substrate Concentrations, Time, Temperature and pH on Activity

Arylsulfatase seemed to show absolute specificity to the substrate (*p*NPS) used. The initial rate of substrate hydrolysis was measured at various substrate concentrations (**Figure 2**). Reaction velocities increased as substrate concentration was increased. At 4 mM the reaction is likely to follow zero-order kinetics. The effect of incubation time on arylsulfatase activity is shown in **Figure 3**. The activity with time was linear for up to two hours, which was used to assess kinetic parameters. The curve then losses curve linearity which indicates enzyme saturation or product inhibition. The *Aerobacter aerogenes* sulfatase was stable between 35˚C to 45˚C. The optimum temperature for the arylsulfatase was at 37˚C is shown in **Figure 4**. The enzyme was active in temperatures approaching 39˚C but was inactivated at temperatures > 40˚C. The kinetic energy of molecules will increase with increasing temperatures, leading to more frequent collisions and

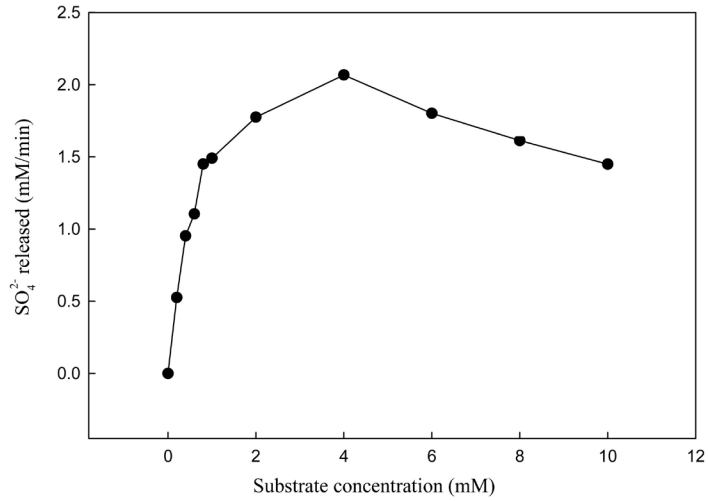


Figure 2. Effect of substrate concentration on arylsulfatase activity.

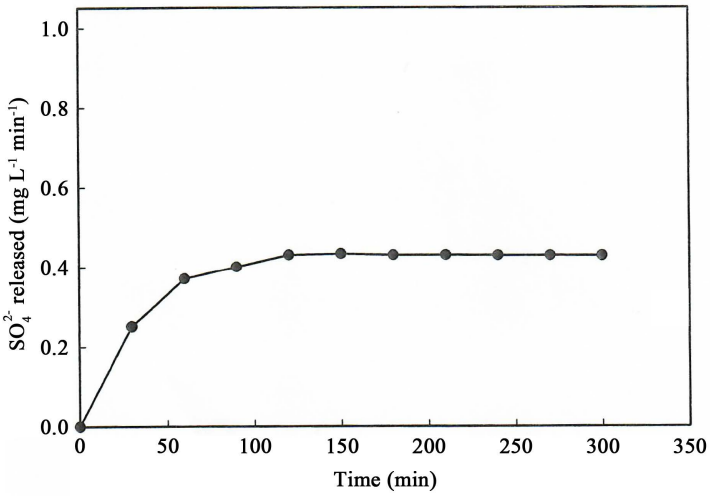


Figure 3. Effect of incubation time on arylsulfatase activity.

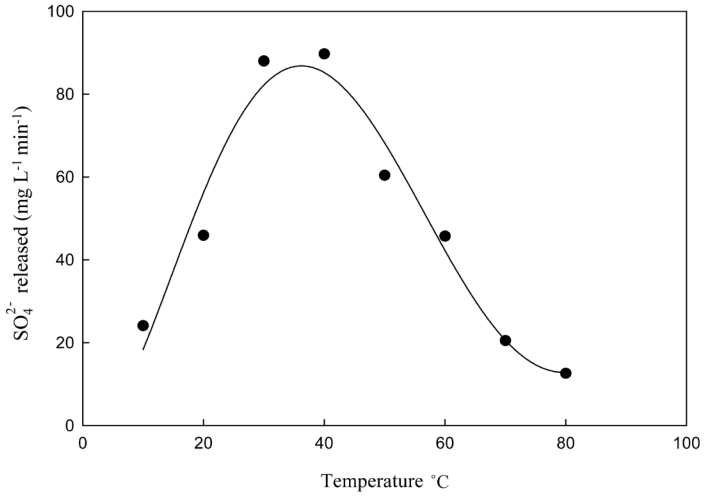


Figure 4. Effect of temperature on arylsulfatase activity.

increase in reaction rates. Increasing the kinetic energy after attaining the optimal temperature causes the hydrogen bonds and hydrophobic interactions to split. The active sites begin to lose shape, and the enzyme denatures. The observation of various optimal temperatures with multiple substrates and the same enzyme may be due to conformational changes induced on the catalyst after the substrate binds. The conformational change induced on the protein may either delay or hasten the collapse of hydrogen and hydrophobic interactions [43].

Variations in pH affect the ionic forms of the enzyme active sites, therefore changing the activities and reactions rates. Enzymes are proteins that contain amino acids, have basic, neutral, or acid side groups which are positively or negatively charged contingent on the pH. As a result, the pH may cause a conformational change in the structure, the maximum reaction rate (K_m), the enzyme stability and the substrate affinity for the enzyme, if the substrate contains ionic groups [44]. The pH optimum of the purified enzyme was determined over a pH range of 3.0 to 9.0, using a concentration 5 times the K_m [45]. The enzyme was active in the pH range 6.0 to 7.5 with the maximal hydrolysis of *p*NPS at the pH of 7.1 (**Figure 5**). At higher pH, the enzyme activity gradually decreased. The pH of arylsulfatase with *p*NPS was two units more elevated than that reported by the supplier.

The activation energy (E_a) was calculated based on the Arrhenius equation (Equation (1.3)).

$$\ln k = \left(-E_a/R\right)\left(1/T\right) + \ln A \tag{1.3}$$

E_a was calculated from the slope of the linear relationship by plotting ln$k$ versus $1/T$ (**Figure 6**). The E_a values are indicative of the energy barrier that must be overcome to drive a reaction forward. The E_a value is nearly equal to the difference in energy between the reactants and the transition state [46]. The calculated *sulfatase* E_a expressed for this study ranged from 18.0 to 36.1 kJ·mole^{-1}.

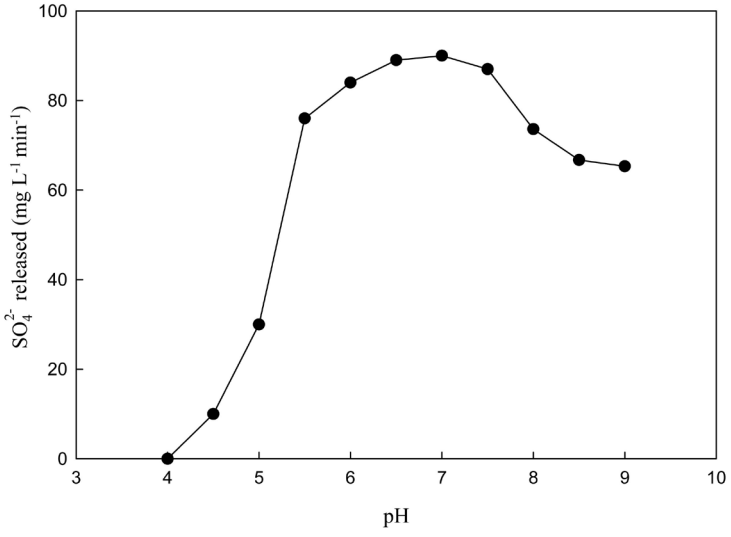


Figure 5. Effect of pH on arylsulfatase activity.

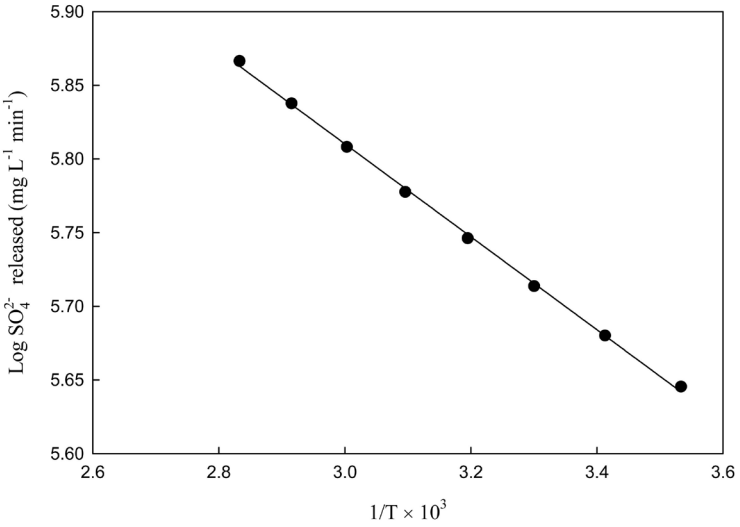


Figure 6. Arrhenius equation plot of arylsulfatase activity.

The temperature coefficient (Q_{10}), is used to measure the rate of change that occurs in biological or chemical systems as a result of 10˚C temperature increases. The Q_{10} in this study was obtained by calculating the effect of 10˚C temperature changes on the activity of the enzyme during incubation. The enzyme activity followed the Equation (1.4) below:

Q_{10} = Activity at given temperature/Activity at given temperature – 10˚C (1.4)

The average Q_{10} for *Aerobacter aerogenes* enzyme between 20˚C and 80˚C ranged from 1.90 - 0.61.

4. Conclusion

Based on this research and other studies, the rate of hydrolysis, catalytic efficiencies, thermal stabilities, and optimal pH values of enzymes may depend on the enzyme sources and the stereochemical or stereoisomeric structures of the substrates. The optimum pH for the sulfatase was observed to be 7.1. The kinetic differences observed, evidenced by other available reports on this topic suggest that enzymes are distinct and have distinct functions. Several microorganisms exhibit multiple arylsulfatase activities, which are generally subject to repression by a variety of S compounds. Little is known of the regulation of the individual enzymes; however, there is evidence that suggests distinct regulatory control of synthesis.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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