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Mining Arylsulfatase from Genome-Scale Metabolic Pathways of Pseudoalteromonas sp. SR43-6 and Its Agar-Based Desulfurization Applications

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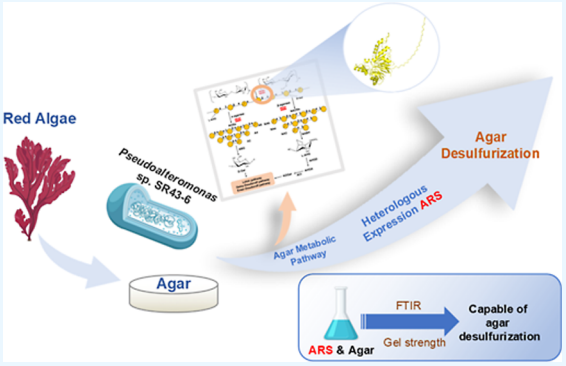
ABSTRACT: Arylsulfatase catalyzes the cleavage of sulfate ester bonds and plays a role in agar desulfation, thereby enhancing agar gel strength and quality. While studying the desulfurization pathway in *Pseudoalteromonas*sp. SR43-6, a sequence encoding a potential arylsulfatase—*Pseudoalteromonas* Ars (*Ps*-Ars)—was found. The enzyme, with *p*-nitrophenyl sulfate as a substrate, exhibited optimal activity at 35 °C and pH 8.0. Its relative activity (206 U/mg) exceeded that of the recently identified arylsulfatases. Four hundred units of the enzyme removed 86.4% of sulfate groups from *Gelidium amansii* agar in 4 h, whereas 800 U of the enzyme removed 71.3% of sulfate groups from *Gracilaria lemaneiformis* agar in 8 h. After enzymatic treatment, *G. amansii* agar gel strength was enhanced by 32%, and a similar improvement was observed in *G. lemaneiformis* agar gel strength. Enzymatic agar desulfurization offers mild, quality-retaining, and environmentally friendly advantages, augmenting industrial application prospects.



INTRODUCTION

The ocean, as one of the most extensive ecosystems on the Earth with rich biodiversity, contains innumerable unique biological components and chemically active substances.[1,2] Red algae, owing to their unique ecological functions and physiological characteristics, are held crucial in the marine system. In addition, their abundant secondary metabolites have attracted much attention. Among them, agar—the main cell wall and intercellular space polysaccharide in red algae[3−5]—has aided in the industrial utilization of marine biological resources due to its excellent gelling properties and a broad range of applications. Agar is composed of agarose and agaropectin.[3−5] Agarose comprises a linear chain of alternating *β*-D-galactose and 3,6-anhydro-*α*-L-galactose units linked through *α*-1,3 and *β*-1,4 glycosidic bonds.[6−8] Agarose is widely used in pharmaceutical and biotechnology-based industries as a chromatographic resin and an electrophoretic medium. Agaropectin contains the same main unit as that of agarose; however, some hydroxyl groups of 3,6-anhydro-*α*-L-galactose residues are substituted by sulfonic acid, methoxy or pyruvic acid residues, etc., forming a sulfated galactan substance.[3] The sulfate group at the C6 position of galactose units forms L-galactose-6 sulfate.[3,9,10] The sulfate groups in agar reduce cross-linking and hence the gel strength of agar.[9−12] Consequently, identifying an effective method to remove sulfate groups from agar and enhance its purity and applications has become a pivotal theme in agar research and applications. Currently, alkali treatment is widely used to remove sulfate groups from agar;[9−13] however, this method presents challenges in controlling agar quality and results in significant energy consumption and environmental pollution during the production process. These drawbacks significantly impede its extensive and long-term application and are not conducive for the current requirement of a low-carbon environment. In contrast, the enzymatic hydrolysis method offers the advantages of product specificity, low energy consumption, and environmental sustainability, thus holding greater developmental potential.[9,14−17]

Arylsulfatase (E.C.3.1.6.1), also known as arylsulfate sulfohydrolase, is a subclass of sulfatases that catalyze the hydrolysis of sulfate ester bonds, yielding inorganic sulfates.[18] This enzyme is instrumental in agar desulfation—a process that markedly enhances agar gel strength and overall quality. Arylsulfatase specifically targets and catalyzes the removal of

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sulfate groups in the agar structure, resulting in a more robust gelling matrix with improved texture and stability. Moreover, the selective nature of arylsulfatase ensures that the intrinsic properties of agar are preserved, making it a superior choice for applications in food, pharmaceutical, and cosmetic industries where quality and consistency are paramount. This enzyme is widely distributed in various microorganisms, such as *Marinomonas* sp.,[14] *Pseudoalteromonas carrageenovora*,[12] *Aspergillus oryzae*.[19] Arylsulfatase is involved in various biodegradation and metabolic processes, such as environmental remediation of complex organic pollutants, removal of sulfated metabolites in organisms, and biotransformation of certain drugs.[20] Arylsulfatase is involved in the sulfur cycle in soil[21] and in agar desulfurization.[14] In red algae resource development and utilization, the potential of arylsulfatase is noteworthy. Wang pointed out that arylsulfatase removes sulfate ester groups at the C6 position of α-L-galactose and the C4 and C6 positions of sulfated galactan of β-D-galactose in red algae.[16] Mining arylsulfatase having high efficiency, stability and specificity promotes the degradation and transformation of complex polysaccharides in red algae and improves the extraction efficiency and purity of products, such as agar and agaropectin.[9,12,14] In addition, it is expected to provide a better method to develop new biocatalysts, drug precursors, and functional food ingredients. Moreover, enzymatic agar desulfurization is an environmentally friendly process.

The genus *Pseudoalteromonas* serves as an epiphytic bacterium of red algae, engaging in symbiotic relationships and participating in their ecological processes.[21,22] Recent studies have indicated that certain *Pseudoalteromonas* strains harbor polysaccharide modification enzymes, playing a pivotal role in polysaccharide degradation and transformation. For instance, specific *Pseudoalteromonas* strains have been found to be capable of degrading algal polysaccharides, thereby generating bioactive secondary metabolites.[21] *P. carrageenovora* Ars uses conserved α/β hydrolase domains to recognize sulfate esters, acting with β-agarase for agarose desulfation.[22] Molecular mechanisms involve substrate-induced gene regulation, such as *P. haloplanktis* TAC125 upregulating arylsulfatase clusters in agar to optimize sulfur metabolism dynamically.[23] Arylsulfatases also show unique substrate specificity for sulfate group conformations in diverse polysaccharides.[24] Yu highlight microbial arylsulfatases (e.g., *Pseudoalteromonas*) as efficient and eco-friendly for agar desulfation.[20] These findings collectively reveal that *Pseudoalteromonas* species harbor agarolytic enzymes and exhibit potential for agar desulfation. In recent years, the research into the metabolic pathways of *Pseudoalteromonas* has been progressively intensifying. Nevertheless, reports regarding the presence and function of arylsulfatase in this bacterium are scarce. Arylsulfatase plays a vital role in numerous biological processes and is potentially closely related to specific metabolic pathways of *Pseudoalteromonas*.[25−27] In the course of exploring agar desulfurization, via the examination of the genome of *Pseudoalteromonas* sp. SR43−6, a sequence was identified which may possess the arylsulfatase function. Moreover, agar desulfurization is of considerable significance. It has the potential to enhance the quality and purity of agar products and facilitates a profound comprehension of sulfur metabolism in related microorganisms as well as its ecological ramifications. Therefore, the excavation of the arylsulfatase gene in *Pseudoalteromonas* and the investigation of its relationship with metabolic pathways as well as the application in agar desulfurization possess significant scientific value.

This paper commenced with the genome of *Pseudoalteromonas* sp. SR43-6, aiming to explore the agar desulfurization pathway in the bacterium. The enzyme functions were verified through heterologous expression, the enzymatic properties of the identified enzyme were explored, and agar desulfurization experiments were conducted to evaluate the desulfurization ability.

MATERIAL AND METHODS

Materials and Culture Conditions. Tryptone and yeast extract were purchased from Oxoid (Basingstoke, United Kingdom). The rapid plasmid miniprep kit was obtained from Tiangen Biotech (Beijing, China). *p*-nitrophenyl sulfate (*p*NPS) was procured from Shanghai Aladdin Biochemical Technology Co. Ltd. (Shanghai, China). Kanamycin, IPTG, Tris, and imidazole were bought from Solarbio (Beijing, China). Other commonly used reagents, such as NaCl, were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). *Escherichia coli* BL21 (DE3) and *E. coli* DH5α strains were purchased from Biomed Biotech Co. Ltd. (Tianjin, China). The gene synthesis was accomplished by Tsingke Biotechnology Co. Ltd.

The LB medium was composed of 1% (w/v) sodium chloride, 1% (w/v) tryptone, and 0.5% (w/v) yeast extract. The strains were cultured in the LB broth containing 1% bacterial suspension, 0.5‰ kanamycin, and 1‰ IPTG.

Sequence Mining and Analysis. Based on the existing research on agar desulfurization pathways, relevant enzymes were identified.[5,28,29] For each enzyme, reference sequences were sought from NCBI and UniProt databases. These reference sequences were then subjected to BLAST alignment against the whole-genome sequence of *Pseudoalteromonas* sp. SR43−6 to identify potential sequences related to the agar desulfurization pathway in this bacterium. Given the focus on arylsulfatase, the arylsulfatase sequence identified in the *Pseudoalteromonas* sp. SR43-6 genome was compared with confirmed arylsulfatase sequences. The phylogenetic tree was constructed using MEGA version 11.0. Subsequently, structural models were predicted using AlphaFold2 for comparison.

Gene Cloning and Plasmid Construction. The sequences that were screened and potentially identified as arylsulfatase were optimized. Codon optimization using JCat[30] improved enzyme expression in *E. coli*, achieving a Codon Adaptation Index (CAI) of 0.85. GC content analysis via EMBOSS CpGPlot[31] revealed the original sequence's 38% GC was adjusted to 50% postoptimization.

Subsequently, Beijing Tsingke Biotech Co. Ltd. was commissioned to synthesize the optimized sequences in the pET-28a (+) vector, thereby obtaining the plasmid harboring the target gene. *E. coli* is the most commonly used host for arylsulfatase. For vector propagation, the ligated products were transformed into *E. coli* DH5α cells in the LB broth at 37 °C with a shaking speed of 220 rpm for 8 h. The recombinant *Ps*-Ars plasmids were purified and verified through DNA sequencing. Subsequently, the recombinant plasmids were transformed into *E. coli* BL21 (*DE3*) to assay protein overexpression.

Transformation and Protein Expression. The recombinant vector pET-28a (+)-*Ps*-Ars was extracted and transformed into *E. coli* BL21 (*DE3*). The recombinant strain was cultured in 5 mL of the LB broth containing 2.5 μL of kanamycin at 37

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°C for 12 h. For scale-up, 1 mL of the above bacterial solution was transferred into 100 mL of the LB broth supplemented with 50 μL of kanamycin and cultured at 37 °C for 4 h. When the OD reached 0.6, 100 μL of IPTG was added, and the culture was continued to grow at 20 °C for 16 h. Thereafter, the culture was collected and centrifuged at 8000 × g at 4 °C for 10 min. The cells were resuspended and disrupted in an ultrasonic homogenizer (Scientz-IID, Ningbo Scientz Biotechnology Co. Ltd.) at 350 W and below 30 °C for 30 min. The disrupted cells were subsequently centrifuged at 4 °C and 8000 × g for 20 min to obtain the supernatant containing the crude enzyme.

Protein Purification. Based on the principle that the His-tag on the expressed protein can bind to the nickel column, the enzyme solution is purified by nickel column purification method and eluted with different concentrations of imidazole to achieve the separation and purification of the target protein. Prior to purification via the Ni-NTA resin column with imidazole eluting buffers at concentrations of 20 mM, 50 mM, 100 mM, 200 mM, and 500 mM, the crude enzyme was filtered through 0.45 μm filters. The protein concentration was ascertained by means of coomassie brilliant blue staining. Subsequently, the eluted fractions were subjected to analysis by SDS-PAGE for the detection of pure *Ps*-Ars. Thereafter, the purified *Ps*-Ars was concentrated using 10 K-molecular weight cut off columns at 4 °C and 4000 × g.

Characterization of Recombinant Enzyme. The Zhu et al. method was modified to measure arylsulfatase activity.[29] The enzyme solution (0.4 μg, 12 μL), potassium *p*NPS substrate solution (25 mM, 50 μL), and tris−HCl buffer solution (113 μL, pH 8.0) were taken. The substrate solution was prepared with Tris−HCl at pH 8.0. After 1 h incubation at 35 °C, 75 μL of 1 M NaOH was added to terminate the reaction. The absorbance was measured at 410 nm in a microplate reader to determine the content of *p*-nitrophenyl (*p*NP) produced.[29,32] The reaction principle is that potassium *p*NPS produces *p*-nitrophenyl under the action of arylsulfatase. 1 U is defined as the amount of enzyme required to produce 1 μmol of *p*NP per minute under the standard conditions.

To investigate the optimal reaction temperature and thermostability of the *Ps*-Ars enzyme, the purified enzyme solution, 25 mM *p*NPS, and Tris−HCl buffer (pH 8.0) were mixed and incubated at various temperatures. To determine the optimal reaction temperature, the mixture was incubated at 25, 35, 40, 45, and 50 °C for 1 h. For assessing thermostability, the mixture was incubated at 4, 25, 35, 45, and 55 °C for 0, 0.5, 1.5, 3.5, 5, 7.5, and 10.5 h in different permutations and combinations. After incubation, *p*NPS was added to the samples and allowed to react for an additional 1 h. The reactions were terminated by adding 1 M NaOH, and the absorbance was measured at 410 nm. This approach allowed the determination of both optimal reaction temperature and the thermostability profile of the *Ps*-Ars enzyme.

To explore the optimal pH and pH stability of the *Ps*-Ars enzyme, the purified enzyme solution and 25 mM *p*NPS were mixed with various buffer solutions: 50 mM citrate buffer solution (pH 3.0−6.0), 50 mM phosphate buffer solution (pH 6.0−8.0), 50 mM Tris−HCl buffer solution (pH 7.0−9.0), and 50 mM glycine-NaOH buffer solution (pH 9.0−11.0). To determine optimal pH, the mixtures were reacted at 35 °C for 1 h. For assessing pH stability, the enzyme solution was incubated in the same buffers at 4 °C for 0, 1, 2.5, 3.5, 5.5, 7.5, and 10.5 h. Subsequently, *p*NPS was added and allowed to react for an additional 1 h. The reactions were terminated by adding 1 M NaOH, and the absorbance was measured at 410 nm. This approach enabled the determination of both optimal pH and the pH stability profile of the *Ps*-Ars enzyme.

The experimental design to investigate the effects of different ions and chemical additives on the *Ps*-Ars enzyme activity is as follows. The pure enzyme was added in equal amounts to solutions containing 1 and 10 mM concentrations of Na^+ (NaCl), K^+ (KCl), Mg^{2+} ($MgCl_2$), Ni^+ ($NiCl_2$), Mn^{2+} ($MnCl_2$), Ba^{2+} ($BaCl_2$), Ca^{2+} ($CaCl_2$), Zn^{2+} ($ZnCl_2$), Co^{2+} ($CoCl_2$), Fe^{3+} ($FeCl_3$), SDS, and Na_2EDTA, and then incubated for 10 min. The control group was the reaction system lacking the above solutions. Using 25 mM *p*NPS as the substrate, the reaction was carried out at 35 °C for 1 h. The absorbance value was measured at 410 nm to calculate the enzyme activity.

*p*NPS substrates with concentrations of 0, 0.1, 0.2, 0.4, 0.8, 1.0, 2.0, 5.0, 10.0, 15.0, 20.0, and 25.0 mM were prepared using 50 mM Tris−HCl buffer (pH 8.0). After incubation at 35 °C for 10 min, 1 μL of pure enzyme was mixed with 149 μL of the above substrates. After reacting for 15 min at 35 °C, 100 μL of 1 M NaOH was added to terminate the reaction and the absorbance was measured at 410 nm. The obtained data was fit using the Michaelis−Menten equation to calculate K_m and V_{max}.

Agar Desulfurization. Literature indicates that arylsulfatase is capable of desulfurizing agar.[9,12,14−16,20,33,34] To examine the desulfurization effect of the *Ps*-Ars enzyme, the following experiment was designed. The agar samples derived from *G. amansii* and *G. lemaneiformis* were prepared in our laboratory through extraction procedures. The detailed extraction protocol is mentioned below.

Dried, pulverized, and sieved *G. amansii* powder (10 g) was dissolved in 150 mL of 6% (w/w) NaOH solution. The resultant mixture was heated at 90 °C with continuous stirring for 3 h. Thereafter, the mixture was washed using a 300-mesh filter bag until the filtrate pH value reached 7.0. Subsequently, the mixture was agitated with an acid washing solution (500 mL H_2O, 0.215 mL H_2SO_4, 0.32 g oxalic acid and 0.065 g $EDTA_2Na$) for 5 min and washed again with a 300-mesh filter bag until the pH value reached 7.0. Thereafter, 0.04% NaClO was incorporated, and the mixture was stirred for an additional 5 min and subsequently washed. Finally, the mixture was heated at 100 °C for 2 h. The agar-containing liquid was filtered using a 300-mesh filter while it was still hot. The cooled liquid was then placed overnight in a freezer at -20 °C.[32−38] On the following day, after thawing, the water was removed, and the remaining residue was dried. To ensure optimal pH conditions (pH 8.0) for subsequent enzymatic reactions, dried agar was resuspended in deionized water and adjusted to pH 7.0−8.0 using 1 M NaOH or HCl, monitored with a calibrated pH meter. The agar from *G. lemaneiformis* was obtained following the same methodology.

The desulfurization process was as follows: The agar powder (0.1 g) was mixed with 20 mL of 50 mM Tris−HCl at pH 8.0 and treated with 100, 200, 400, and 800 U of the purified enzyme solution. The specific experimental steps to explore desulfurization with different reaction times and enzyme dosages using *G. amansii* and *G. lemaneiformis* agar as substrates are as follows. The reaction time gradients were set as 4, 8, 12, and 24 h, whereas the enzyme dosage gradients were set as 100, 200, 400, and 800 U. The agar was stirred with arylsulfatase, and the reaction was terminated by heating in a

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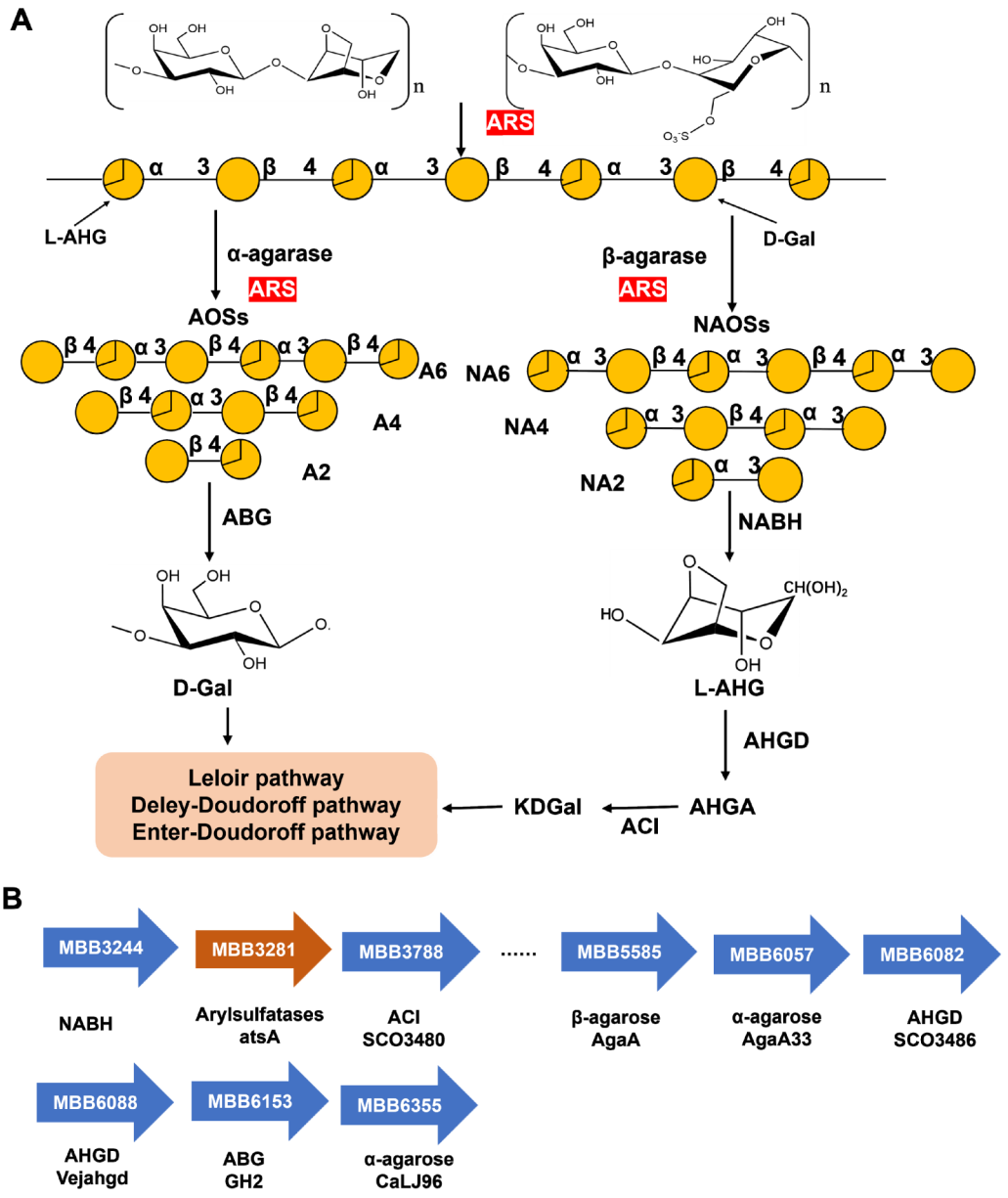

Figure 1. (A) Possible agar metabolic pathways of *Pseudoalteromonas*sp. SR43−6. NABH: α-neoagarobiose hydrolase; ACI: 3,6-anhydrogalactonate cycloisomerase; AHGD: 3,6-anhydro-L-galactose dehydrogenase; ABG: agarolytic β-galactosidase; ARS: arylsulfatase. (B) Predicted polysaccharide utilization loci in *Pseudoalteromonas*sp. SR43-6.

70 °C water bath for 30 min. Then, through freezing, thawing, centrifugation, and rinsing with pure water, the reaction solution was washed off to obtain the residue. Subsequently, the samples were placed overnight in a −80 °C freezer. On the following day, the samples were subjected to freeze-drying for 48 h to obtain the final product. The change in the sulfate content in agar was determined by Fourier transform infrared (FTIR) spectroscopy and the barium sulfate turbidimetric method.[33,39−47] The same steps were followed for *G. lemaneiformis*agar.

FTIR Spectroscopic Determination of Products. Dried KBr was ground with the samples and then pressed into pellets. The pellets were placed in the FTIR spectrometer to measure the relevant values.

Barium Sulfate Turbidimetric Method. The gelatin−barium chloride solution was prepared and the standard curve was constructed. The freeze-dried sample was weighed and hydrolyzed with 10 mL of 1 M HCl at 100 °C for 4 h. After cooling to room temperature, 0.8 g of activated carbon was added, and the mixture was stirred and allowed to stand for 1 h. The solution was filtered through a 0.45 μm filter. The gelatin−barium chloride solution (3 mL) was added to the filtrate (1 mL), mixed, and allowed to stand for 10 min. The absorbance was measured at 360 nm. The sulfate content in the sample was obtained by comparing the obtained value with the standard curve values.[32,34,48−56]

Gel Strength Determination. Pure water was added to appropriate amounts of control and enzymatically hydrolyzed agar samples to form 2% (w/v) *G. amansii* agar and 3% (w/v) *G. lemaneiformis* agar. The mixtures were heated in a microwave oven and dissolved by shaking. While still hot, 3 mL of agar solution was poured in a cylindrical container (diameter × height = 2 × 1.5 cm). After cooling, the containers were placed overnight in a 4 °C refrigerator for aging. The next

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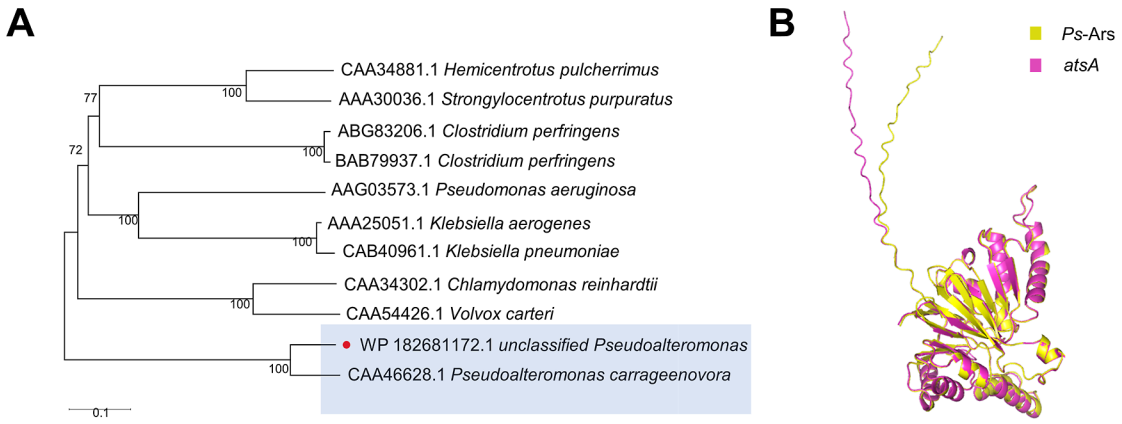

Figure 2. (A) *Ps*-Ars phylogenetic tree analysis. (B) Structure alignment diagram of *Ps*-Ars (yellow) and atsA (pink).

day, the gel strength was measured using a texture analyzer.[57,60,61] The trigger force was set at 0.1 N, the test and return speed were 60 mm/min, and the puncture distance was 4 mm. A cylindrical probe with a diameter of 5 mm was used for the measurement, and its cross-sectional area was taken as S. The maximum force required to squeeze the agar until it ruptures was recorded as F. The calculation formula for gel strength is as follows[61]

$$\text{Gel strength } (\text{g/cm}^2) = \frac{F(N)}{g \times S(\text{cm}^2)} \times 1000$$

where "*g*" represents acceleration due to gravity, with a value of 9.8 N/kg.

Statistical Analysis. The study findings are presented as means ± standard deviations. Moreover, the kinetic parameters pertaining to the reaction between the enzyme and its substrates were analyzed using Origin 2024 software.

RESULTS AND DISCUSSION

Sequence Mining and Analysis. Based on the existing research, several agar metabolism-related enzymes have been reported: arylsulfatase (E.C.3.1.6.1), α-agarase (E.C.3.2.1.158), β-agarase (E.C.3.2.1.81), α-neoagarobiose hydrolase (NABH), 3,6-anhydrogalactonate cycloisomerase (ACI), 3,6-anhydro-L-galactose dehydrogenase (AHGD), and agarolytic β-galactosidase (ABG).[5,24,26,32] Arylsulfatase catalyzes the hydrolysis of sulfate ester bonds in agar, thereby increasing the agarose content in agar. α-and β-agarase selectively cleave α-1,3 and β-1,4 glycosidic bonds, respectively, yielding agar oligosaccharides (AOSs) with agarotetraose as the main product and generating neoagarooligosaccharides.[5,35−38] NABHs act on neoagarobiose, further reducing the degree of oligosaccharide polymerization and generating L-anhydrous galactose (L-AHG).[5,27,34,39] ABGs hydrolyze oligosaccharides or polysaccharides containing β-galactosidic bonds, degrading AOSs into D-galactose (D-gal).[5,34,40,41,49] AHGDs participate in the redox reactions of L-AHG, catalyzing its dehydrogenation to generate 3,6-anhydrogalacturonic acid (AHGA).[5,40,49] ACIs catalyze AHGA cycloisomerization, altering the structure of metabolic intermediates and converting AHGA to KDGal, thereby propelling subsequent metabolic pathways.[5,49] D-gal and KDGal participate in Leloir, DeLey-Doudoroff, and Entner−Doudoroff pathways, generating glucose and other intermediates that contribute to energy metabolism (Figure 1A). Through BLAST alignment of the *Pseudoalteromonas* sp. SR43-6 genome with reference sequences of various agar desulfurization-related enzymes, Figure 1B was obtained, which illustrates the prediction of polysaccharide utilization loci. Compared with other agar-degrading microorganisms including *Streptomyces coelicolor*A3(2),[5,50] *Vibrio* sp. EJY3,[5,34] or *Saccharophagus degradans* 2-40,[5,51] the metabolic pathway of *Pseudoalteromonas*sp. SR43-6, which includes an arylsulfatase, represents a more complete agar metabolic process. This may facilitate the adaptation of *Pseudoalteromonas* to the phycosphere of red algae. In addition, the genes in this pathway are separately located in different gene clusters in the *Pseudoalteromonas* sp. SR43-6 genome, suggesting a highly flexible agar metabolism where gene expression may be regulated by the availability of substrates. Notably, *Zobellia galactanivorans*[5,52] has a similar agar metabolic cluster with *Pseudoalteromonas*sp. SR43-6, including sulfatases convincing the roles of this enzyme in agar degradation. Furthermore, environmental signals such as the presence of agar oligosaccharides may dynamically modulate the transcriptional activity of the gene cluster via two-component signal transduction systems (TCS)[54] or cAMP-CRP regulatory networks,[54] enabling adaptive responses to changes in carbon source availability.

The arylsulfatase sequence mined from *Pseudoalteromonas sp.* SR 43−6 was WP 182681172.1, which exhibited 84.5% sequence similarity to the reference sequence CAA46628.1 (arylsulfatase [P. carrageenovora]-Protein-NCBI). A phylogenetic tree was constructed using WP 182681172.1 and other reference sequences of arylsulfatases (Figure 2A), demonstrating that it clusters with CAA46628.1 within the same clade. Further structural predictions were made using AlphaFold2, as shown in Figure 2B, revealing a high degree of match between the two. The consistency between the phylogenetic tree and structural predictions provides additional support for the hypothesis that this enzyme likely possesses arylsulfatase function. To further verify whether this sequence indeed possesses arylsulfatase function, we proceeded with heterologous expression of the sequence. The protein expressed from WP 182681172.1 was designated as *Ps*-Ars.

Expression and Purification of *Ps*-Ars. The *Ps*-Ars gene is constituted by 993 base pairs and encodes a protein comprising 330 amino acids, possessing a predicted molecular weight of 36.2 kDa.

The arylsulfatase designated as *Ps*-Ars was successfully expressed in *E. coli* BL21 (*DE3*) and underwent SDS-PAGE analysis. Through SDS-PAGE experiments (Figure 3), when the enzyme was eluted with 100 mM imidazole, the protein

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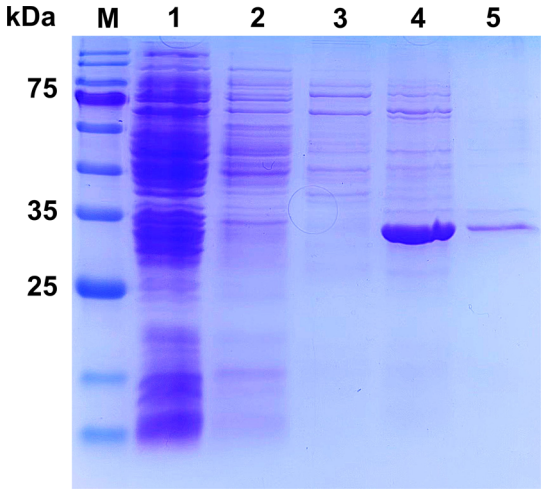

Figure 3. Protein electrophoresis. M: Molecular mass marker of proteins; Lane 1: Crude enzyme extract; Lane 2: Column penetration fluid; Lane 3:50 mM imidazole eluent; Lane 4:100 mM imidazole eluent; Lane 5:200 mM imidazole eluent.

band was single, and enzyme activity was determined. The molecular weight of the enzyme was 34 kDa. This is consistent with the molecular weight prediction of 36.2 kDa by Expasy (Expasy-ProtParam). The remaining 100 mM imidazole eluate was used to obtain pure enzyme solution through ultrafiltration.

The molecular weight of *Ps*-Ars was similar to that of *Marinomonas* sp. FW-1 (33 kDa).[14] However, it was significantly smaller than those of *Naja nigricolis* (50 kDa),[58] *Flammeovirga pacifica* (56 kDa),[56] *Sphingomonas sp.* AS6330 (62 kDa),[46] OUC-FARS6 (63 kDa),[50] *Thermotoga maritima* (65 kDa),[15] and *Pyrococcus furiosus* (90 kDa)[47] (Table 1).

Physicochemical Properties of *Ps*-Ars. As shown in Figure 4A, when *Ps*-Ars used *p*NPS as the substrate, the optimum temperature was 35 °C. At a temperature range of 25−35 °C, the enzyme activity increased with the increase of temperature. More than 80% enzyme activity was exhibited at a temperature range of 25−40 °C. Thereafter, as the temperature continues to rise, the enzyme activity rapidly decreases. The optimal temperature of *Ps*-Ars was relatively low compared with that of other arylsulfatases, for example, *Marinomonas* sp. FW-1 (45 °C),[14] *F. pacifica* (40 °C),[56] *Sphingomonas* sp. AS6330 (45 °C),[46] OUC-FARS6 (45 °C),[50] *P. furiosus* (45 °C),[47] and *T. maritima* (80 °C)[15] (Table 1). The high relative activity of *Ps*-Ars at 25−45 °C (notably at 35 °C; Figure 4A) aligns with industrial requirements for low-energy biocatalysis. For instance, conventional enzymatic and microbial desulfurization processes typically operate at 50−65 °C. In contrast, *Ps*-Ars maintains stable performance under mild conditions, reducing the need for costly thermal management systems, which makes *Ps*-Ars a potential candidate for industrial applications such as agar refining and biopharmaceutical production.

As shown in Figure 4B, the enzyme was incubated at temperatures of 4, 25, 35, 45, and 55 °C for 0, 1, 2.5, 3.5, 5.5, 7.5, and 10 h. When the enzyme was incubated at 4 and 25 °C, the *Ps*-Ars enzyme activity was stable when using *p*NPS as the substrate. At 35 °C, the enzyme activity gradually decreased. After 10.5 h, the enzyme was nearly completely inactivated. At other temperatures, the enzyme activity dropped sharply to nearly 0 after 1 h of incubation. However, the thermostability of other arylsulfatases was relatively good, such as *F. pacifica*[56] could still maintain 70% enzyme activity after incubation at 50 °C for 12 h. Overall, *Ps*-Ars is suitable for storage at low and normal temperatures; however, its thermal stability needs to be improved. In the future, there's great potential to boost *Ps*-Ars enzyme's thermal stability with protein engineering. Site-directed mutagenesis on key amino acids can modify its 3D structure for better thermotolerance. Also, adding stabilizers like saccharides and polyols creates a protective microenvironment, reducing heat - induced denaturation and enhancing thermal stability.

At the optimum temperature, optimum pH was determined. As shown in Figure 4C, when arylsulfatase used *p*NPS as the substrate, the optimum pH system was 50 mM Tris−HCl buffer (pH 8.0). The optimal pH of *Ps*-Ars was alkaline, which is similar to most existing arylsulfatases, such as *T. maritima* (pH 7.0),[15] *Sphingomonas*sp. AS6330 (pH 7.0),[46] *Citrobactoer braakii* (pH 7.5),[57] *F. pacifica* (pH 8.0),[56] and *Marinomonas*sp. FW-1 (pH 9.0).[14] This indicates that a weakly alkaline environment is most suitable for *Ps*-Ars. As shown in Figure 4D, the relative enzyme activity under the Tris−HCl buffer system was significantly higher than that under the CPBS, PBS, and Glycine-NaOH buffer systems. The enzyme exhibited better stability under medium alkaline conditions (pH 7.0−10.0). At this pH range, the enzyme activity was maintained above 70% for 3.5 h, whereas it rapidly decreased under acidic conditions.

At 35 °C and pH 8.0, the effect of various metal ions and their concentrations on enzyme activity was determined. Most metal ions had a strong inhibitory effect on this enzyme at low and high concentrations (Figure 5). Ba^{2+} promoted the enzyme reaction at a concentration of 10 mM. Ca^{2+} had a weak promoting effect at a low concentration. Co^{2+} promoted enzyme activity at a low concentration and inhibited enzyme activity at a high concentration. Cu^{2+}, Zn^{2+}, Fe^{3+}, SDS, and Na_2EDTA had significant inhibitory effects on enzyme activity at both low and high concentrations.

The data were nonlinearly fitted by using the Michaelis−Menten equation. The K_m value of calcium and magnesium was 0.57 mM and the V_{max} value was 29.95 μM/min. When compared with other enzymes, a wide variation in K_m and V_{max} values was observed, for example, *Sphingomonas*sp. AS6330 (K_m 54.9 μM, V_{max} 113 mM/min),[46] *P. carrageenovora* (K_m 0.66 mM, V_{max} 11.47 U/mg),[9] *Marinomonas*sp. FW-1 (K_m 13.73, V_{max} 270.27 μM/min),[14] and *N. nigricolis* (K_m 110 μM, V_{max} 225 μmol/min)[58] (Table 1). *Ps*-Ars exhibited poor molecular dynamics, with a relatively high K_m value and a relatively low V_{max} value.

At 35 °C and pH 8.0 conditions, the relative enzyme activity can reach 206 U/mg. Currently, the relative activity of most arylsulfatases is below 100 U/mg, for example, OUC-FARS6's relative enzyme activity is 0.19 U/mg,[50] and the relative enzyme activity from *F. pacifica* is 64.8 U/mg.[56] The highest relative enzyme activity currently known is from *P. carrageenovora*—468 U/mg (Table 1).[12] The enzyme activity of *Ps*-Ars was relatively higher than those of other arylsulfatases against *p*NPS. Compared to the arylsulfatase from *F. pacifica* (optimal at 40 °C, pH 8.0)[57], *Ps*-Ars shares similar optimal reaction conditions but achieves 3-fold higher relative activity at lower temperatures. This further highlights *Ps*-Ars' advantage of high activity under mild conditions.

While *Ps*-Ars demonstrates a relatively high relative enzyme activity, its elevated K_m value suggests suboptimal substrate

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Table 1. Physicochemical Properties and Desulfurization Effects of Different Arylsulfatases

source	molecular mass	optimal T/pH	K_m/V_{max}	activity	reaction conditions	enzyme dosage	desulfurization rate	reference
Pseudoalteromonas carrageenovora	33.1 kDa	40 °C/8.5	NA	NA	40 °C, pH 8.5, 12 h	7 U	73%	8
Pseudoalteromonas carrageenovora	33.6 kDa	55 °C/8.0	K_m = 0.66 mM; V_{max} = 11.47 U/mg	10.0 U/mg	45 °C, pH 7.5, 4 h (modified by random mutagenesis)	90 U	82.1% (from *G. lemaneiformis*)	9
Pseudoalteromonas carrageenovora	33 kDa	40−45 °C/7.0	K_m = 1.15 mM; k_{cat} = 1000 s^{-1}	468 U/mg	NA	NA	NA	12
Marinomonas sp. FW-1	33 kDa	45 °C/9.0	K_m = 13.73; V_{max} = 270.27 μM/min	NA	45 °C, pH 9.0, 12 h	200 U	86.11% (from *G. amansii*) 89.61% (from *G. lemaneiformis*)	14
Thermotoga maritima	65 kDa	80 °C/7.0	NA	20.63 U/mg	70 °C, pH 7.0, 12 h	49.5 U	60%	15
Sphingomonas sp. AS6330	62 kDa	45 °C/7.0	K_m = 54.9 μM; V_{max} = 113 mM/min	94.2 U	45 °C, pH 7.0, 8 h	100 U	97.7%	46
Pyrococcus furiosus	35 kDa	45 °C/9.5	NA	NA	50 °C, pH 9.0, 12 h	50 U	75%	47
Formosa agariphila	63 kDa	45 °C/9.0	K_m = 32.63 μM; V_{max} = 9.25 μM/min	0.19 U/mg	35 °C, pH 9.0, 24 h	NA	15.6%	50
Flammeovirga pacifica	56 kDa	40 °C/8.0	NA	64.8 U/mg	NA	NA	NA	34
Citrobacter braakii	61.6 kDa	45 °C/7.5	K_m = 6.9 mM	47.7 U/mg	NA	NA	NA	51
Naja nigricolis	50 kDa	40 °C/5.0	K_m = 110 μM; V_{max} = 225 μmol/min	NA	NA	NA	NA	53
Pseudoalteromonas	34 kDa	35 °C/8.0	K_m = 0.57 mM; V_{max} = 29.95 μM/min	206 U/mg	25 °C, pH 8.0, 4 h 25 °C, pH 8.0, 8 h	400 U 800 U	86.4% (from *G. amansii*) 71.3% (from *G. lemaneiformis*)	present study

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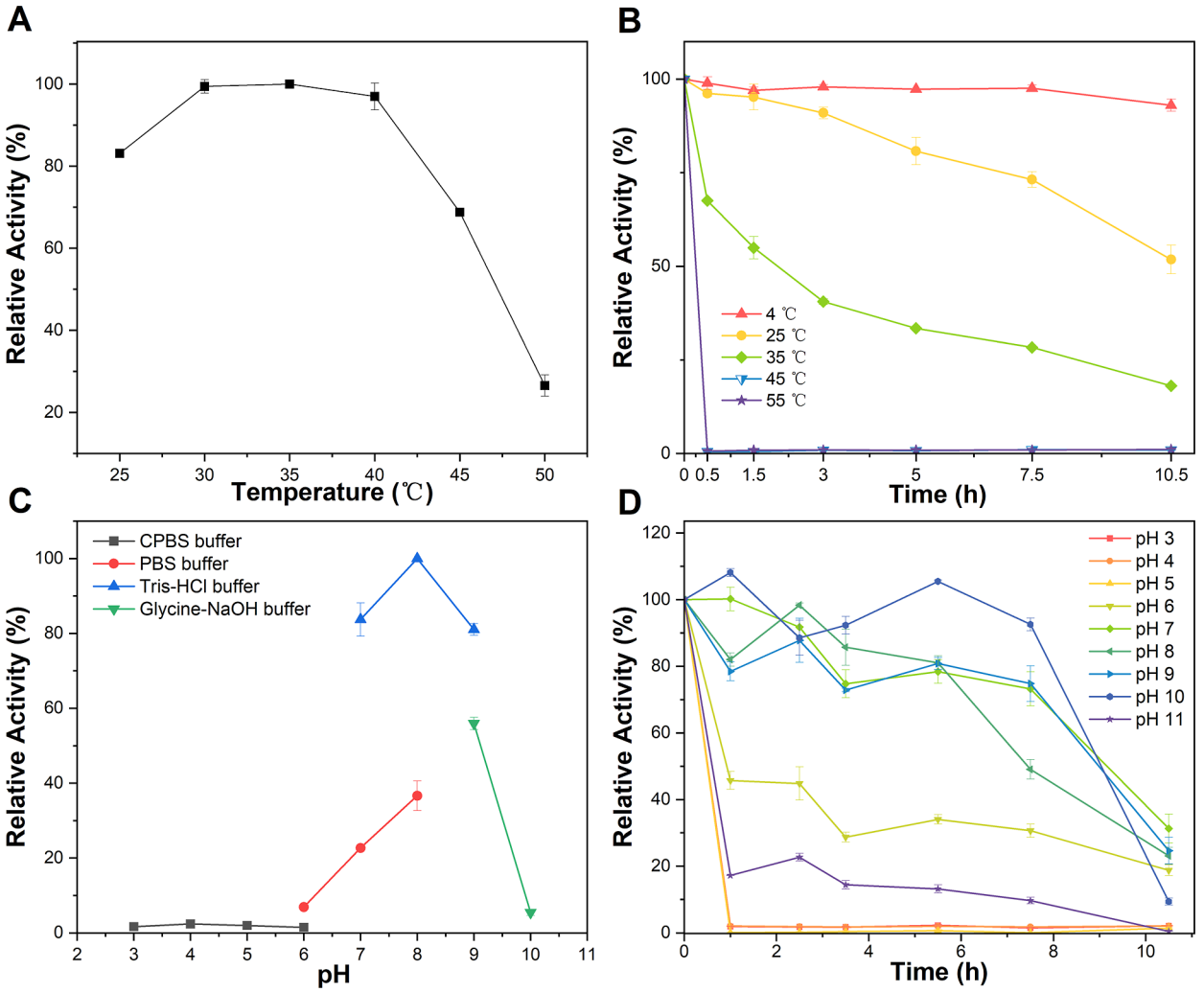

Figure 4. Influence of temperature and pH on enzyme activity (A) Change in enzyme activity at a temperature range of 25−50 °C in the presence of Tris−HCl (pH 8.0) (B) Under optimal pH conditions, the change in enzyme activity was observed at different temperatures (C) At 35 °C, the change in enzyme activity at a pH range of 3−10 is presented (D) Change in enzyme activity at 35 °C at different durations and different pH values.

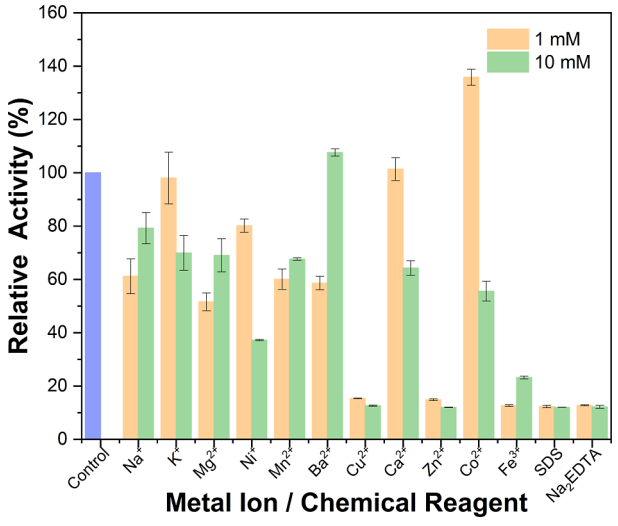

Figure 5. Effects of different concentrations of metal ions (1 mM and 10 mM) and organic solvents on enzyme activity.

binding efficiency. Implementing computational tools like 'Hotspot Wizard'[59] enables systematic structural analysis to precisely identify functionally critical hotspot regions. Strategic site-directed mutagenesis at these positions may optimize enzyme−substrate binding conformations, potentially enhancing both catalytic efficiency and substrate specificity for comprehensive performance improvement.

Agar Desulfurization. Table 1 summarizes some of the published arylsulfatases with desulfurization functions. Only a few arylsulfatase studies have explored its role in agar desulfurization. As can be observed from Table 1, studies on agar desulfurization can be further categorized based on the type of substrate used: commercial and laboratory-prepared agar. The sulfur content in commercial agar powder was lower than that in laboratory-prepared *G. amansii* agar and *G. lemaneiformis* agar. Therefore, *G. amansii* and *G. lemaneiformis* were employed as substrates to investigate agar desulfurization.

As shown in Figure 3B, at an optimal temperature of 35 °C, the enzyme activity decreased to 50% after 1.5 h, indicating that the enzyme is not suitable for long-term desulfurization experiments. However, at 25 °C, the enzyme maintained a relatively high activity for 8 h. The desulfurization reaction

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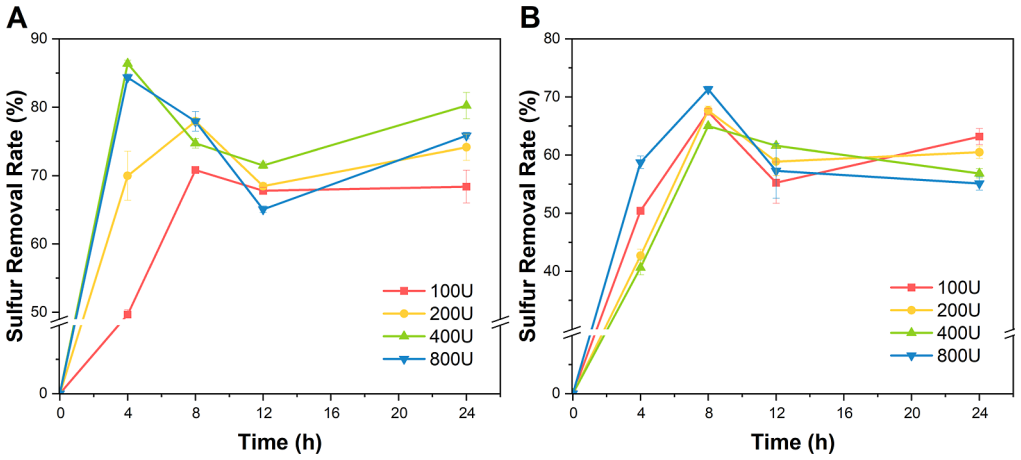

Figure 6. Desulfurization rate of agar under different reaction times and enzyme amounts. (A) *G. amansii* agar. (B) *G. lemaneiformis* agar.

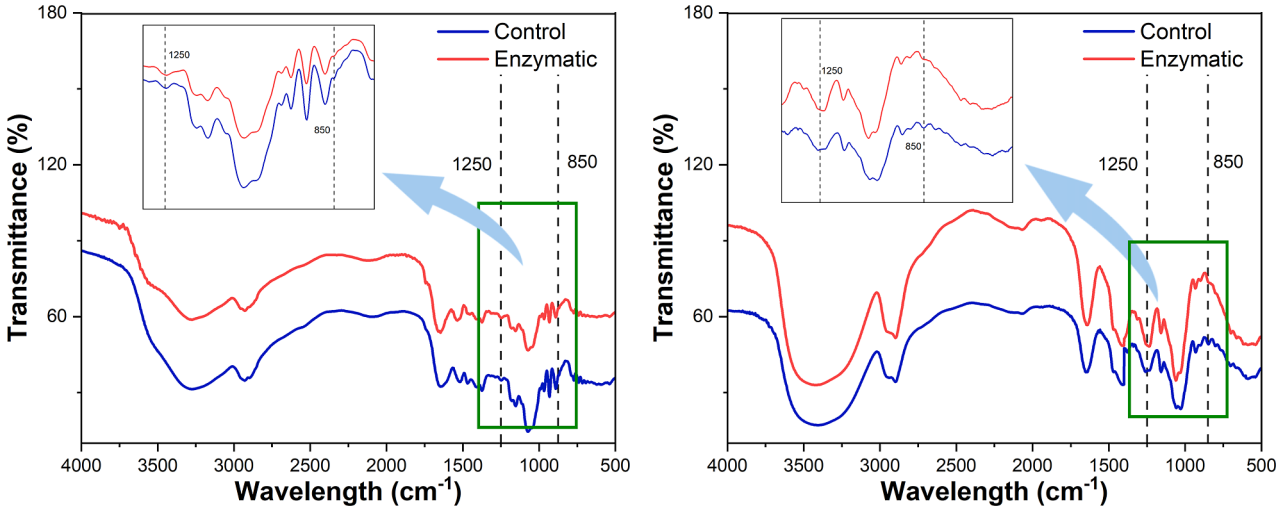

Figure 7. FTIR spectra of agar after enzyme treatment. (A) FTIR spectrum of *G. amansii* agar. (B) FTIR spectrum of *G. lemaneiformis* agar.

conditions of this enzyme were milder than those of most arylsulfatases, such as *Marinomonas*sp. FW-1 (45 °C)[14] and *T. maritima* (80 °C).[15]

The optimal desulfurization ratio of *G. amansii* agar was achieved in a reaction time of 4 h and with an enzyme dosage of 400 U, resulting in a desulfurization ratio of 86.4% (Figure 6A). Under the conditions of 8 h reaction time and an enzyme dosage of 800 U, the desulfurization ratio of *G. lemaneiformis* agar was the highest—71.3% (Figure 6B). According to the survey results (Table 1), the desulfurization ratios of arylsulfatases on agar typically ranged from 60% to 90%. The desulfurization ratio of arylsulfatase used in this study was at moderately high. However, the desulfurization ratio of *G. lemaneiformis* agar was relatively lower than the desulfurization ratios of *G. amansii* agar and *G. lemaneiformis* agar observed in other studies. The desulfurization ratio of *G. amansii* agar was higher than that of *G. lemaneiformis* agar and showed a certain disparity, which could be related to the species themselves and their sulfur content.

The FTIR spectra of *G. amansii* agar and *G. lemaneiformis* agar before and after enzyme treatment are presented in Figure 7. A significant decrease in the peak at 850 cm^{-1} and a slight variation in the peak at 1250 cm^{-1} were observed. The absorption peak at 850 cm^{-1} is known to represent the presence of a sulfate group at the C4 position of 4-sulfo-D-galactose, and the peak at 1250 cm^{-1} is attributed to the presence of a sulfate group.[16,43−45] This indicates that the enzyme can be utilized to remove the sulfate group at the C4 position of agarose 4-sulfo-D-galactose.

The gel strength of samples in the enzymatically hydrolyzed and enzyme-inactivated groups was measured using a texture analyzer. The gel strength of *G. amansii* agar and *G. lemaneiformis* agar was found to be enhanced. For the *G. lemaneiformis* agar, the untreated samples failed to reach the trigger force on the texture analyzer. However, the samples in the enzymatically hydrolyzed group could reach the trigger force of 0.1 N. Although the gel strength was low, there was still an improvement compared to the control. In contrast, the *G. amansii* agar showed significant improvement before and after treatment. The "F" was 1.56 N, and the gel strength of the enzyme-inactivated group was 613 g/cm^2, whereas "F" and gel strength of the enzymatically hydrolyzed group was 1.18 N and 811 g/cm^2, respectively, representing a 32% increase in gel strength. Compared to existing enzymatic methods, *Ps*-Ars showed a slight advantage in enhancing gel strength. For instance, when agar was enzymatically modified using the mutant arylsulfatase K253Q derived from *Pseudoalteromonas cervicornis*, the performance characteristics revealed that the gel strength boosted from 783 to 966.6 g/cm^2, marking a 23.4% increase.[59] Compared with the microbial agar desulfurization

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method, there was a gap in enhancing gel strength. The agar-making desulfurization technology established and optimized by Song achieved gel strength of 1104.25 g/cm^2, representing an increase of 170.76%.[60,61]

This article reports the identification and characterization of an arylsulfatase, designated as *Ps*-Ars, from *Pseudoalteromonas sp.* SR43-6. The WP 182681172.1 sequence was identified and confirmed as an arylsulfatase through heterologous expression and purification. When *p*NPS was used as the substrate, the enzyme exhibited optimal activity at 35 °C and pH 8.0 (50 mM Tris−HCl). High concentrations of Ba^{2+} enhanced the enzymatic reaction, whereas low concentrations of Ca^{2+} and Co^{2+} promoted enzyme activity. The enzyme demonstrated a specific activity of 206 U/mg, which was higher than that of most arylsulfatases. *Ps*-Ars effectively desulfurized agar as evidenced by changes in the FTIR spectrum at 850 cm^{-1}. The barium sulfate precipitation method showed a desulfurization rate of 86.4% for *G. amansii* agar and 71.3% for *G. lemaneiformis* agar. Additionally, the gel strength of the enzyme treated 2% (w/v) *G. amansii* agar increased by 32% compared to untreated samples.

■ ASSOCIATED CONTENT

Data Availability Statement

The data sets generated and analyzed during the current study are publicly available on figshare with the DOI: 10.6084/m9.figshare.28596944. Additionally, they can also be obtained from the corresponding author upon reasonable request.

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Author Contributions

⊥Songzhi Li and Yang Guo contributed equally to this work. Songzhi Li designed and executed the experimental work and conducted data analysis. Based on the suggestions from all the authors, Songzhi Li and Guo drafted the manuscript. Jiayu Li and Chen provided experimental assistance. Associate Dr. Jiang and Researcher Wang coordinated this research project. Dr. Jiang, Guo, Zhang, Li Wang and Prof. Mao were responsible for the writing-review and editing of the paper. All the authors read and approved the final manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

GH	glycoside hydrolases
LB	Luria−Bertani
IPTG	isopropyl-*β*-D-thiogalactopyranoside
NCBI	National Center for Biotechnology Information
BLAST	Basic Local Alignment Search Tool

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