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BIODEGRADATION

A Novel Esterase, $DacA_{pva}$, from *Comamonas* sp. Strain NyZ500 with Deacetylation Activity for the Acetylated Polymer Polyvinyl Alcohol

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ABSTRACT As a water-soluble polymer, the widely used polyvinyl alcohol (PVA) is produced from hydrolysis of polyvinyl acetate. Microbial PVA carbon backbone cleavage via a two-step reaction of dehydrogenation and hydrolysis has been well studied. The content of the acetyl group is a pivotal factor affecting performance of PVA derivatives in industrial applications, and deacetylation is a nonnegligible part of PVA degradation. However, the genetics and biochemistry of its deacetylation remain largely elusive. Here, *Comamonas* sp. strain NyZ500 was isolated for its capability of growing on acetylated PVA from activated sludge. The spontaneous PVA utilization-deficient mutant strain NyZ501 was obtained when strain NyZ500 was cultured in rich media. Comparative analysis between the genomes of these two strains revealed a fragment (containing a putative hydrolase gene, $dacA_{pva}$) deletion in strain NyZ501, and in the $dacA_{pva}$-complemented strain NyZ501 the ability to grow on PVA was restored. $DacA_{pva}$, which shares 21% identity with xylan esterase AxeA1 from *Prevotella ruminicola* 23, is a unique deacetylase catalyzing the conversion of acetylated PVA and its derivatives to deacetylated counterparts. This indicates that strain NyZ500 utilizes acetylated PVA via acetate as a carbon source to grow. $DacA_{pva}$ also possesses the ability to deacetylate acetylated xylan and the antibiotic intermediate 7-aminocephalosporanic acid (7ACA), but the enzymes responsible for the conversion of those two compounds have no activity against PVA derivatives. This study enhanced our understanding of the diversity of microbial degradation of PVA, and $DacA_{pva}$ characterized here is also a potential biocatalyst for the eco-friendly biotransformation of PVA derivatives and other acetylated compounds.

IMPORTANCE Water-soluble PVA, which possesses a very robust ability to accumulate in the environment, has a very grave environmental impact due to its widespread use in industrial and household applications. On the other hand, chemical transformation of PVA derivatives is currently being carried out under high-energy-consumption and high-pollution conditions using hazardous chemicals (such as NaOH and methanol) under high temperatures. The $DacA_{pva}$ reported here performs PVA deacetylation under mild conditions, so it has great potential to be developed into an eco-friendly biocatalyst for biotransformation of PVA derivatives. $DacA_{pva}$ also has deacetylation activity for compounds other than PVA derivatives, which facilitates its development into a broad-spectrum deacetylation biocatalyst for production of certain desired compounds.

KEYWORDS *Comamonas* sp. strain NyZ500, deacetylation, deacetylase, esterase, gene expression, polyvinyl alcohol, PVA

Polyvinyl alcohol (PVA) and its derivatives are widely used in the manufacturing of papers, textiles, adhesives, coatings, and membranes as well as in drug delivery

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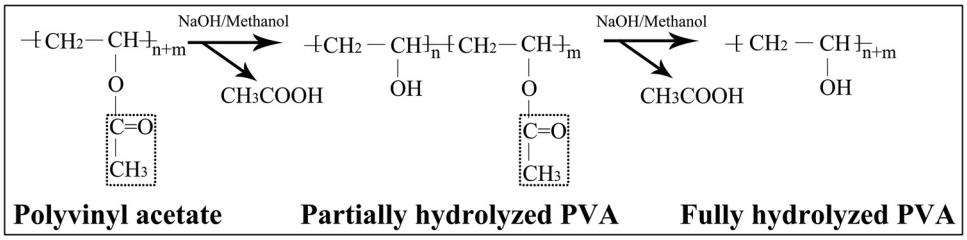

FIG 1 Chemical production of polyvinyl alcohol (PVA). PVA, a water-soluble polymer, is produced from polyvinyl acetate by hydrolysis. The partially or fully hydrolyzed PVA is produced under the control of the degree of polyvinyl acetate hydrolysis, which can be achieved by controlling the ratio of catalyst (NaOH), solvent (menthol), and substrate (polyvinyl acetate).

(1–3). Thousands of kilometric tons of PVA are produced annually (1). The countries in Asia (especially China and Japan) and Western Europe, as well as the United States, are the most significant contributors of PVA production and consumption (4). PVA is distinctly different from other polymers because of its water solubility (5, 6). Industrial-scale production of PVA was conducted by hydrolysis of polyvinyl acetate as shown in Fig. 1. PVA with different contents of acetyl groups was produced with the control set as the degree of polyvinyl acetate hydrolysis. The content of acetyl groups is an important factor affecting the physicochemical properties of PVA derivatives. A case in point is that fully hydrolyzed PVA (the degree of hydrolysis [DH] is about 98% to 99.8%) can only be dissolved in water at temperatures over 80°C, while partially hydrolyzed PVA can often be dissolved in water at lower temperatures (7). Another good example termed "stickies control" is used in the pulp and paper industry, where polyvinyl acetate serving as "stickies" is hydrolyzed to a less sticky form by removing the acetyl groups (8). Commonly used PVA derivatives and their property parameters are listed in Table 1.

PVA was considered a "green polymer" and truly biodegradable material because of its excellent hydrophilicity and bioavailability. Previous reports have clearly revealed the mechanism of bacterial PVA carbon backbone cleavage, which was accomplished by a two-step reaction (4, 9, 10). First, PVA was catalyzed by an oxidase (11, 12) or dehydrogenase (13–15) to form a breakpoint structure of β-diketone or β-hydroxyl ketone, and then the carbon backbone was cleaved by a β-diketone hydrolase (11, 16, 17) or aldolase (13) at the breakpoint to form fragmented organic ketone, aldehyde, or acid. The content of acetyl groups in PVA is one of the factors affecting its degradation (9, 10). It was generally considered that a content of less than 20% of acetyl groups has no significant effect on PVA degradation (10, 18). However, it has also been shown that PVA with 72% DH caused significant growth delay in PVA-degrading microorganisms compared to that with higher DH (19). A similar result was that PVA with high DH is better for growth of, and PVA-degrading enzyme production by, *Streptomyces venezuelae* GY1 compared with PVA with low DH (20). The presence of acetyl groups requires PVA-degrading microorganisms to possess specific hydrolytic enzymes to remove the acetyl groups. However, most reported PVA degraders do not have such enzymes, thus hampering its degradation (9). Interestingly, an esterase that functioned in PVA deacetylation was purified from PVA utilizer *Pseudomonas vesicularis* PD and 30 residues at its N terminus were determined (21). On the other hand, commercial cutinases (22) and an inefficient esterase from *Pseudomonas putida* mt-2 (23) also showed modest deacetylation activities toward polyvinyl acetate. So far, reports on biochemical characterization of deacetylation of PVA derivatives are extremely scarce, and no gene encoding such an enzyme was characterized. In this study, we initially aimed to isolate a bacterial strain capable of utilizing PVA via "C-C" backbone cleavage, but it turned out that the isolated PVA degrader *Comamonas* sp. strain NyZ500 was grown on acetate sourced from PVA hydrolysis catalyzed by a novel enzyme. We report here its characterization as an acetyl esterase with activity for deacetylation of partially hydrolyzed

TABLE 1 PVA and derivatives used in this study

PVA or derivative	Degree of polymerization	Degree of hydrolysis (%)[b]
Polyvinyl acetate	NA[a]	0
PVA1788	1,700	88
PVA1799	1,700	99
PVA105	500	99
PVAxx78	NA[a]	78

[a]NA, not available.
[b]Degree of hydrolysis (DH) is a parameter representing the content of acetyl groups (or hydroxyl groups) in PVA. The higher the hydrolysis degree is, the less acetyl groups (or the more hydroxyl groups) are in PVA.

PVA, its derivatives, and other acetyl compounds. It will enhance our understanding of microbial diversity in PVA degradation and may provide a potential biocatalyst for the conversion of other acetyl compounds.

RESULTS

Isolation and characterization of strain NyZ500 grown on PVA1788. Selective enrichment with PVA1788 (PVA derivatives and their property parameters are listed in Table 1) as the sole carbon source yielded an isolate designated strain NyZ500. Taxonomical classification based on its 16S rRNA gene sequence revealed that strain NyZ500 comes from the genus *Comamonas*, and it was identified as *Comamonas* sp. strain NyZ500, which was deposited to the China Center for Type Culture Collection (CCTCC); its catalog number is CCTCC M 2021114. This strain utilized acetylated PVA1788 and PVAxx78 for growth but was unable to grow with PVA105 or PVA1799, both with a higher degree of hydrolysis (99%) than PVA1788 (88%) and PVAxx78 (78%) (Fig. 2a). The sequenced draft genome (5.4 Mb) of strain NyZ500 consists of 66 scaffolds in total.

Spontaneous deletion of gene *dacA*$_{pva}$ in strain NyZ500 resulted in PVA1788 utilization deficiency. Generally, the degradation phenotypes of xenobiotic-metabolizing bacteria are not stable since many catabolic genes are part of mobile genetic elements (e.g., catabolic transposons), which facilitate interspecies or intraspecies transmissions of catabolic genes (24, 25). Consistent with the expectation, the phenotype of PVA1788 utilization in strain NyZ500 was unstable. One such mutant, which was designated strain NyZ501 and lost the ability to utilize PVA1788 for its growth (Fig. 2b), was obtained by continuous cultivation of wild-type strain NyZ500 on lysogeny broth (LB) medium. In order to explore the cause of its growth deficiency on PVA1788, all 14 annotated transposases in the genome of strain NyZ500 were screened against strain NyZ501 by PCR. A segment deletion in the genome of strain NyZ501 was observed compared to its locus in the chromosome of strain NyZ500. A scaffold that included an annotated putative hydrolase gene designated *dacA*$_{pva}$ and an adjacent IS*5* family transposase gene was lost in the genome of NyZ501. To further characterize the significance of the *dacA*$_{pva}$ gene to utilize PVA1788, gene *dacA*$_{pva}$ was transformed into strain NyZ501. The complementation of the *dacA*$_{pva}$ gene in strain NyZ501 restored its ability to utilize PVA1788 (Fig. 2b). In addition, gene *dacA*$_{pva}$ transcription levels were increased by 5 to 10 times when incubated with PVA1788 as well as other compounds with acetyl groups (xylan and 7-aminocephalosporanic acid [7ACA]), as revealed by reverse transcription-quantitative PCR (qRT-PCR) analysis. These results indicated that the *dacA*$_{pva}$ gene was essential for PVA1788 utilization in strain NyZ500.

DacA$_{pva}$ is a novel member of SGNH/GDSL family hydrolases. The GDSL/SGNH hydrolase family was featured as the presence of four strictly conserved residues, Ser-Gly-Asn-His, in four conserved blocks, I, II, III, and V, respectively (26, 27). Sequence analysis showed that the *dacA*$_{pva}$ gene consists of 1,284 bp and encodes a protein of 427 amino acids. A putative signal peptide was at the N terminus of DacA$_{pva}$, with the most likely cleavage site located between residues Gly$_{30}$ and Cys$_{31}$, theoretically resulting in a 397-residue mature protein. A BLASTp search against the nonredundant (nr) protein sequence database revealed that DacA$_{pva}$ exhibited moderate sequence

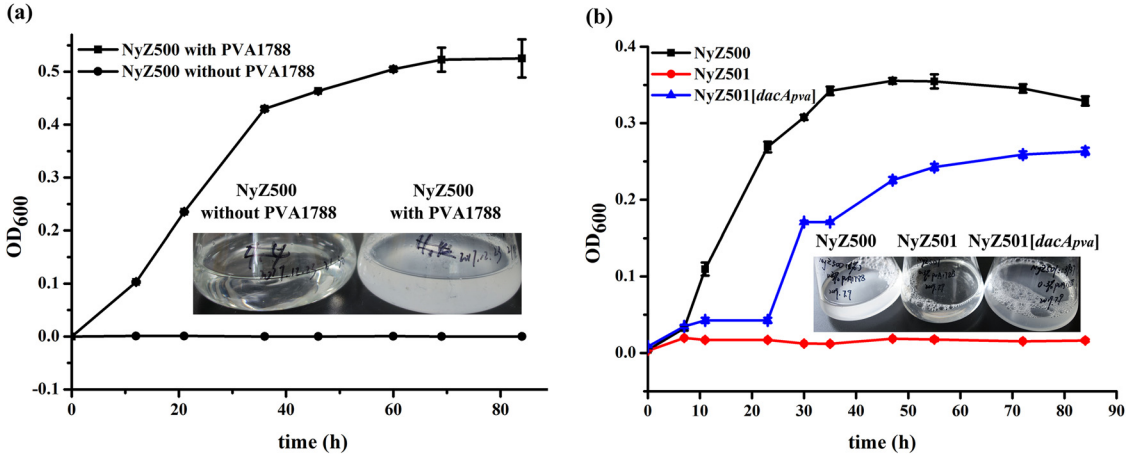

FIG 2 Growth curves of strains NyZ500, NyZ501, and *dacA*$_{pva}$-complemented NyZ501. (a) Strain NyZ500 was grown on PVA1788 as the sole carbon and energy source. (b) Complementation of the *dacA*$_{pva}$ gene in NyZ501 restored its ability to utilize PVA1788. Cultivation was carried out in 100 ml of liquid carbon-free basal medium (LCFBM) with 0.3% (wt/vol) PVA1788 serving as the carbon and energy source. Data points represent the mean values of triplicate trials, and error bars indicate standard deviations.

identity (highest identity is 61%) with many hypothetical SGNH/GDSL family proteins whose secondary structures are composed of a typical SGNH_hydro domain. A search for manually annotated and reviewed sequences in Swiss-Prot with the BLASTp program was unsuccessful because of low identity, and then a domain enhanced lookup time accelerated BLAST (DELTA-BLAST) was used instead to search for distant homologues. Among the functionally characterized proteins, DacA$_{pva}$ shared the highest identify (21%) with AxeA1 (GenPept accession number D5EV35.1), which catalyzes the hydrolysis of xylan analogs. Amino acid sequence alignment showed the presence of the conserved residues of SGNH/GDSL family hydrolases in DacA$_{pva}$, with Ser$_{227}$, Gly$_{267}$, Asn$_{303}$, and His$_{408}$ residing on blocks I, II, III, and V, respectively (Fig. 3a). Apart from containing a signature SGNH_hydro domain at the C terminus, a stretch of approximately 180 residues without evident domains was found to be situated on the N terminus of DacA$_{pva}$, but it is absent in all other characterized SGNH family hydrolases (Fig. 3b). The truncated DacA$_{pva}$ omitting this stretch was not successfully expressed (data not shown). Furthermore, phylogenetic analysis between DacA$_{pva}$ and other reported SGNH hydrolases showed that DacA$_{pva}$ occupied a distinct branch (Fig. 3c). Collectively, these analyses indicated that DacA$_{pva}$ seemed to be a novel member of SGNH/GDSL family hydrolases and that the N-terminal domain of DacA$_{pva}$ may be involved in protein scaffold construction and substrate specificity maintenance via interacting with the SGNH_hydro domain.

DacA$_{pva}$ catalyzes deacetylation of partially hydrolyzed PVA and polyvinyl acetate. Expressed recombinant DacA$_{pva}$ was purified from *Escherichia coli* BL21(DE3) as an N-terminally His$_6$-tagged fusion protein, and its purity and expected size (44 kDa) were confirmed by SDS-PAGE analysis. Deacetylation activity on PVA was characterized by measuring the amount of released acetate and acetyl groups that remained in PVA via high-performance liquid chromatography (HPLC) and Fourier transform infrared spectroscopy (FTIR), respectively. With the extension of the reaction period, the yield of acetate product was increased, while the substrate PVA was completely deacetylated within 40 min (Fig. 4) under conditions specified in Materials and Methods. The difference between the acetyl content of PVA and the value of acetate released is likely due to the different analytic methods applied. As expected, the insoluble substrate polyvinyl acetate was more difficult to be hydrolyzed than the soluble substrate PVA (specific activities of DacA$_{pva}$ for PVA1788, PVAxx78, and polyvinyl acetate were 272.79 $\pm$ 12.02 U/mg, 308.47 $\pm$ 12.25 U/mg, and 12.71 $\pm$ 2.71 U/mg, respectively), consistent with the fact that strain NyZ500 was incapable of growing on polyvinyl acetate. The possibility of inhibitors being present in polyvinyl acetate was excluded by

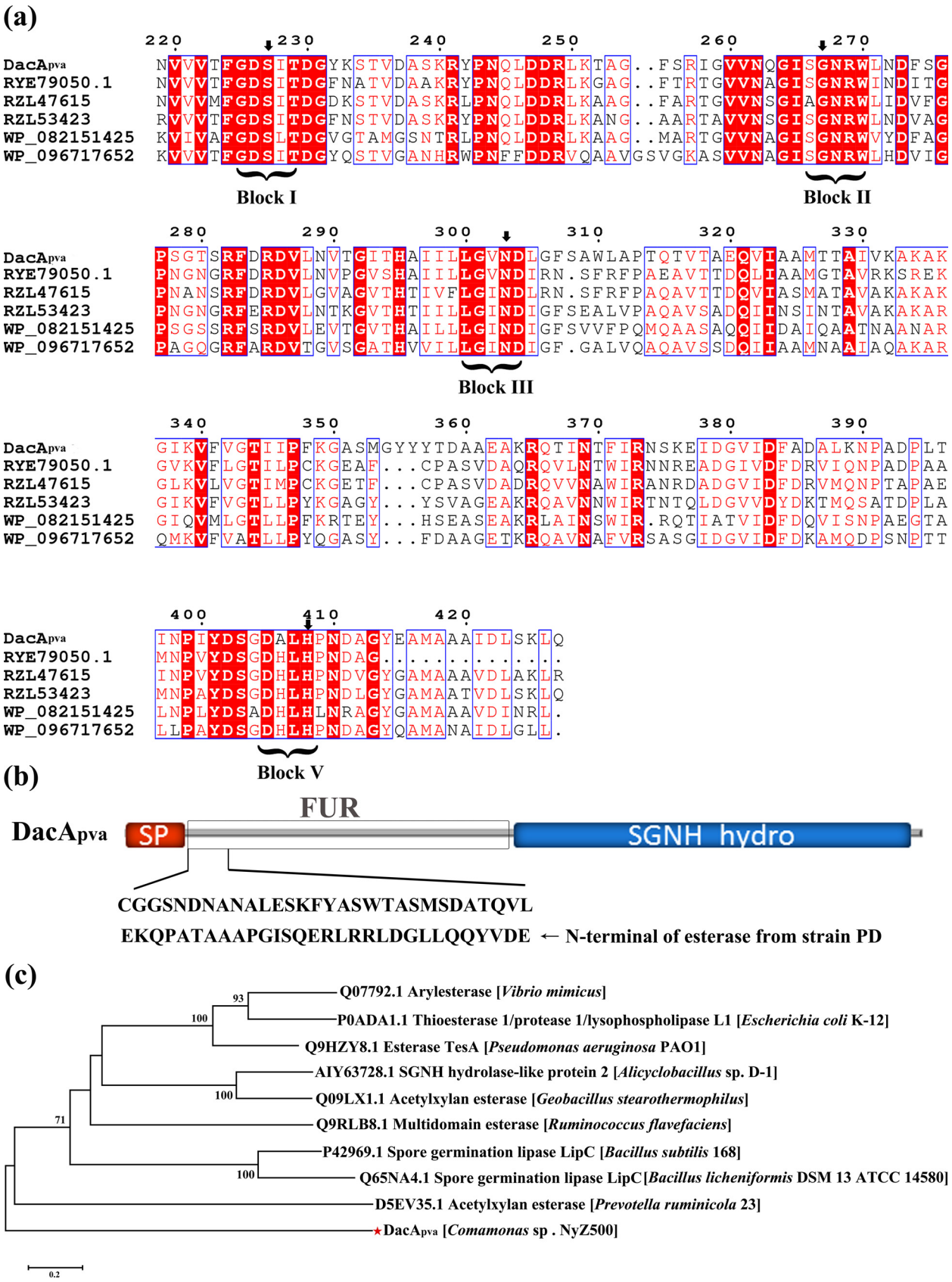

FIG 3 Sequence analysis of $DacA_{pva}$. (a) Amino acid sequence alignment of $DacA_{pva}$ with other proteins belonging to the SGNH/GDSL family. The sequences used for alignment were retrieved from NCBI, including proteins from *Oxalobacteraceae* bacterium (GenPept accession number RYE79050.1),

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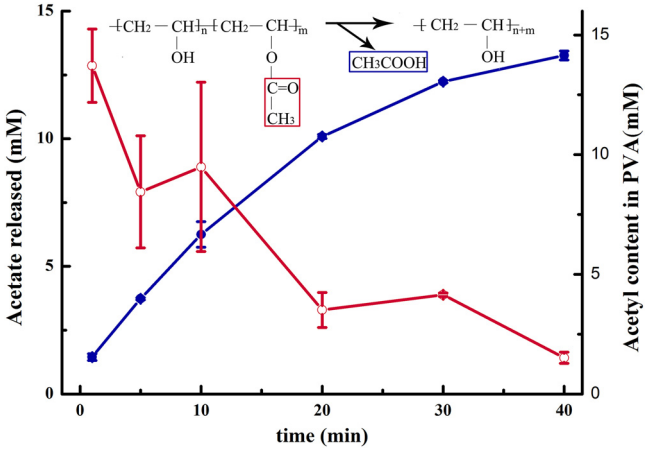

FIG 4 Time course of PVA1788 deacetylation by $DacA_{pva}$. $DacA_{pva}$ was added into 500 μl PVA1788 solution (3%, wt/vol) dissolved in PB buffer (100 mM, pH 7.4), and the reaction for the appropriate time was at 37°C; the reactions were stopped by boiling at 85°C for 10 min, and the released acetate (solid circle) from PVA in the reaction fluid was analyzed by HPLC. The acetyl content (empty circle) in PVA1788 was analyzed by FTIR (Fourier transform infrared spectroscopy) after drying thoroughly. Data points represent the mean values of triplicate trials, and error bars indicate standard deviations.

observing normal growth of strain NyZ500 after adding sodium acetate (2 mM) to the medium with polyvinyl acetate.

The substrate specificity of the purified $DacA_{pva}$ was examined with other acetylated compounds, including cellulose acetate, ethylene vinyl acetate (EVA), acetylated xylan, and 7ACA. Among these compounds, only acetylated xylan and 7ACA can be deacetylated by $DacA_{pva}$, as shown in Fig. 5. On the other hand, two identified SGNH hydrolases, EstD1 (GenBank accession number AIY63728.1) (28) and AxeA1 (GenBank accession number D5EV35.1) (29), closely related to $DacA_{pva}$ in the phylogenetic analysis (Fig. 3c), happened to be the active enzymes for 7ACA and acetylated xylan. Then, a comparative study of these three enzymes was performed to detect their activity against PVA and its derivatives. It turned out that only $DacA_{pva}$ was capable of deacetylating all PVA derivatives tested. However, neither AxeA1 nor EstD1 exhibited detectable activities toward the above PVA derivatives.

DISCUSSION

The global production of PVA and its environmental problems have been reviewed previously (28). Microbial degradation of PVA has been investigated in several PVA utilizers at the biochemical and molecular levels, but they are limited to degradation via carbon backbone cleavage through a two-step reaction of dehydrogenation and hydrolysis (9, 10). On the other hand, PVA with different contents of acetyl groups as a side chain is very common in industrial applications (4), and the presence of acetyl groups has been shown to give rise to an increase in the difficulty of PVA degradation to some extent (19, 20). However, studies on PVA deacetylation are limited. The esterase activities were only detected intracellularly from several PVA-utilizing strains (20,

FIG 3 Legend (Continued)

Variovorax sp. (GenPept accession numbers RZL47615 and RZL53423), *Caenimonas* sp. strain SL110 (WP_082151425), and *Pseudomonas acidophila* (WP_096717652). All the sequences were aligned by ClustalW and rendered with ESPript (38). The blue box highlights relatively conserved residues, and the red background describes absolutely conserved residues. Four conserved blocks, I, II, III, and V, in GDSL family proteins are bracketed, and four conserved residues, S-G-N-H, located in the four blocks are marked with black arrows. (b) Domain analyses of $DacA_{pva}$ showing an N-terminal signal peptide (SP), a C-terminal SGNH_hydro domain, and a large functionally unknown region (FUR) at the N terminus. The N-terminal amino acid sequences of $DacA_{pva}$ and esterase from *Pseudomonas vesicularis* strain PD are displayed for comparison. (c) Evolutionary relationship of $DacA_{pva}$ with its homologs. The evolutionary history was inferred using the neighbor-joining method (39). The bootstrap consensus tree inferred from 1,000 replicates represents the evolutionary history of the taxa analyzed (40). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary analyses were conducted in MEGA5 (41). The enzyme $DacA_{pva}$ characterized in this study is labeled with a star.

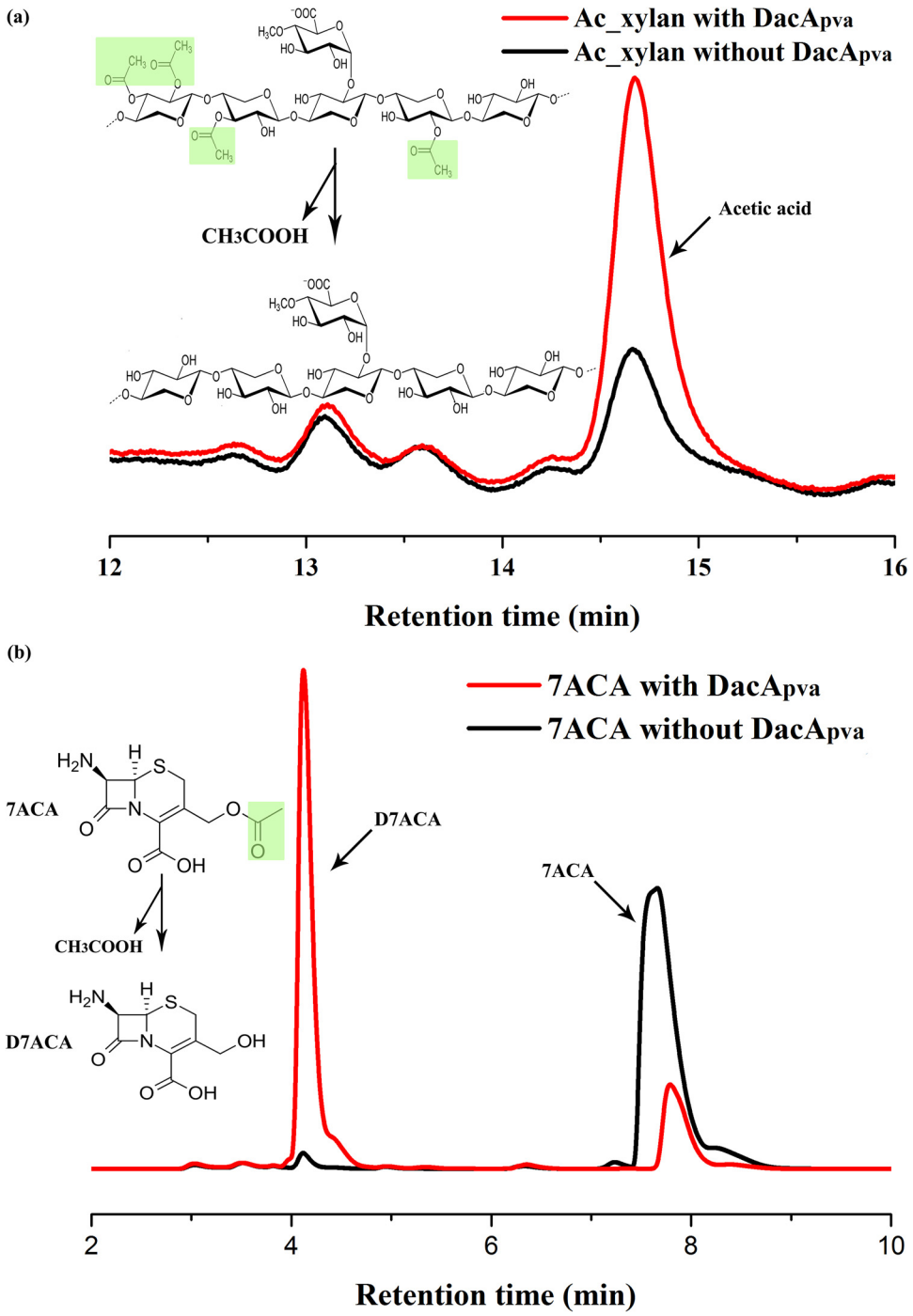

FIG 5 Deacetylation of acetylated xylan and 7ACA by $DacA_{pva}$. HPLC traces of the metabolites from $DacA_{pva}$-catalyzed deacetylation of acetylated xylan (Ac_xylan) (a) and 7ACA (b). Deacetylation of acetylated xylan was monitored by detection of acetate using HPLC. Substrate 7ACA and product D7ACA were analyzed by HPLC. The same reaction system without $DacA_{pva}$ served as a negative control for both reactions.

21), but there are no reports on the genes encoding enzymes involved in PVA deacetylation. Since it shares only 21% and 20% identity with its closest characterized homologs xylan esterase AxeA1 and 7ACA esterase EstD1, respectively, but has exclusive PVA deacetylation activity compared to its counterparts, $DacA_{pva}$ from *Comamonas* sp. strain NyZ500 in this study represents a novel esterase catalyzing the deacetylation of

PVA to produce acetate, which serves as the carbon source for the growth of this PVA utilizer.

Horizontal gene transfer (HGT) could circumvent the slow step of complete gene creation and accelerate genome innovation by rapidly introducing new genes into existing genomes (24, 29). To survive in an environment where PVA is abundant, one of the ubiquitous *Comamonas* strains (the predecessor of strain NyZ500) may have recruited $dacA_{pva}$ through HGT to liberate acetate from inert PVA as a carbon source for its growth, resulting in the birth of strain NyZ500. This is supported by the presence of an IS*5* family transposon adjacent to $dacA_{pva}$ and absence of homologs of $dacA_{pva}$ among available genomes of *Comamonas* strains.

So far, the only identified esterase specific for PVA deacetylation comes from PVA utilizer *Pseudomonas vesicularis* PD (21). For PVA deacetylation, this purified native enzyme showed a specific activity of 6.52 U/mg for PVA500 (0.5% [wt/vol]; DP, 500; DH, 86.5 to 89.0%) at 30°C. The corresponding activities of $DacA_{pva}$ for PVA1788 (3% [wt/vol]; DP, 1,700; DH, 88%) and PVAxx78 (3% [wt/vol]; DP, unknown; DH, 78%) were 272.79 and 308.47 U/mg, respectively, at 37°C. $DacA_{pva}$ reported here was more efficient than the esterase of *Pseudomonas vesicularis* PD at deacetylation of PVA. In contrast to the esterase which was localized in the cytoplasm of strain PD (21), $DacA_{pva}$ was predicted to function in the periplasmic space. Furthermore, the available sequence of 30 amino acids at the N terminus of purified esterase from strain PD significantly differs from its counterpart of $DacA_{pva}$, as shown in Fig. 3b. Despite both having deacetylation activity against PVA, the differences in their subcellular locations, catalytic activities, and N-terminal sequences clearly indicate that the esterases from strains PD and $DacA_{pva}$ are not the same deacetylase.

$DacA_{pva}$ identified in this study is also a promising biocatalyst for the deacetylation of PVA derivatives and other acetylated compounds which are useful in industrial applications (8, 30). In addition to the efficient deacetylation of PVA derivatives, $DacA_{pva}$ also exhibits deacetylation activity toward other acetylated compounds, such as acetyl xylan and 7ACA. Its property of extended activity is favorable for its development as a potential broad-spectrum deacetylation biocatalyst. On the other hand, $DacA_{pva}$ also holds promise for conversion of polyvinyl acetate to PVA with various degrees of hydrolysis under mild reaction conditions, but with the drawback of limited ability to catalyze the water-insoluble substrate polyvinyl acetate. This could be improved by protein engineering (31). The advantage of enzymatic conversion of PVA and its derivatives is that it could bypass the use of toxic chemicals and reduce energy consumption, generating an eco-friendly conversion route.

MATERIALS AND METHODS

Chemicals, media, plasmids, primers, and bacterial strains used in this study. Polyvinyl acetate was purchased from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China, and its average molecular weight is 30 to 50 kDa. PVA with different degrees of polymerization and hydrolysis (listed in Table 1) is from Aladdin Industrial Corporation, Shanghai, China. Acetylated xylan from corn cob is from Meryer Chemical Technology Co., Ltd. Shanghai, China. The chemicals 7-aminocephalosporanic acid (7ACA) and 7-amino-deacetylcephalosporanic acid (D7ACA) are from Bide Pharmatech Ltd., Shanghai, China.

Lysogeny broth (LB) (32) and liquid carbon-free basal medium (LCFBM) (33) were prepared as previously reported, and their corresponding agar media were prepared by adding 1.5% (wt/vol) agar. Plasmids, primers, and bacterial strains used in this study are listed in Table 2.

Isolation of PVA1788 degrader. Activated sludge from sewage treatment plants was added into LCFBM containing 0.3% (wt/vol) PVA1788 and served as an enrichment medium, which was incubated at 30°C with shaking at 180 rpm. A fraction of turbid culture was subcultured into fresh medium as above for second-round enrichment. After three rounds of such enrichments, a PVA degrader was obtained by spreading the enrichment culture on agar LCFBM containing 0.3% PVA1788 (wt/vol), and an emerged pure colony was subjected to taxonomic classification based on its 16S rRNA gene amplified using universal primers 27F and 1492R (34).

Draft genome sequencing of PVA degrader. A single colony of PVA degrader strain NyZ500 was cultured with 100 ml LB medium plus 0.3% (wt/vol) PVA1788 until the optical density at 600 nm (OD_{600}) reached 0.8. The cells were washed and harvested for genome extraction before being sequenced using an Illumina HiSeq 4000 system (Illumina, San Diego, CA, USA) at the Beijing Genomics Institute.

Acquisition of a PVA1788 utilization mutant derived from strain NyZ500. A spontaneous mutant (designated strain NyZ501) deficient in PVA1788 utilization was obtained when strain NyZ500 was

TABLE 2 Plasmid, primers, and bacterial strains used in this study

Plasmid, primer, or strain	Description or sequence (5′ to 3′)	Source or reference no.
Plasmids		
pET-28a(+)	Overexpression vector for *E. coli*, Km^r	Novagen
pBBR1MCS-2	Broad-host-range vector, Km^r	42
Primers		
F(*dacA*$_{pva\text{-}MCS}$)	AGGGAACAAAAGCTGGGTACCGATGTATAAACTAAAGCCCAATCCATTT	This study
R(*dacA*$_{pva\text{-}MCS}$)	CAGGAATTCGATATCAAGCTTCTATTGTAACTTGCTAAGATCAATTGCC	This study
F(*dacA*$_{pva\text{-}pET}$)	GTGCCGCGCGGCAGCCATATGGGAAGCAACGATAACGCCAA	This study
R(*dacA*$_{pva\text{-}pET}$)	ACGGAGCTCGAATTCGGATCCCTATTGTAACTTGCTAAGATCAATTGCC	This study
F(16S-qPCR)	AGCAACTAATGGCAAGGG	This study
R(16S-qPCR)	GCGGTGGATGATGTGGT	This study
F(*dacA*$_{pva}$-qPCR)	AAACTAAAGCCCAATCCA	This study
R(*dacA*$_{pva}$-qPCR)	GGTCGCATCAGACATCG	This study
Bacterial strains		
E. coli DH5a	*supE44* Δ*lacU169* (*φ80dlacZ*ΔM15) *hsdR17 recA1 endA1 thi-1 gyrA96 relA1*	Novagen
E. coli BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r$_B^-$m$_B^-$) *gal* (*λcI*857 *ind1 Sam7 nin5 lac* UV5-T7 gene 1) *dcm* (DE3)	Novagen
Comamonas sp. strain NyZ500	Wild type, PVA1788$^+$	This study
Comamonas sp. strain NyZ501	Spontaneous mutant of strain NyZ500, PVA1788$^-$	This study
Comamonas sp. strain NyZ501 (*dacA*$_{pva}$)	Strain NyZ501 complemented with *dacA*$_{pva}$ gene with pBBR1MCS-2	This study

cultured on an LB plate. Considering that spontaneous mutations are most likely caused by transposition, all annotated transposase-encoding genes in the genome of strain NyZ500 were screened by PCR to locate the possible mutation site in strain NyZ501. A fragment containing such a mutation site, as well as its flanking sequence, was PCR amplified from strain NyZ500 and sequenced to reveal the genes spontaneously deleted.

Complementation of PVA1788 utilization-deficient mutant strain NyZ501 with *dacA*$_{pva}$. The entire *dacA*$_{pva}$ gene was amplified from the strain NyZ500 genome with primers F(*dacA*$_{pva\text{-}MCS}$) and R (*dacA*$_{pva\text{-}MCS}$) (listed in Table 2) and then fused into pBBR1MCS-2 by using a One Step cloning kit (Vazyme, Nanjing, China). The sequence-validated plasmid was introduced into mutant strain NyZ501 by electrotransformation with 2.5 kV. The positive transformants were screened on LB plates with kanamycin (50 μg/ml), and the obtained recombinant strain was subjected to a growth test on PVA1788.

Bioinformatics analysis of DacA$_{pva}$. Functional prediction of DacA$_{pva}$ was conducted by BLASTp against nonredundant protein sequences (nr) and Swiss-Prot databases. Manually curated protein sequences were aligned and a phylogenetic tree constructed by MEGA 5.0 using the neighbor-joining method (1,000 bootstrap replicates). Multiple-sequence alignment was also used to analyze the protein signatures with DNAman software. Signal peptides and subcellular localization of DacA$_{pva}$ were predicted with SignalP 5.0 (35) and the Gneg-mPLoc (36) Web server, respectively.

Expression and purification of DacA$_{pva}$. The *dacA*$_{pva}$ gene-omitted nucleic acid sequence corresponding to signal peptides was amplified from strain NyZ500 genome with primers F(*dacA*$_{pva\text{-}pET}$) and R (*dacA*$_{pva\text{-}pET}$) (listed in Table 2) and then fused into pET-28a(+) (digested with NdeI and BamHI) to produce His$_6$-DacA$_{pva}$ overexpression plasmid pET-28a(+)-*dacA*$_{pva}$ by using a One Step cloning kit. The sequence-validated plasmid was transformed into *E. coli* BL21(DE3) by a standard procedure (37). The generated strain BL21(DE3)[pET-28a(+)-*dacA*$_{pva}$] was cultured in LB with kanamycin (50 μg/ml) at 180 rpm and 37°C until an OD$_{600}$ of 0.6 was reached and then induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 150 rpm and 16°C overnight. After being washed and resuspended with PB buffer (100 mM, pH 7.4; 200 mM NaCl, 10% [vol/vol] glycerol) following ultrasonic fragmentation, the cell extracts were obtained through centrifugation at 13,000 $\times$ *g* at 4°C for 40 min and then filtered with 0.45-μm filter membranes. Protein purification was conducted using the ÄKTA start system (GE Healthcare) equipped with a 5-ml HisTrap HP column (GE Healthcare). The recombinant His$_6$-DacA$_{pva}$ was eluted with 250 mM imidazole dissolved in PB buffer, and imidazole was then removed from the protein solution through an ultrafiltration tube. The resulting recombinant His$_6$-DacA$_{pva}$ was assessed by SDS-PAGE, and the concentration was measured by a Nano-300 spectrophotometer (Allsheng Instruments Co., Ltd. Hangzhou, China). The proteins AxeA1 and EstD1 were expressed and purified with the same methods.

HPLC analysis of reaction products. The HPLC (Waters) equipped with an organic acid analysis column (Aminex HPX-87H, 300 by 7.8 mm, 9 μm; Bio-Rad) was used to analyze the acetate produced by deacetylation of PVA derivatives and acetylated xylan. Ten microliters of sample was injected and analyzed after filtration with a 0.22-μm filter membrane. The single mobile phase of 5 mM H_2SO_4 was used to elute products with a flow rate of 0.6 ml/min at 50°C. The detection wavelength was 210 nm. Under these conditions, the retention time of acetate was 14.80 min.

The conversion of 7ACA to D7ACA was measured using HPLC equipped with a C_{18} column (Zorbax SB-C_{18}, 250 $\times$ 4.6 mm, 5 μm; Agilent). Ten microliters of sample was injected and analyzed by isocratic elution with a mobile phase consisting of 20 mM sodium acetate (pH 5.5) and acetonitrile (93:7, vol/vol)

at a flow rate of 0.5 ml/min and 30°C. The detection wavelength was 254 nm. Under these conditions, the retention times of 7ACA and D7ACA were 7.64 min and 4.14 min, respectively.

Enzyme activity assay. The substrates PVA1788, PVAxx78, acetylated xylan, and 7ACA were dissolved in PB buffer (100 mM, pH 7.4) with concentrations of 3% (wt/vol), PVA1788 and PVAxx78, 10% (wt/vol) acetylated xylan, and 5 mM 7ACA. The reaction was started by adding 14 μg $DacA_{pva}$ into 500 μl of each substrate at 37°C and maintained for appropriate reaction times. The samples were acidified by adding H_2SO_4 at a final concentration of 5 mM and boiled at 85°C for 10 min to stop the reaction before being subjected to HPLC analysis. The same reaction system was used without enzyme as a control. For 7ACA deacetylation, 0.60 μg $DacA_{pva}$ was used instead to slow down the reaction, and the boiling step was omitted for 7ACA due to its instability at high temperature. For water-insoluble polyvinyl acetate, 28 μg $DacA_{pva}$ and 20 mg polyvinyl acetate (average molecular weight was 30 to 50 kDa) were added into 500 μl PB buffer to initiate the reaction; the rest of the procedures were the same as for other substrates mentioned above. One unit of activity was defined as the amount of enzyme required to produce 1 μmol acetate per minute at 37°C.

FTIR analysis of acetyl contents in PVA. Acetyl group contents in PVA were calculated from the ratio of absorbances at wavenumbers of 1,251 cm^{-1} and 1,093 cm^{-1} in FTIR spectra by a method reported previously (21).

qRT-PCR. Cells of strain NyZ500 was cultured on LB agar plates at 30°C overnight, and a formed single colony was inoculated into fresh LB medium until an OD_{600} of 0.6 was reached. Then, the cells were washed and inoculated into 5-ml volumes of LCFBM containing sodium succinate (2 mM), 7ACA (2 mM), acetylated xylan (0.3%, wt/vol), or PVA1788 (0.3%, wt/vol). After incubation at 30°C and 180 rpm for 4.5 h, 3 ml cells was harvested and their RNAs extracted using RNA isolation kit (Sangon Biotech, Shanghai, China). After measurement of the concentration of isolated RNA using a microplate reader (Epoch 2; BioTek, USA), an equal amount of RNA (540 ng) for each sample was reverse transcribed to cDNA using a reverse transcription kit (HiScript III RT SuperMix for qPCR; Vazyme). The 16S rRNA gene from strain NyZ500 served as an internal reference, and the primers for the 16S rRNA gene and $dacA_{pva}$ gene are listed in Table 2. The qRT-PCR proceeded per the instructions of TB Green Premix Ex Taq kit (TaKaRa) using a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA).

Data availability. The draft genome (accession number JAEIJE000000000) of strain NyZ500 containing $dacA_{pva}$ and the 16S rRNA gene (accession number MW356895) have been deposited in GenBank.

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REFERENCES

1. Aslam M, Kalyar MA, Raza ZA. 2018. Polyvinyl alcohol: a review of research status and use of polyvinyl alcohol based nanocomposites. Polym Eng Sci 58:2119–2132. https://doi.org/10.1002/pen.24855.
2. Kim JW, Chang JH. 2020. Syntheses of colorless and transparent polyimide membranes for microfiltration. Polymers (Basel) 12:1610. https://doi.org/10.3390/polym12071610.
3. Hosseini MS, Nabid MR. 2020. Synthesis of chemically cross-linked hydrogel films based on basil seed (*Ocimum basilicum* L.) mucilage for wound dressing drug delivery applications. Int J Biol Macromol 163:336–347. https://doi.org/10.1016/j.ijbiomac.2020.06.252.
4. Amann M, Minge O. 2012. Biodegradability of poly(vinyl acetate) and related polymers. Synth Biodegradable Polym 245:137–172. https://doi.org/10.1007/12_2011_153.
5. Hallensleben ML, Fuss R, Mummy F. 2000. Polyvinyl compounds, others, p 1–23. *In* Ullmann's encyclopedia of industrial chemistry. Wiley-VCH, Weinheim, Germany.
6. Barui A. 2018. Synthetic polymeric gel, p 55–90. *In* Pal K, Banerjee I (ed), Polymeric gels: characterization, properties and biomedical applications. Woodhead Publishing, Elsevier, Cambridge, UK. https://doi.org/10.1016/b978-0-08-102179-8.00003-x.
7. Fan Q. 2008. Fabric chemical testing, p 125–147. *In* Hu J (ed), Fabric testing. Woodhead Publishing, Elsevier, Cambridge, UK. https://doi.org/10.1533/9781845695064.125.
8. Skals PB, Krabek A, Nielsen PH, Wenzel H. 2008. Environmental assessment of enzyme assisted processing in pulp and paper industry. Int J Life Cycle Assess 13:124–132. https://doi.org/10.1065/lca2007.11.366.
9. Fusako K, Xiaoping H. 2009. Biochemistry of microbial polyvinyl alcohol degradation. Appl Microbiol Biotechnol 84:227–237. https://doi.org/10.1007/s00253-009-2113-6.
10. Chiellini E, Corti A, D'Antone S, Solaro R. 2003. Biodegradation of poly (vinyl alcohol) based materials. Prog Polym Sci 28:963–1014. https://doi.org/10.1016/S0079-6700(02)00149-1.
11. Sakai K, Hamada N, Watanabe Y. 1986. Studies on the poly(vinyl alcohol) degrading enzyme. Part VI. Degradation mechanism of poly(vinyl alcohol) by successive reactions of secondary alcohol oxidase and β-diketone hydrolase from *Pseudomonas* sp. Agric Biol Chem 50:989–996. https://doi.org/10.1271/bbb1961.50.989.
12. Suzuki T. 1976. Purification and Some properties of polyvinyl alcohol-degrading enzyme produced by *Pseudomonas* O-3. Agric Biol Chem 40:497–504. https://doi.org/10.1080/00021369.1976.10862089.
13. Matsumura S, Tomizawa N, Toki A, Nishikawa K, Toshima K. 1999. Novel poly(vinyl alcohol)-degrading enzyme and the degradation mechanism. Macromolecules 32:7753–7761. https://doi.org/10.1021/ma990727b.
14. Shimao M, Tamogami T, Nishi K, Harayama S. 1996. Cloning and characterization of the gene encoding pyrroloquinoline quinone-dependent poly(vinyl alcohol) dehydrogenase of *Pseudomonas* sp. strain VM15C. Biosci Biotechnol Biochem 60:1056–1062. https://doi.org/10.1271/bbb.60.1056.
15. Wei Y, Fu J, Wu J, Jia X, Zhou Y, Li C, Dong M, Wang S, Zhang J, Chen F. 2017. Bioinformatics analysis and characterization of highly efficient polyvinyl alcohol (PVA)-degrading enzymes from the novel PVA degrader *Stenotrophomonas rhizophila* QL-P4. Appl Environ Microbiol 84:e01898-17. https://doi.org/10.1128/AEM.01898-17.
16. Shimao M, Tamogami T, Kishida S, Harayama S. 2000. The gene *pvaB* encodes oxidized polyvinyl alcohol hydrolase of *Pseudomonas* sp. strain VM15C and forms an operon with the polyvinyl alcohol dehydrogenase gene *pvaA*. Microbiology 146:649–657. https://doi.org/10.1099/00221287-146-3-649.
17. Klomklang W, Tani A, Kimbara K, Mamoto R, Ueda T, Shimao M, Kawai F. 2005. Biochemical and molecular characterization of a periplasmic hydrolase for oxidized polyvinyl alcohol from *Sphingomonas* sp. strain 113P3. Microbiology (Reading) 151:1255–1262. https://doi.org/10.1099/mic.0.27655-0.
18. Suzuki T, Ichihara Y, Yamada M, Tonomura K. 1973. Some characteristics of *Pseudomonas* O-3 which utilizes polyvinyl alcohol. Agric Biol Chem 37:747–756. https://doi.org/10.1271/bbb1961.37.747.

19. Corti A, Solaro R, Chiellini E. 2002. Biodegradation of poly(vinyl alcohol) in selected mixed microbial culture and relevant culture filtrate. Polym Degrad Stab 75:447–458. https://doi.org/10.1016/S0141-3910(01)00247-6.
20. Zhang Y, Du G, Fan X, Chen J. 2008. Effects and statistical optimization of fermentation conditions on growth and poly(vinyl alcohol)-degrading enzyme production of *Streptomyces venezuelae* GY1. Biocatal Biotransformation 26:430–436. https://doi.org/10.1080/10242420802364957.
21. Sakai K, Fukuba M, Hasui Y, Moriyoshi K, Ohmoto T, Fujita T, Ohe T. 1998. Purification and characterization of an esterase involved in poly(vinyl alcohol) degradation by *Pseudomonas vesicularis* PD. Biosci Biotechnol Biochem 62:2000–2007. https://doi.org/10.1271/bbb.62.2000.
22. Ronkvist AM, Lu WH, Feder D, Gross RA. 2009. Cutinase-catalyzed deacetylation of poly(vinyl acetate). Macromolecules 42:6086–6097. https://doi.org/10.1021/ma900530j.
23. Millar R, Rahmanpour R, Yuan EWJ, White C, Bugg TDH. 2017. Esterase EstK from *Pseudomonas putida* mt-2: an enantioselective acetylesterase with activity for deacetylation of xylan and poly(vinylacetate). Biotechnol Appl Biochem 64:803–809. https://doi.org/10.1002/bab.1536.
24. McAdams HH, Srinivasan B, Arkin AP. 2004. The evolution of genetic regulatory systems in bacteria. Nat Rev Genet 5:169–178. https://doi.org/10.1038/nrg1292.
25. Di Gioia D, Peel M, Fava F, Wyndham RC. 1998. Structures of homologous composite transposons carrying *cbaABC* genes from Europe and North America. Appl Environ Microbiol 64:1940–1946. https://doi.org/10.1128/AEM.64.5.1940-1946.1998.
26. Kim Y, Ryu BH, Kim J, Yoo W, An DR, Kim B-Y, Kwon S, Lee S, Wang Y, Kim KK, Kim TD. 2017. Characterization of a novel SGNH-type esterase from *Lactobacillus plantarum*. Int J Biol Macromol 96:560–568. https://doi.org/10.1016/j.ijbiomac.2016.12.061.
27. Akoh CC, Lee GC, Liaw YC, Huang TH, Shaw JF. 2004. GDSL family of serine esterases/lipases. Prog Lipid Res 43:534–552. https://doi.org/10.1016/j.plipres.2004.09.002.
28. Julinová M, Vaňharová L, Jurča M. 2018. Water-soluble polymeric xenobiotics–polyvinyl alcohol and polyvinylpyrrolidon–and potential solutions to environmental issues: a brief review. J Environ Manage 228:213–222. https://doi.org/10.1016/j.jenvman.2018.09.010.
29. Daubin V, Szöllősi GJ. 2016. Horizontal gene transfer and the history of life. Cold Spring Harb Perspect Biol 8:a018036. https://doi.org/10.1101/cshperspect.a018036.
30. Nuttelman CR, Henry SM, Anseth KS. 2002. Synthesis and characterization of photocrosslinkable, degradable poly(vinyl alcohol)-based tissue engineering scaffolds. Biomaterials 23:3617–3626. https://doi.org/10.1016/s0142-9612(02)00093-5.
31. Biundo A, Ribitsch D, Guebitz GM. 2018. Surface engineering of polyester-degrading enzymes to improve efficiency and tune specificity. Appl Microbiol Biotechnol 102:3551–3559. https://doi.org/10.1007/s00253-018-8850-7.
32. Bertani G. 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. J Bacteriol 62:293–300. https://doi.org/10.1128/JB.62.3.293-300.1951.
33. Yang J, Yang Y, Wu W-M, Zhao J, Jiang L. 2014. Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms. Environ Sci Technol 48:13776–13784. https://doi.org/10.1021/es504038a.
34. Lane D. 1991. 16S/23S rRNA sequencing, p 115–175. *In* Stackebrandt E, Goodfellow M (ed), Nucleic acids techniques in bacterial systematics. John Wiley and Sons, New York, NY.
35. Almagro Armenteros JJ, Tsirigos KD, Sonderby CK, Petersen TN, Winther O, Brunak S, von Heijne G, Nielsen H. 2019. SignalP 5.0 improves signal peptide predictions using deep neural networks. Nat Biotechnol 37:420–423. https://doi.org/10.1038/s41587-019-0036-z.
36. Shen HB, Chou KC. 2010. Gneg-mPLoc: a top-down strategy to enhance the quality of predicting subcellular localization of Gram-negative bacterial proteins. J Theor Biol 264:326–333. https://doi.org/10.1016/j.jtbi.2010.01.018.
37. Sambrook J, Fritsch EF, Maniatis T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
38. Gouet P, Courcelle E, Stuart DI, Metoz F. 1999. ESPript: analysis of multiple sequence alignments in PostScript. Bioinformatics 15:305–308. https://doi.org/10.1093/bioinformatics/15.4.305.
39. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454.
40. Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791. https://doi.org/10.1111/j.1558-5646.1985.tb00420.x.
41. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28:2731–2739. https://doi.org/10.1093/molbev/msr121.
42. Kovach ME, Phillips RW, Elzer PH, Roop RM, II, Peterson KM. 1994. pBBR1MCS: a broad-host-range cloning vector. Biotechniques 16:800–802.