Promiscuous Arylsulfatase Activity in Chlamydomonas reinhardtii

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Abstract: Sulfate is an essential macroelement for all living organisms. However, sulfate can be limited in agricultural settings. Microorganisms change their gene expression to acclimate to sulfate deficient conditions. Green microalga *Chlamydomonas reinhardtii* expresses and secretes extracellular arylsulfatase (ARS) under sulfate deficient conditions. Our results show that *C. reinhardtii* ARS can hydrolyze both sulfate monoester (5-bromo-4-chloro-3 indolyl sulfate; X-SO_4^{2-}) and phosphate monoester (5-bromo-4-chloro-3 indolyl phosphate; X-PO_4^{3-}) providing evidence that ARS enzyme has promiscuous activity. *C. reinhardtii* is found in soil and fresh water habitats in nature. This promiscuous activity can be beneficial in making both sulfate and phosphate bioavailable for uptake by soil organisms and plant roots.

Keywords: *Chlamydomonas reinhardtii*, arylsulfatase, promiscuous enzyme, sulfate

Chlamydomonas reinhardtii'de Seçici Olmayan Arilsülfataz Aktivitesi

Öz: *Sülfat canlılar için zorunlu bir makroelementtir. Buna rağmen, sülfat tarımsal alanlarda eksik olabilir. Mikroorganizmalar gen ekspresyonlarını değiştirerek sülfat eksikliğine aklimasyon gösterirler. Yeşil mikroalg Chlamydomonas reinhardtii sülfat eksikliği altında arilsülfataz (ARS) sentezler ve hücre dışına salgılar. Bu çalışmadaki sonuçlarımız, C. reinhardtii ARS'ın hem sülfat monoesterlerini (5-bromo-4-chloro-3 indolyl sulfate; X-SO_4^{2-}) hem de fosfat monoesterlerini (5-bromo-4-chloro-3 indolyl phosphate; X-PO_4^{3-}) hidrolize ettiğini göstermiştir. Bu da ARS'ın seçici olmayan aktiviteye sahip olduğuna kanıt sunmaktadır. C. reinhardtii toprak ve taze su ortamlarında yaşamaktadır. Sahip olduğu seçici olmayan ARS aktivitesi hem sülfatın hem de fosfatın bitki tarafından kullanılabilir forma getirilmesine katkı sağlayabilir.*

Anahtar Kelimeler: *Chlamydomonas reinhardtii, arilsülfataz, seçici olmayan enzim, sülfat*

INTRODUCTION

Sulfatases (EC 3.1.6.1) are a class of enzymes involved in the hydrolysis of sulfate ester bonds in different molecules. Arylsulfatases hydrolyze O-S bond of C-O-S ester linkage, whereas alkylsulfatases hydrolize C-O bond of the C-O-S ester linkage (Cloves, 1977). Arylsulfatases contain the conserved consensus motif, C/S-X-P-X-S-X_4-TG (Kertesz, 1999). The lead cysteine/serine residue of this motif is posttranslationally modified (oxidation of Cys or Ser) into a catalytically active C-formylglycine (FGly) by formylglycine generating enzyme in the endoplasmic reticulum (Schmidt et al., 1995; Dierks et al., 1999 Boltes et al., 2001). Defects in posttranslational modification of this residue to FGly in the active site results in a rare autosomal recessive disease called multiple sulfatase deficiency in humans (MSD) (Schmidt et al., 1995). Eukaryotic enzymes have a cysteine whereas prokaryotic enzymes have either cysteine or serine as their first residue in this motif. Depending on the catalytic residue, serine-type sulfatases are located in the periplasmic space and cysteine-type sulfatases are located in the cytosol (Cloves et al., 1977; Murooka et al., 1990; Marquordt et al., 2003; Toesch et al., 2014).

Catalytic promiscuity is the ability of an enzyme to catalyze different chemical reactions which depends on enzyme's ability to form or break distinct chemical bonds (Marino et al., 2013). In the literature, it is proposed that this ability may have been involved in the development of new enzymatic functions. *Pseudomonas aeruginosa* arylsulfatase have been shown to hydrolase both sulfate monoesters and phosphate monoesters (Olguin, 2008). In *P. aeruginosa* arylsulfatase, only some of the steps between sulfate hydrolysis and phosphate hydrolysis is identical. For hydrolysis of p-nitrophenyl-sulfate (PNPS) His115 acts as a base to accept proton by the O atom of the FGly51, whereas in hydrolysis of p-nitrophenyl-phosphate (PNPP) promiscuous reaction, Asp317 protonated residue works as a general acid to deliver a proton by a water molecule to the oxygen atom of the C-O bond (Marino et al., 2013). *Rhizobium leguminasorium* phosphonate monoester hydrolase/phosphodiesterase was shown to be the first non-sulfatase that uses formylglycine in catalysis (Jonas et al., 2008).

C. reinhardtii is found in soil and fresh water habitats in nature. In the genome of this alga there are nineteen genes annotated as arylsulfatase (ARS) (Salarvan, 2021). Among these genes, *ARS1* and *ARS2* were studied extensively and they encode for periplasmic arylsulfatases (ARS) which were shown to be highly upregulated under sulfate deficiency (de Hostos et al., 1988; de Hostos et al., 1989; Aksoy et al., 2013). Arylsulfatase is translated under sulfate deficiency and secreted to extracellular space where it hydrolyzes sulfate esters to make sulfate bioavailable for cellular uptake (Kagiwada et al., 2004). This change in gene expression

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allows *C. reinhardtii* to acclimate to nutrient deficient conditions. Under phosphate deficiency, *C. reinhardtii* upregulates alkaline phosphatase PHOX (Moseley et al., 2009; Aksoy et al., 2014). Activities of ARS and PHOX enzymes can be shown using a simple colorimetric assay (Davies et al., 1994; Shimogawara et al., 1999).

In this study, *C. reinhardtii* cells which kept under sulfate or phosphate deficiency were tested for their ability to hydrolyze sulfate monoester (5-bromo-4-chloro-3 indolyl sulfate; X-SO_4^{2-}) or phosphate monoester (5-bromo-4-chloro-3 indolyl phosphate; X-PO_4^{3-}) to determine if this alga has promiscuous sulfatase activity.

MATERIALS AND METHODS

Cell Culture and Nutrient Deprivation

C. reinhardtii wild type strain (CC124) was obtained from The Chlamydomonas Resource Center (https://www.chlamycollection.org/). Cells were grown mixotrophically on solid Tris Acetate Phosphate (TAP) medium (Gorman and Levine, 1965) under white fluorescent light bulbs. Light intensity was approximately 40 μmol photons/m^2/sec. To induce phosphate or sulfate deficiency, cells were kept on solid TA (TAP medium without phosphate) or TAP-S (TAP medium without sulfate) media for one week, respectively. TA and TAP-S media were prepared according to the recipes from the Chlamydomonas Resource Center.

Sulfatase and Phosphatase Assays

Cells were kept on solid TAP-S or TA media containing Petri plates for one week to induce sulfate or phosphate starvation, respectively. On each plate two colonies were grown, one for (upper colony) 5-bromo-4-chloro-3 indolyl sulfate (X-SO_4^{2-}) and the other (bottom colony) for 5-bromo-4-chloro-3 indolyl phosphate (X-PO_4) application. As a control, cells were grown on TAP media. To determine sulfatase activity, 10 mM solution of X-SO_4^{2-} in 0.1 M Tris-Cl, pH 7.5 was applied onto the colonies (Davies et al., 1994). To determine phosphatase activity, 10 mM solution of X-PO_4^{3-} in water was applied onto the colonies (Shimogawara et al., 1999). The appearance of blue color indicates presence of active enzyme; no coloration indicates absence of enzyme activity. After the applications, plates were observed visually and photographed at different time points.

Homology Modeling

Since structural similarity might suggest functional similarity, we generated homology model of *C. reinhardtii* ARS1 and ARS2 polypeptide sequences. Phyre2 web portal (Kelley et al., 2015) used *Ruegeria pomeroyi* sulfatase (PDB id, 4upi; https://www.rcsb.org) as the template for modeling of both ARS1 and ARS2. Phyre2 program generated both models with 100% confidence. The models generated were visualized in three dimention using Jmol, an open-source Java viewer (http://www.jmol.org/). To see if there is similarity between the models and a known sulfatase, human ARSA crystal structure (PDB id, 1n2l; Chruszcz et al., 2003) was also visualized with Jmol.

Conserved Motif Analysis

Polypeptide sequences were aligned with Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and conserved residues were highlighted manually. NCBI and Protein Databank accession numbers (PDB id) are as follows: *Chlamydomonas reinhardtii* ARS1 (XP_042915859.1), *Chlamydomonas reinhardtii* ARS2 (XP_001691972.1), *Homo sapiens* arylsulfatase A (NP_000478.3, PDB id 1n2l), *Pseudomonas aeruginosa* sulfatase (WP_003106692.1; PDB id 1hdh), *Rhizobium leguminosarum* alkaline phosphatase (WP_011649751.1, PDB id 2vqr), *Ruegeria pomeroyi* sulfatase (WP_011241904.1, PDB id 4upi).

RESULTS

Arylsulfatase can hydrolyze both sulfate and phosphate esters

C. reinhardtii cells were kept on solid media that contained all the nutrients (Plate 1, TAP), no phosphate (Plate 2, TA) or no sulfate (Plate 3, TAP-S) for one week (Figure 1A). Figure 1A shows the plates prior to enzyme activity tests. Cells that were kept on TAP and TA media were dark green after one week, however they turned light green on TAP-S (Figure 1A). These plates were then used for colorimetric enzyme activity tests; on each plate, upper colony was tested for sulfatase activity (X-SO_4^{2-} application) and lower colony was tested for phosphatase activity (X-PO_4^{3-} application). As expected, on Plate 1 (having full nutrients) there was no sulfatase or phosphatase activity (Figure 1B, 1C). On plate 2, upper colony shows no enzyme activity (implicating there was no sulfatase activity), whereas lower colony has enzyme activity (implicating there was phosphatase activity). Interestingly, on Plate 3 both colonies showed enzyme activity. Upper colony has sulfatase activity; this is expected because cells were starved for sulfate on Plate 3 and expected to secrete sulfatase. However, lower colony also shows enzyme activity; this implicates sulfatase can also hydrolyze X-PO_4^{3-} (Figure 1B and 1C, Plate 3). This result shows that, arylsulfatases which are secreted under sulfate deficient conditions can hydrolyze both X-SO_4^{2-} and X-PO_4^{3-}, proving arylsulfatase has promiscuous activity. However, color in promiscuous reaction (hydrolysis of X-PO_4^{3-} by arylsulfatase) is not as strong as the reaction seen in X-SO_4^{2-} hydrolysis (compare upper and lower colonies on Plate 3, Figure 1B and 1C). Notably, phosphatase doesn't hydrolyze X-SO_4^{2-} (Figure 1B and 1C, Plate 2 lower colony). This provides evidence that phosphatase doesn't have promiscuous activity; it only hydrolyzes X-PO_4^{3-} (it doesn't hydrolyze X-SO_4^{2-}).

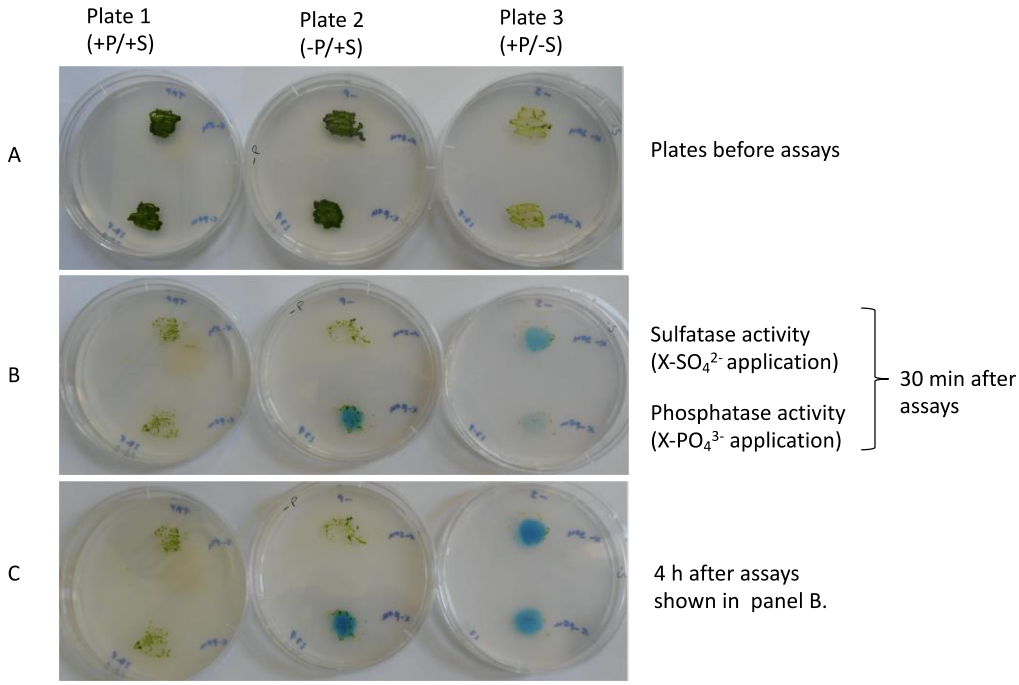

Figure 1. Colorimetric phosphatase and sulfatase assays. A. Cells were kept for 1 week on the plates indicated and pictures were taken before the assays. B. Pictures of plates 30 min after incubation with substrates. C. Same plates seen in B after 4 h incubation with substrates

***C. reinhardtti* ARS1 and ARS2 both have the conserved motifs found in known sulfatases**

The polypeptide sequences of CrARS1 and CrARS2 were aligned with a known sulfatase from human, ARSA and conserved residues are highlighted (Figure 2). As shown in Figure 2, both CrARS1 and CrARS2 have the conserved motifs and residues. Notably, they both have the conserved cysteine residue which is oxidized to catalytically active formyl glycine (FGly) (Cyc73 in both CrARS1 and CrARS2, shown in red color) (Figure 2 and Figure 3).

Homology Modeling of *C. reinhardtii ARS1 and ARS2*

Homology modelling results show that the crystal structure of human ARSA (PDB id 1n2l) (Figure 4A) resembles the homology models of *C. reinhardtii* ARS1 (Figure 4B) and ARS2 (Figure 4C). The FGly69 is at the center of the human ARSA (Figure 4A). Interestingly, cystein 73 of CrARS1 and CrARS2 is also at the center (Figure 4B and 4C). These results suggest that ARS1 and ARS2 has similar structural fold with human ARSA.

DISCUSSION

Sulfur and phosphorous are essential for organisms and deficiency of both effects plant development and productivity (Liang et al. 2014; Bouranis et al. 2020). However, only a small portion of chemical fertilizers that are applied to soil can be taken up by plants. Because, these nutrients can bound to other molecules and may not be bioavailable in soil for plant uptake (Sharma et al. 2013; López-Arredondo et al. 2014). Sulfatases and phosphatases secreted by microbes make these nutrients bioavailable and there are efforts to isolate strains with higher biofertilizer potentials. According to the literature, plants don't have any arylsulfatase activity (Knauff et al. 2003; Günal et al., 2019) and therefore they rely on microbial sulfatase activities for bioavailable sulfate for uptake through their roots. *C. reinhardtii* is a soil and fresh water microalgae and also a model organism for plants (Sasso et al., 2018). It has phosphatase and sulfatase enzymes that are upregulated under phosphate and sulfate deficiency, respectively (de Hostos et al., 1989; Moseley et al., 2009).

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CLUSTAL O(1.2.4) multiple sequence alignment

HsARSA      MSMGAPRSLLLA------LAAGLAVARPPNIVLIFADDLGYGDLGCYGHPSSTTPNLDQL    54
CrARS1      --MGALAVLAVACLAAVASVAHAADTKKPNFVVIFTDDQDAIQ--NSTHPHYMPSLEKYI    56
CrARS2      --MGALAVFAVACLAAVASVAHAADTKKPNFVVIFTDDQDAIQ--NSTHPHYMPSLEKYI    56
              ***   : :*       .*  * :: **:*:**:** .  :     **       . :

HsARSA      AAGGLRFTDFYVPVSLCTPSRAALLTGRLPVRMGMYPGVLVPS-----SRGGLPLEEVTVA   110
CrARS1      RYPGVELSQYFVTTPVCCPSRTNLWRGQFAHNTNFT-SVLPPYGGWAKWKGLGIDQSYLP   115
CrARS2      RYPGVELSQYFVTTPVCCPSRTNLWRGQFAHNTNFT-SVLPPYGGWAKWKGLGIDQSYLP   115
              *:.::::* . :* ***: *  *::  . .:  .** *     .  ** :::  :

HsARSA      EVLAARGYLTGMAGKWHLGVGPEGAFLPPHQGFHR--FLGIPYSHDQGPC------QNLT   162
CrARS1      LWLKDQGYNTYYVGKFLVDYSVSNY-QQVPAGWDDIDALVTPYTFDYNTPGFSRNGATPN   174
CrARS2      LWLKDQGYNTYYVGKFLVDYSVSNY-QQVPAGWDDIDALVTPYTFDYNTPGFSRNGATPN   174
              *  :** *  .**: :. . ..        *:.    *  **:.* .          . .

HsARSA      CFPPATPCDGGCDQGLVPIPLLANLSVEAQPPWLPGLEARYMAFAHDLM-ADAQRQDRPF   221
CrARS1      IYPGEYSTDVIRDKGVAQIKS----AVAAGKPFYAQISPIAPHTSTQISTNPATGVTRSY   230
CrARS2      IYPGEYSTDVIRDKGIAQIKS----AVAAGKPFYAQISPIAPHTSTQISTDPVTGVTRSF   230
             :*     *   *:*:. *      :* *  *:   :.       : ::    .    * :

HsARSA      FLYYASHHTHYPQFSGQSF--------------------------AERSGRGP-------   248
CrARS1      FFPPIPAPRHWQLFSDANLPGGTPNKNLYEVDVSDKPAWIRALPLAQQNNRTYLEEIYRL   290
CrARS2      FYPPIPAPRHWQLFSDANLPGGTPNKNLYEVDVSDKPAWVRALPLAQQNNRTYLEEIYRL   290
            *        *:  **. .:                          *::..*

HsARSA      FGDSLMELDAAVGTLMTAIGDLGLLEETLVIFTADNGPETMRMSRGGCSGLLRCGKGTTY   308
CrARS1      RLRSLAAVDELIEQVVKTLDEAGVLDNTYIIYSADNGYHVGAH-------RFGAGKTTGY   343
CrARS2      RLRSLAAVDELIEQVVKTLDEAGVLDNTYIIYSADNGYHVGAH-------RFGAGKTTGY   343
               **  :*  :  ::.::.: *:*::* :*::**** ..           : .** * *

HsARSA      EGGVREPALAFWPGHIAPGVTH----ELASSLDLLPTLAALAGAPL--PNVTLDGFDLSP   362
CrARS1      EEDLRVPFLIRGPGIKASKSDKPQNSKVGLHVDFAPTILSLAGASHLLGDKGLDGTPLGL   403
CrARS2      EEDLRVPFLIRGPGIKASQSDKPQNSKVGLHVDFAPTILSLAGASHLLGDKGLDGTPLGL   403
            * .:* * *   **  *    :    ::.  :*: **: :****     :  ***  *.

HsARSA      LLLGTGKS----PR--Q-----SLFFYPSYPDEVRGVFAVR-TGKYKAHFFTQGSAHSDT   410
CrARS1      YANDDGTLRSDYPRPEQHRQQFQGEFWGGWSDELLQNLRSQPNNTWKVV----------R   453
CrARS2      YANDDGTLRSDYPRPEQHRQQFQGEFWGGWSDELLQNLRSQPNNTWKVV----------R   453
               . *.     **  *     .  *: .: **:   :  : ...:*.

HsARSA      TADPACHA--SSSLTAHEPPLLYDLSKDPGENYNLLGGVAGATPEVLQALKQLQLLKAQL   468
CrARS1      TYDESSKQGWKLIAQCTNERELYDLRKDPGELYNIYDKAKPAVRSRLEGLLA--------   505
CrARS2      TYDESSKQGWKLIAQCTNERELYDLRKDPGELYNIYDKAKPAVRSRLEGLLA--------   505
            * * :.:   .    . :   **** ***** **: . .  *. . *:.*

HsARSA      DAAVTFGPSQVARGEDPALQICCHPGCTPRPACCHCPDPHA-------------------   509
CrARS1      -----------------VLAVCKGESCSNPWKI-LHPDGTVKNFTQALNSKYDRIYNAIR   547
CrARS2      -----------------VLAVCKGESCSNPWKI-LHPDGTVKNFTQALNSKYDRIYNAIR   547
                          .* :*   .*:        **  .

HsARSA      ------------------------------------------------------------   509
CrARS1      PFTYKRCLPYLDWDNEDSQFKTQIRGANPAAGVGHERLLTAASERAIATRRRAQAAVSAE   607
CrARS2      PFTYKTCLQYLDWDNEDSQFKTQIRGANPAAGVGHERLLTAASERAIATRRRAQAAVSAE   607

HsARSA      ---------------------------------------------    509
CrARS1      LAERPAVFQAKVEEKSVPVPQDILKADVEKWFAFNNAEYYLA     649
CrARS2      LAERPAVFQAKVEEKSVPVPQDILKADVEKWFAFNNAEYYLA     649
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Figure 2. Alignment of *C. reinhardtii* ARS1, ARS2 and *Homo sapiens* arylsulfatase A. Conserved sulfatase motifs are shown in rectangles and conserved residues are colored

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CLUSTAL 2.1 multiple sequence alignment


Rl-2VQR          CGPARASLLTG      67
HsARSA-1N2L      CTPSRAALLTG      79
Pa-1HDH          CSPTRSMLLTG      61
Rp-4UPI          CGPSRMSAYTG      63
CrARS1           CCPSRTNLWRG      83
CrARS2           CCPSRTNLWRG      83
                 * *:*      *
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Figure 3. Sulfatase motif in known PDB structures and *C. reinhardtii* ARS1 and ARS2. Lead cysteine residue which is oxidized to FGly is shown in red color (Cyc73 in both CrARS1 and CrARS2)

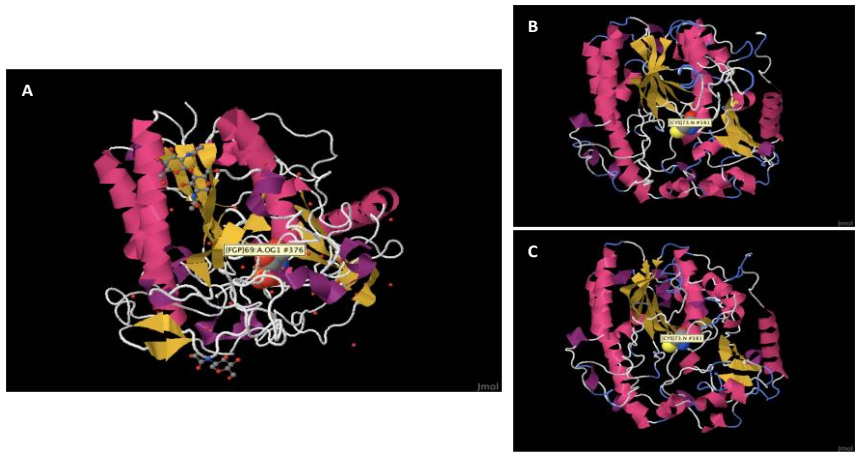

Figure 4. Three Dimentional Model of *C. reinhardtii* ARS1 and ARS2. A. Jmol view of HsARSA (PDB id 1n2l). FGly69 is at the center of the molecule, shown in spacefill. B. Homology model of CrARS1. C. Homology model of CrARS2. In B and C, Cys73 is shown at the center in spacefill

Therefore, it has a potential to be used as biofertilizer. Our results show that *C. reinhardtii* arylsulfatases (ARS) have promiscuous activity; they can hydrolyze both sulfate and phosphate monoesters. However, phosphatase does not have promiscuous activity; it can hydrolyze only phosphate esters (Figure 1). Microorganisms which have enzymes with promiscuous activity might be beneficial in soil for generation of bioavailable sulfate and phosphate for plant nutrition. Also, heterologous expression of *C. reinhardtii ARS* genes in plant roots may be beneficial for plants under nutrient deficient conditions.

Although reaction kinetics of *P. aerigunosa* sulfatase for PNPS and PNPP were found to be similar (Marino et al., 2013) our results suggest that the kinetics of *C. reinhardtii* ARS may be different (Figure 1). Colorimetric assay results suggest ARS has different kinetic activity for hydrolysis of X-SO_4^{2-} and X-PO_4^{3-}; enzyme turnover number may be lower for X-PO4 hydrolysis. Quantitative analyses are needed to make a conclusion on this matter.

CONCLUSION

C. reinhardtii is a green microalga that is found in soil and fresh water habitats. Our results show that *C. reinhardtii* ARS can hydrolyze both sulfate and phosphate monoesters. This activity can be beneficial for making both sulfate and phosphate bioavailable in soil.

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