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Carboxylic ester hydrolases from Antarctic psychrophilic *Psychrobacter* strains: From genome prospecting to biotreatment of polyester plastics

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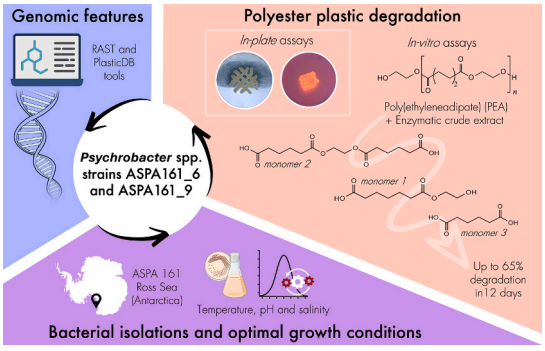
HIGHLIGHTS

- Two new *Psychrobacter* strains were isolated from Terra Nova Bay, Antarctica.
- Genomes perspecting for polyester material degradation.
- Up to 65 % degradation of PEA with averaged M_n 1000 was achieved in 12 days.
- Cold-active enzymes offer promising tools for sustainable plastic biotreatment.
- First report on isolated plastic-degrading bacteria from Antarctic Specially Protected Areas.

GRAPHICAL ABSTRACT



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ABSTRACT

Background: Antarctica can serve as the source of novel extremophilic microorganisms and biomolecules, including extremozymes which could find applications in bioremediation processes for the presence of enzymes able to degrade plastic polymers. Carboxylic ester hydrolases (EC 3.1.1) catalyze the hydrolysis of linear and cyclic ester bonds. Among these, esterases (EC 3.1.1.1) and triacylglycerol lipases (EC 3.1.1.3) have been shown effective in degrading polyester polymers.

Abbreviations: ANI, average nucleotide identity; ASPA, Antarctic Specially Protected Area; ASPAs, Antarctic Specially Protected Areas; CAPS, 3-(cyclohexylamino) propane-1-sulfonic acid; CEH, carboxylic ester hydrolases; dDDH, digital DNA–DNA hybridization; GBDP, Genome BLAST Distance Phylogeny; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid; MES, 2-morpholinoethanesulfonic acid; M_n, molar mass; MOPS, 3-morpholinopropanesulfonic acid; PBS, polybutylene succinate; PCL, polycaprolactone; PCR, polymerase chain reaction; PE, polyethylene; PEA, polyethylene adipate; PEG, polyethylene glycol; PET, polyethylene terephthalate; PETase, poly(ethylene terephthalate) hydrolase; PHA, polyhydroxyalkanoate; PHB, polyhydroxybutyrate; PLA, polylactic acid; PlasticDB, Plastic Biodegradation Database; *p*NP, *p*-nitrophenyl; PU, polyurethane; RASTtk, RAST tool kit; TOC, total organic content; TSB, tryptic soy broth; TYGS, Type (Strain) Genome Server.

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Polyester depolymerization
Bioplastic degradation
Polyethylene adipate

Investigation and methodologies: Two psychrophilic bacterial strains ASPA161_6 and ASPA161_9 were isolated from environmental samples, and their optimal growth conditions were determined. The marine sediment samples were collected at a depth of 20 m in the Antarctic Specially Protected Area n. 161 (74°42′ S, 164°07′ E, Ross Sea) during the 2017/2018 XXXIII Italian Antarctic expedition. Genomes were sequenced, assembled and annotated. Genes encoding for proteins involved in cold adaptation were searched with an *in-silico* search approach. Moreover, the sequences of genes involved in the degradation of polyester plastics were studied through the annotation via PlaticDB tool. Esterase and lipase activities were also detected *in-plate* and quantitatively evaluated at different temperatures on crude protein extracts from the extracellular fraction of the microorganisms using a specific assay based on the hydrolysis of *p*-nitrophenyl esters. Furthermore, the same fractions were tested onto the polymer polyethylene adipate as a model of biodegradable polyester plastic. The degradation studies with polyethylene adipate were performed following the reaction progress with ^{1}H NMR.
Results and discussion: Based on digital DNA–DNA hybridization and average nucleotide identity values, the two new isolates were assigned to the *Psychrobacter* genus. A comprehensive annotation using PlasticDB revealed substantial degree of similarity in the gene sequences coding for a variety of plastic-degrading enzymes, particularly carboxylic ester hydrolases. However, some differences were observed in the enzymatic profiles of the two bacterial strains, highlighting their versatility and potential for effective biodegradation of a range of plastic polymers. Subsequently, carboxylic ester hydrolases were detected with qualitative *in-plate* assays with olive oil and tributyrin as substrates. The enzymatic activity of psychrotolerant extracellular esterases and lipases was quantified using chromogenic-spectrophotometric assays. The presence of esterases was assayed against *p*NP-acetate, and for both strains an optimal activity was detected at 35 °C (0.5 and 0.8 nmol *p*NP released/mg protein in 30 min for strain ASPA161_6 and strain ASPA161_9, respectively). A significant activity was maintained at lower temperatures, ranging from 50.1 and 71.8 % at 15 and 25 °C for strain ASPA161_6 and from 49.6 and 68.1 % for strain ASPA161_9. These results suggested psychrotolerant characteristics of these enzymes. The presence of lipases was determined with *p*NP-dodecanoate, resulting in 2.6 and 3.5 nmol *p*NP released/mg protein at 25 °C in 30 min for the two strains. Most of the activity was retained at 25 °C (73.4 and 66.3 %, respectively), with a decrease at 15 °C (45.3 and 38.6 %), which may be attributed to the reduced kinetic activity. Finally, the degradation experiments with PEA with averaged molar mass 1000 g/mol as a model polyester plastic demonstrated a 55–65 % degradation rate after 12 days of incubation.
Conclusions: The obtained data underlined the ability of the investigated strains to express carboxylic ester hydrolases under the specified growth conditions. These findings highlight their biotechnological potential in bioremediation, particularly for sustainable plastic waste management in cold or temperate environments. Future investigations could focus on optimizing enzymatic activity through heterologous expression or utilizing whole-cell systems for direct polymer degradation.

1. Introduction

Extreme environments such as the poles are increasingly explored for novel microorganisms and biomolecules adapted to combined stresses like low temperatures, desiccation, nutrient scarcity, and solar radiation (Lo Giudice and Fani, 2015). Bioprospecting of cold-active bacteria and other extremophiles is a promising biotechnological frontier to discover extremozymes and bioproducts applicable to pharmaceutical, food, chemical, and cosmetic sectors, as well as for bioremediation purposes (da Silva et al., 2022; Finore et al., 2024). Cold-adapted enzymes from psychrophilic and psychrotolerant bacteria are efficient at low temperatures due to their structural flexibility and low activation energies (Liu et al., 2023).

Antarctic ecosystems are vulnerable to both local and global pressures, including human activity and the risk of invasive species (Burrows et al., 2023). Antarctic Specially Protected Areas (ASPAs) play a key role in minimizing contamination, also regulating scientific activities. Microbial communities in ASPAs have been studied for their ecological and biotechnological roles. Antarctic Specially Protected Area (ASPA) 161 samples revealed cold-active hydrocarbon-degrading bacteria (Lo Giudice et al., 2010), ASPA 165 showed bacteria involved in organic matter turnover (Papale et al., 2018), and ASPA 125 was studied for plastic-associated microbiomes (Cappello et al., 2021). These microbial consortia are potential sources of extracellular enzymes capable of polymer degradation into biodegradable fragments (De Santi et al., 2016), particularly cold-active carboxylic ester hydrolases (CEHs) relevant for low-temperature bioremediation (Wu et al., 2015).

CEHs catalyze ester bond hydrolysis, producing alcohols and carboxylic acids. Main classes include esterases and lipases (Chen et al., 2016). CHEs are ubiquitous to all domains of life, and bacterial CEHs play roles in nutrient assimilation and pathogenesis, with activity depending on substrate chain length (Oh et al., 2019). Lipases act at lipid-water interfaces despite being water-soluble (Hasan et al., 2009), and their industrial value lies in cofactor independence, broad substrate range, and chemo-/regioselectivity (Wu et al., 2015). Studies since the 1990s have advanced their isolation and characterization (de Pascale et al., 2009), with variable responses to culture conditions, salt, and pH.

Bacterial strains *Psychrobacter* spp. ASPA161_6 and ASPA161_9 (Di Donato et al., 2024) were isolated from marine sediment samples collected at 20 m depth in the Antarctic Specially Protected Area (ASPA) n. 161 (74°42′ S, 164°07′ E, Ross Sea) as part of the 2017/2018 XXXIII Italian Antarctic expedition (Semprucci et al., 2021).

Several *Psychrobacter* strains have been reported for their ability to produce CEHs, including esterases, triacylglycerol lipases, and generic lipases. Ou et al. (2024) identified *Psychrobacter* species in microplastic-rich sediments of Shenzhen Bay (China), suggesting their key role in plastic-degrading enzyme activity, supported by metagenomic analysis using KEGG pathway and enzyme databases. Multiple enzymes implicated in plastic biodegradation were detected, mainly oxidoreductases, hydrolases, transferases, ligases, lyases, and isomerases. Recently, Paun et al. (2024) cloned and characterized a cold-active lipase from *Psychrobacter* SC65A.3, isolated from the perennial ice of Scarisoava Cave (Romania), highlighting its biocatalytic role in silybin acetylation to improve bioavailability.

The main objective of this study was to address the pressing issue of plastic pollution by characterizing genomic and enzymatic potentials of two newly isolated Antarctic strains, *Psychrobacter* spp. ASPA161_6 and ASPA161_9. Given the limited data on cold-adapted microbial enzymes involved in polyester degradation, the work focused on identifying genes encoding carboxylic ester hydrolases. Genomic annotation was performed using the RAST server and the specialized PlasticDB to evaluate the strains' enzymatic capabilities.

To investigate enzymatic functionality, optimal growth parameters for both strains were established and enzymatic crude extracts were

Table 1
Metrics of the genome assemblies. Metrics of the genome assemblies for *Psychrobacter* spp. strains ASPA161_6 and ASPA161_9.

Assembly details	ASPA161_6	ASPA161_9
Genome length (bp)	3,506,154	3,343,950
GC content (%)	43.31	41.84
Contigs	23	26
Contig N50	771,101	370,592
Contig L50	2	3

recovered in these conditions. Subsequently, esterase and lipase activities were evaluated using qualitative *in-plate* assays and quantitative spectrophotometric methods to assess their activity profiles across a range of conditions. Enzymatic crude extracts were tested as biocatalysts in the hydrolytic degradation of low-molecular weight polyethylene adipate, chosen as a model substrate.

This paper represents the first study of isolation of plastic degrading microorganisms from an Antarctic Specially Protected Area of the continent since the previous papers described Antarctic bacterial communities and gene annotation involved in plastics biodegradation only by metagenomic approaches. By demonstrating the presence and activity of cold-active hydrolases in these strains, the study also expands the understanding of biodiversity and functional capabilities of psychrophilic microorganisms. Future applications could involve deploying these enzymes or microbial consortia in cold or temperate ecosystems for sustainable plastic waste management.

2. Materials and methods

2.1. General

Chemicals were purchased from Sigma-Aldrich (U.S.A.) and used as received. Dihydroxy terminated poly(ethyleneadipate) (PEA) with averaged molar mass (M_n) 1000 g/mol was purchased from Aldrich (Catalogue number 494933). ^{1}H- and ^{13}C NMR spectra were recorded on a Bruker Avance™ 400 instrument at 400.13 and 100.03 MHz respectively, and chemical shifts (δ) are relative to the residual solvent peak.

2.2. Sampling and culture conditions

The two bacterial strains studied in this work were indicated as strains ASPA161_6 and ASPA161_9 (Di Donato et al., 2024). They were isolated from marine sediment samples collected at 20 m depth in ASPA n. 161 (74°42′ S, 164°07′ E, Ross Sea) as part of the 2017/2018 XXXIII Italian Antarctic expedition. The physicochemical details of the sampling site were previously described (Semprucci et al., 2021). In general, these sediments appeared composed mainly by coarse sand with pH value of 8.33 and the presence of 5.7 mL/L of O_2. The presence of bivalent cations as Zn^{2+} (41.9 nM), Pb^{2+} (17.5 nM) and Cu^{2+} (50.17 nM) was found, and the total organic content (TOC) was estimated 1.7 % (Semprucci et al., 2021). Bacterial strains were stored as cellular pellets at −20 °C and as lyophilized pellets in 10 % (w/v) nonfat dried milk at room temperature. They are now part of the extremophilic bacterial collection of the Institute of Biomolecular Chemistry, part of the SUSMIRRI network (https://susmirri-catalog.di.unito.it/collections). The strains were maintained in liquid medium tryptic soy broth (TSB) at 15 °C, or solid medium obtained with the addition of agar 1.8 % (w/v). Sub-cultures on TSB at 15 °C for 24 h were prepared upon use.

Table 3
Search in the annotated genome for cold tolerance genes. The coding sequences associated to these adaptations were reported for *Psychrobacter* spp. strains ASPA161_6 (A) and ASPA161_9 (B).

	Gene	Length (bp)	COG	Product	EC number
A	cspV	213	COG1278	Cold shock protein CspV	N/A
	cspA_1	210	COG1278	Major cold shock protein CspA	N/A
	cspA_2	216	COG1278	Major cold shock protein CspA	N/A
	deaD	1668	COG0513	ATP-dependent RNA helicase DeaD	3.6.4.13
	desA3	1335	COG3239	NADPH-dependent stearoyl-CoA 9-desaturase	1.14.19
B	cspV	213	COG1278	Cold shock protein CspV	N/A
	cspA_1	210	COG1278	Major cold shock protein CspA	N/A
	cspA_2	216	COG1278	Major cold shock protein CspA	N/A
	deaD	1680	COG0513	ATP-dependent RNA helicase DeaD	3.6.4.13

COG: Clusters of Orthologous Genes; N/A: not available.

Table 2
Digital DNA-DNA hybridization value and Average Nucleotide Identity values. dDDH (%) and ANI (%) values calculated between *Psychrobacter* spp. strain ASPA161_6 (A) and strain ASPA161_9 (B) considering the nine strains closely related to each. dDDH values with confidence interval (%), and ANIb and ANIm values with conserved aligned DNA (%) were reported.

	Genomes compared	Accession number	dDDH [confidence interval] (%)	ANIb [aligned nucleotide] (%)	ANIm [aligned nucleotide] (%)
A	Reference genome: *Psychrobacter* sp. strain ASPA161_6				
	P. glaciei KCTC 42280	GCA_014652895	67.7 [64.8–70.6]	95.7 [82.6]	96.4 [84.1]
	P. okhotskensis MD17	GCA_904846405	30.2 [27.8–32.7]	85.1 [66.9]	88.0 [60.2]
	P. fjordensis BSw21516B	GCA_904845995	25.7 [23.4–28.2]	81.0 [59.9]	87.0 [33.9]
	P. fozii CECT 5889	GCA_003217155.1	25.5 [23.2–28.0]	81.1 [60.8]	86.8 [35.3]
	P. cibarius DSM 16327	GCA_016107535	24.6 [22.2–27.0]	81.1 [60.1]	86.0 [38.1]
	P. aquimaris DSM 16329	GCA_016107525	24.6 [22.3–27.1]	81.1 [62.5]	86.1 [38.6]
	P. immobilis DSM 7229	GCA_003148585	24.5 [22.2–27.0]	81.1 [60.8]	86.1 [38.4]
	P. nivimaris 88/2–7	GCA_904846365.1	24.3 [22.0–26.7]	80.3 [60.1]	86.1 [32.6]
	P. adeliensis SJ 14	GCA_904845895	24.3 [22.0–26.8]	80.3 [59.7]	86.1 [32.0]
B	Reference genome: *Psychrobacter* sp. strain ASPA161_9				
	P. okhotskensis MD17	GCA_904846405	28.8 [22.5–27.3]	79.7 [55.7]	86.3 [26.7]
	P. fulvigenes KC40	GCA_904846155	24.3 [22.0–26.7]	79.9 [57.1]	85.6 [29.1]
	P. maritimus Pi2-20	GCA_904846295	24.2 [21.9–26.7]	79.4 [54.2]	86.0 [24.7]
	P. luti CECT 5885	GCA_014192115	23.9 [21.6–26.4]	79.2 [52.7]	85.7 [23.2]
	P. glaciei KCTC 42280	GCA_014652895	23.9 [21.6–26.3]	79.2 [56.4]	85.6 [24.1]
	P. aquimaris DSM 16329	GCA_016107525	23.4 [21.1–25.8]	79.0 [56.2]	85.3 [22.8]
	P. fjordensis BSw21516B	GCA_904845995	23.4 [21.1–25.8]	78.5 [54.4]	85.0 [21.0]
	P. cryohalolentis K5	GCA_000013905	23.3 [21.1–25.7]	78.4 [55.3]	85.6 [19.3]
	P. frozii CECT 5889	GCA_003217155.1	23.2 [20.9–25.6]	78.6 [54.8]	85.2 [20.7]

Table 4
Gene sequences coding for esterase and lipase in the annotate genomes of *Psychrobacter* spp. strain ASPA161_6 (A) and strain ASPA161_9 (B). Moreover, genes coding for enzymes involved in the degradation of different polyester plastic polymers were reported.

	Gene	Length (bp)	COG	Product	EC number
A	estB	672	COG0400	Carboxylesterase 2	EC 3.1.1.1
	lip1	987	COG1075	Lipase 1	EC 3.1.1.3
	lip2	1302	COG1075	Lipase 2	EC 3.1.1.3
	lip3	945	COG1075	Lipase 3	EC 3.1.1.3
	lip	1077	COG1075	Triacylglycerol lipase	EC 3.1.1.3
	bdhA	801	COG1028	D-beta-hydroxybutyrate dehydrogenase	EC 1.1.1.30
	alkB	1260	N/A	Alkane-1 monooxygenase	EC 1.14.15.3
B	estB	672	COG0400	Carboxylesterase 2	EC 3.1.1.1
	lip2	948	COG1075	Lipase 2	EC 3.1.1.3
	lip3	1548	COG1075	Lipase 3	EC 3.1.1.3
	lip	1053	COG1075	Triacylglycerol lipase	EC 3.1.1.3
	bdhA	801	COG1028	D-beta-hydroxybutyrate dehydrogenase	EC 1.1.1.30

COG: Clusters of Orthologous Genes; N/A: not available.

2.3. Genome sequencing, assembly, annotation and comparative genomics

Sequencing of the whole genomes was provided by MicrobesNG (U. K.) through lllumina NovaSeq 6000 (Illumina, U.S.A.) starting from the bacterial cellular pellets prepared accordingly to the company recommendations. The service provided the adapter trimming of the reads and quality control, which was performed with Trimmomatic version 0.30. Comprehensive genome analysis was made in Bacterial and Viral Bioinformatics Resource Center (BV-BRC; https://www.bv-brc.org/) (Olson et al., 2023).

Assembly of the samples was done using Unicycler Version 0.5.0 (Wick et al., 2016). For functional annotation was used RAST tool kit (RASTtk) in combination with Plastic Biodegradation Database (PlasticDB; https://plasticdb.org) with E-value set at 1E-6 and 30 % identity of the alignments (Brettin et al., 2015; Gambarini et al., 2022). For the annotation of the genes involved in bioplastic synthesis and degradation, the matching results were visualized in a Krona pie chart tool (Ondov et al., 2011). The assembled genomes have been determined to be of good quality, featuring key details on gene content and functional annotation.

Whole Genome Shotgun projects of strain ASPA161_6 and strain ASPA161_9 was deposited at DDBJ/ENA/GenBank under the accession number JBEFNJ000000000 for ASPA161_6 and JBEFNI000000000 for ASPA161_9.

Proteome Comparison Service (https://www.bv-brc.org/app/SeqComparison) was used to compare the protein sequences against the taxonomical closest *Psychrobacter* strains, chosen as described below. This analysis employs bidirectional BLASTP with the following parameters: a minimum coverage of 30 %, a BLAST E-value threshold of 1E-5, and a minimum identity of 10 %.

2.4. Genome-based phylogenetic tree, digital DNA–DNA hybridization, and average nucleotide identity

To conduct the genome-based phylogenetic analysis, the genome sequence data were uploaded to the Type (Strain) Genome Server (TYGS; https://tygs.dsmz.de). First, all user genomes were compared against all those available in the database via the MASH algorithm, which is a rapid approximation of intergenomic relatedness (Ondov et al., 2016). Subsequently, the nine with the smallest MASH distances were selected for each user genome. Secondly, an additional set of nine

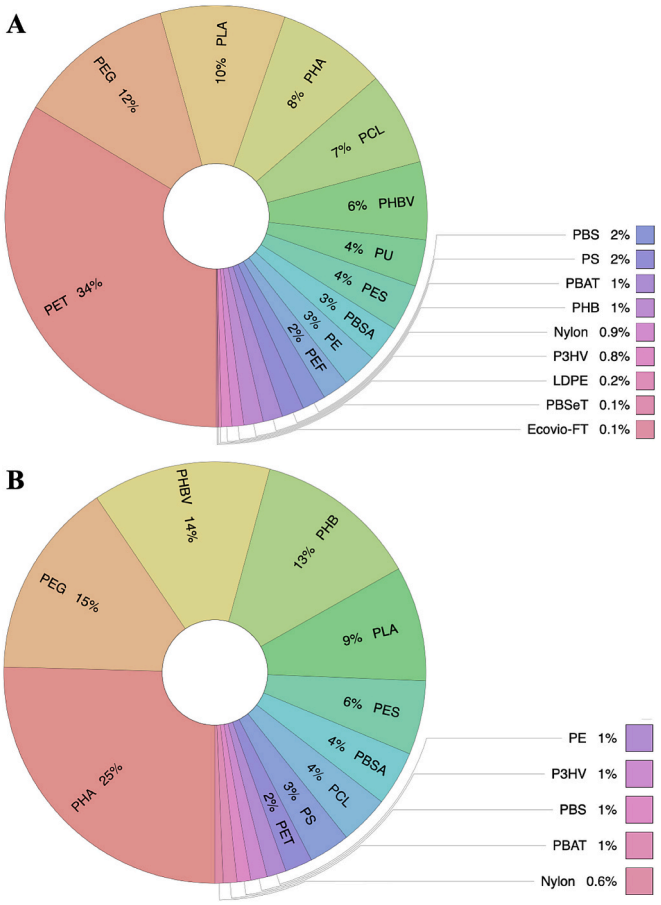

Fig. 1. Genome annotation for plastic degradation. Krona display showing the results of the genome annotation for genes with a potential role in the degradation of different plastic polymers for *Psychrobacter* strain ASPA161_6 (A) and strain ASPA161_9 (B). The percentages indicate the fraction of hits for each class of enzymes with known activity on a plastic polymer.

closely related Type strains was identified through the analysis of the 16S rDNA gene sequences. These sequences were extracted from the user genomes using RNAmmer, and each sequence was subsequently subjected to a BLAST search against the 16S rDNA gene sequence of each of the currently 21,176 type strains available in the TYGS database (Camacho et al., 2009; Lagesen et al., 2007). This was employed as a proxy to identify the optimal 50 matching Type strains (according to the bitscore) for each user genome and to subsequently calculate precise distances using the Genome BLAST Distance Phylogeny approach (GBDP) under the algorithm coverage and distance formula d5 (Meier-Kolthoff et al., 2013). The resulting intergenomic distances were utilized to infer a balanced minimum evolution tree with branch support via FastME 2.1.6.1, including SPR post-processing (Lefort et al., 2015). Branch support was inferred from 100 pseudo-bootstrap replicates.

Digital DNA-DNA hybridization (dDDH) values and confidence intervals were calculated using the recommended settings of the GGDC 4.0 (Meier-Kolthoff et al, 2013). According to these factors, for strain ASPA161_6 the reference genomes considered were *Psychrobacter glaciei* KCTC 42280, *P. okhotskensis* MD17, *P. fjordensis* BSw21516B, *P. fozii* CECT 5889, *P. cibarius* DSM 16327, *P. aquimaris* DSM 16329, *P. immobilis* DSM 7229, *P. nivimaris* 88/2-7, and *P. adeliensis* SJ 14. For strain ASPA161_9 they were *P. okhotskensis* MD17, *P. fulvigenes* KC40, *P. maritimus* Pi2-20, *P. luti* CECT 5885, *P. glaciei* KCTC 42280, *P. aquimaris* DSM 16329, *P. fjordensis* BSw21516B, *P. cryohalolentis* K5, and *P. frozii* CECT 5889.

Reference genomes were downloaded from NCBI (www.ncbi.nlm.nih.gov/refseq) and to calculate the average nucleotide identity (ANI),

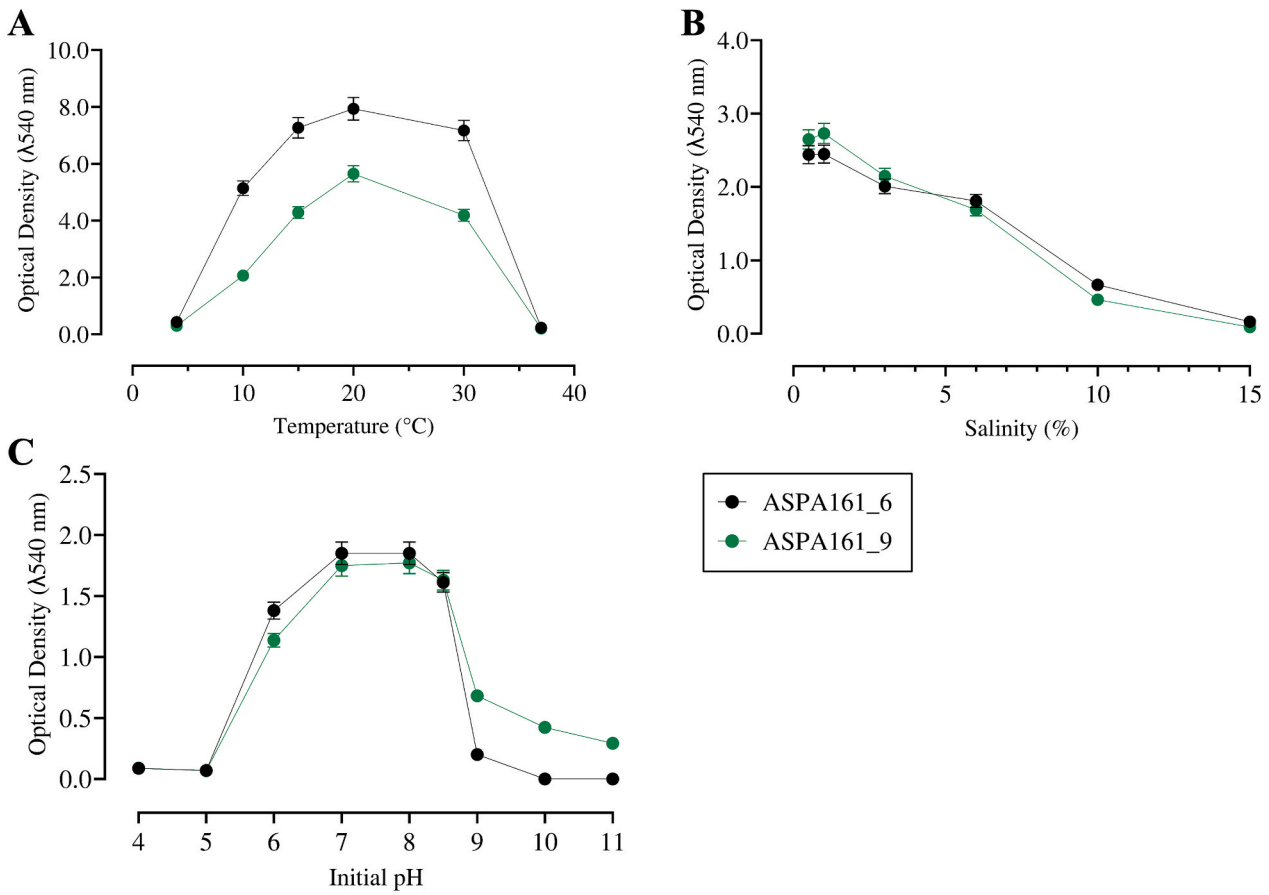

Fig. 2. Curve for optima growth conditions. Curves in liquid cultures for the optima growth parameters of *Psychrobacter* spp. strain ASPA161_6 and strain ASPA161_9, evaluating temperature (°C) of incubation (A), salinity (% NaCl) of the medium (B), and initial pH (C). Optical density measured at λ540 nm after 24 h of incubation. Growths were performed in triplicates and mean values were reported.

each of the two ASPA161 strain genomes was compared with the nine reference genomes using JSpeciesWS server (www.jspecies.ribohost.com/jspeciesws) with two alignment algorithms, namely MUMmer and BLASTN, which returned ANIm and ANIb values, respectively (da Silva et al., 2022; Richter et al., 2016). Subspecies clustering was done using a 79 % dDDH threshold as previously introduced (Meier-Kolthoff et al., 2014). Trees were rooted at the midpoint and visualized with PhyD3 (Farris, 1972; Kreft et al., 2017).

2.5. Growth curves for optimal growth conditions

Growth curves in liquid culture were assessed by testing in sequence different temperatures (4, 10, 15 20, 30 and 37 °C), NaCl concentrations (0.5, 1.0, 3.0, 6.0, 10.0, and 15.0 %, w/v), and starting buffered pH, maintaining the previously identified optimum parameter. The pH was regulated using the following buffers, each 50 mmol L^{-1}: citrate buffer (pH 4.0 ± 0.1), acetate buffer (5.0 ± 0.1), 2-morpholinoethanesulfonic acid (MES; 6.0 ± 0.1), 3-morpholinopropanesulfonic acid (MOPS; 7.0 ± 0.1), 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic acid (HEPES; 8.0 ± 0.1), and 3-(cyclohexylamino)propane-1-sulfonic acid (CAPS; regulated at 8.5 ± 0.1, 9.0 ± 0.1, 10.0 ± 0.1 and 11.0 ± 0.1). Bacterial growth was conducted under agitation (120 rpm). Following 24 h of incubation, optical density at λ540 nm (Genesys 150 UV–vis spectrophotometer; ThermoFisher Scientific, U.S.A.) and pH value (PHM210 pH meter; Radiometer, Denmark) were determined. Growths were performed in triplicates and mean values were reported.

2.6. Qualitative in-plate assays for carboxylic ester hydrolases activities

To confirm the expression of carboxylic ester hydrolase activities previously identified in the genomes, qualitative *in-plate* assays were set up. Solidified TSB plates enriched by 1.0 % (v/v) tributyrin or by 2.5 % (v/v) olive oil plus 0.001 % (w/v) rhodamine B, were prepared. Triglycerides were added after the media sterilization in autoclave, and they were emulsified before pouring into plates with magnetic stirring. Rhodamine B aqueous solution was sterilized through 0.22 μm filtration. The plates were inoculated with 50 μL of fresh liquid cultures in the exponential phase ($4\bullet10^{11}$ UFC/mL for strain ASPA161_6; $7\bullet10^{10}$ UFC/mL for strain ASPA161_9) and they were incubated at 22 °C for 2 weeks. For tributyrin hydrolysis, the presence of esterase and lipase activities produced by the bacterial strains was showed by formation of a clear halo around the colonies because of the release of glycerol and butyric acid which are soluble in an aqueous medium; hydrolysis and release of fatty acid chains from olive oil were visualized with fluorescent dye Rhodamine B, whose orange halo was observed under UV light (Hasan et al., 2009).

2.7. Recovery of the crude extract and protein quantification

Enzymatic activities were investigated in crude extract of cell-free supernatants following growth under optimal conditions, obtained through partial purification. Bacterial strains were cultured in 500 mL TSB (buffered with HEPES 60 mmol L^{-1}, pH 8), and optical density was monitored at λ540 nm. Following bacterial growth, the broths were centrifuged with a Beckman Coulter JA-14 centrifuge at 6,000 rpm for 45 min at 4 °C, then the cell-free supernatants were concentrated with

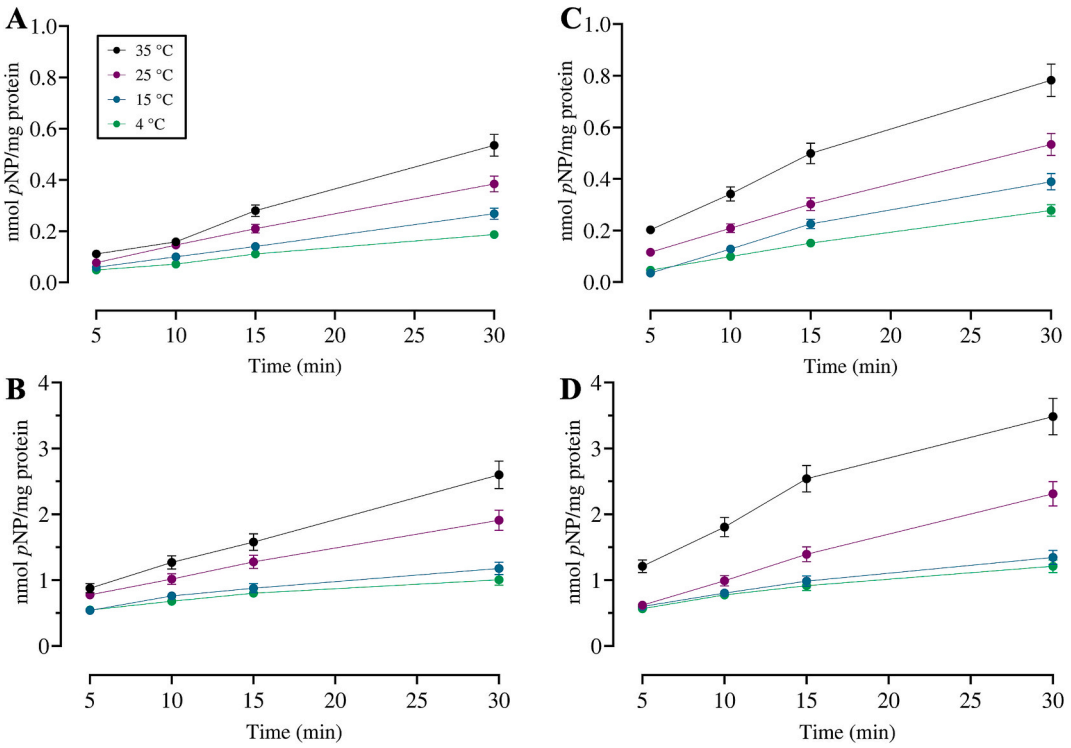

Fig. 3. Time courses of *p*-nitrophenol release by the crude extracts (nmol *p*-NP/mg of protein). Extracellular crude extracts were tested on *p*NP-esters at varying temperatures and incubation times. Results for *Psychrobacter* sp. strain ASPA161_6 on *p*NP-acetate (A) and *p*NP-dodecanoate (B), and *Psychrobacter* sp. strain ASPA161_9 on *p*NP-acetate (C) and *p*NP-dodecanoate (D) were reported. Protein solutions and substrate blanks were prepared. Assays were performed in triplicates and mean values were reported.

80 % $(NH_4)_2SO_4$ and dialyzed (12–14 kDa dialysis tubes; Medicell membranes, U.K.) against 20 mM phosphate buffer pH 8.0 (Na_2HPO_4•7 (H_2O), 4.95 g/L; NaH_2PO_4•2(H_2O), 0.25 g/L) for 24 h at 4 °C (buffer changed at 15 and 17.5 h). Dialyzed enzymatic solutions were assayed for their protein content with the Bio-Rad protein assay kit (Bio-Rad, U.S.A.) as described in Bradford (1976), using bovine serum albumin as the standard protein. These enzymatic solutions were stored at −20 °C (0.164 and 0.171 mg protein/mL, respectively for strain ASPA161_6 and ASPA161_9) or lyophilized to give raw enzymatic preparation to be used in the hydrolytic biodegradation of PEA (11.3 and 11.7 mg protein/mg lyophilized powder, respectively).

2.8. Quantitative in-vitro assays for esterase and lipase activities

Esterase and lipase activities in the partially purified extracellular fractions were assessed with spectrophotometric-colorimetric assays based on the hydrolysis of selected *p*-nitrophenyl (*p*NP) esters of fatty acids with two different chain lengths (Sigma-Aldrich, U.S.A.) as substrates. The reaction mixture consisted of 50 μL substrate solution (0.2 mM in the mix) and 22.5 mg of protein enzymatic solution in 500 μL (total volume) of 20 mM phosphate buffer pH 8.0. *p*NP-acetate was dissolved in ethanol; *p*NP-dodecanoate was dissolved in 2-propanol, prepared in starting stock solutions of 2 mM. The reaction mixtures were incubated at different temperatures (4, 15, 25 and 35 °C), static, and absorbance was measured at λ405 nm to quantify the release of *p*-nitrophenol after 5, 10, 15 and 30 min, according to a calibration curve developed with a pure *p*-nitrophenol standard. Assays were performed in triplicates and mean values were reported. Appropriate blanks were also set up.

2.9. Degradation of poly(ethyleneadipate) with crude extracts

To a suspension of PEA (10 mg) in 50 mM potassium phosphate buffer pH 8.5 (3 mL) lyophilized enzymatic preparation from ASPA161_6 or ASPA 161_9 was added (80 mg for each strain). The suspension was magnetically stirred at temperature of 29 °C and the reaction stopped at suitable time (4 d or 12 d) by addition of 0.1 M HCl up to pH 4.5. The reaction mixture was then extracted with EtOAc: MeOH 90:10 v/v (3 × 2 mL) and the organic layers separated and collected. Pooled organic extracts were dried over Na_2SO_4, filtered and taken to dryness under reduced pressure to give a residue that was analyzed by ^{1}H NMR ($CDCl_3$/CD_3OD).

Other reactions, carried out in parallel for 12 days, were subjected to a different work-up protocol based on sequential extractions of the reaction mixture with two solvents of different polarity. After acidification with 0.1 M HCl at pH 4.5, the reaction mixture was first extracted with CH_2Cl_2 (3 × 2 mL) and the organic layer separated. Then, the aqueous phase was again extracted with EtOAc:MeOH 90:10 v/v (3 × 2 mL) and the organic phase collected. The two organic extracts were separately taken to dryness, weighted and analyzed by ^{1}H NMR ($CDCl_3$ and CD_3OD, respectively).

3. Results and discussion

3.1. In-silico screening of the genome and annotation

3.1.1. Genome sequences: General data

Two new Antarctic psychrophilic strains ASPA161_6 and ASPA161_9, which were isolated from a marine sediment sample collected in Terra Nova Bay (Ross Sea), were identified as *Psychrobacter*

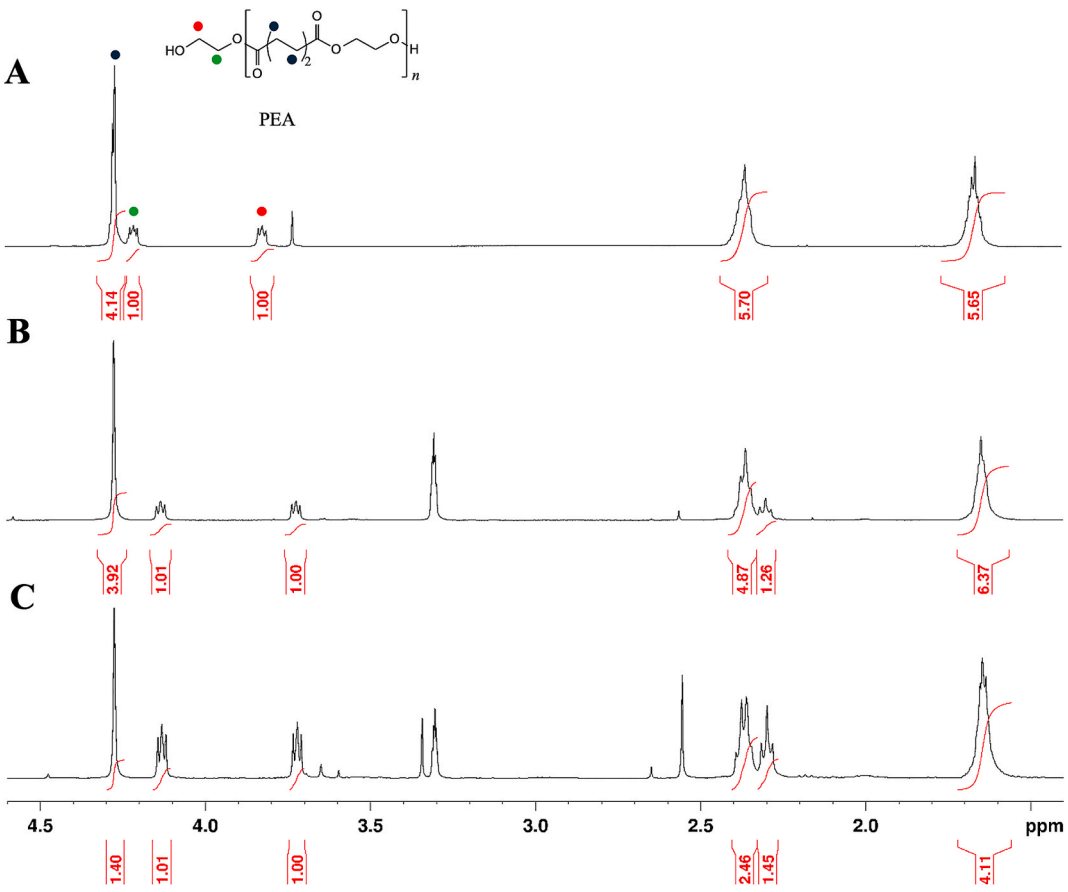

Fig. 4. ^{1}H NMR spectra of PEA starting polymer (A); extract of hydrolysis reaction catalyzed by crude extract from ASPA161_6 after 4 days (B) and after 12 days (C).

species based on the sequencing of the genomes. This finding is consistent with the hypothesis that bacteria of this genus possess unique adaptations for survival in extreme cold environments (Kondakova et al., 2012). Their draft genomes were obtained, and the metrics of the assemblies were reported in Table 1.

3.1.2. Digital DNA–DNA hybridization, and average nucleotide identity

Taxonomy of strain ASPA161_6 and strain ASPA161_9 was evaluated considering the nine strains closely related to each based on the genome sequences (Table 2). For strain ASPA161_6 the results of the dDDH values overlapped with the 70 % threshold used to distinguish between different species among Bacteria, while for strain ASPA161_9 they were significantly lower (Meier-Kolthoff et al., 2013). In the case of strain ASPA161_6, the highest value was reported from the comparison with *Psychrobacter glaciei* KCTC 42280 (67.7 %, confidence interval 64.8–70.6 %), while for ASPA161_9 it resulted from the comparison with *Psychrobacter okhotskensis* MD17 (28.8 %, confidence interval 22.5–27.3 %). For strain ASPA161_6, the ANI results were again on the proposed boundaries for species delimitation of 95–96 % in comparison with *Psychrobacter glaciei* KCTC 42280 (95.7 % ANIb and 96 % ANIm) (Richter and Rosselló-Móra, 2009). In the case of ASPA161_9, the ANI results were lower, varying between 78.4 and 79.9 % for ANIb, and between 85.2 and 86.3 % for ANIm. Based on these results, strain ASPA161_6 could belong to the species *Psychrobacter glaciei*, while strain ASPA161_9 could be considered as new species of the genus *Psychrobacter*. Further studies are needed to confirm these results.

3.1.3. Genome-based phylogenetic tree and comparative genomics

An overview of the annotation data was reported, together with genome-based phylogenetic trees and the results of the Proteome Comparison Service, comparing the protein sequences of strain ASPA161_6 and ASPA161_9 with the nine closest strains for each, as previously indicated (see supplementary materials).

It is evident that the sequence identity of strain ASPA161_6 with the first track in the plot, which is *Psychrobacter glaciei* KCTC 42280, is high. For both ASPA161_6 and ASPA161_9 strains, the remaining tracks exhibit a similarity between 80 and 95 %, indicating that their proteins share a substantial degree of homology with those of the other *Psychrobacter* strains included in the comparison. This is consistent with dDDH and ANI values found.

3.1.4. Adaptation to cold environments

In the annotated genomes of the two bacteria, the coding sequences related to cold adaptation mechanisms were found and they were reported in Table 3. In both strains, cold shock proteins coded by genes cspV, cspA_1 and cspA_2 were noted, together with the ATP-dependent RNA helicase DeaD which has a role in cold tolerance. In strain ASPA161_6, gene desA3 was present, coding for a NADPH-dependent stearoyl-CoA 9-desaturase (EC 1.14.19), a key enzyme for the insertion of double bond in the delta position of fatty acids, with a role in the variation of the fluidity of membranes (da Silva et al., 2022).

3.1.5. Polyester plastic polymer degradation

The potential for polyester plastic degradation by means of enzymes is promising, especially to address environmental challenges posed by this kind of waste. Enzymatic activities such as laccase (EC 1.10.3.2), PETase, alkane 1-monooxygenase (EC 1.14.15.3), cutin hydrolase (EC 3.1.1.74), and triacylglycerol lipases (EC 3.1.1.3) have shown varying degrees of effectiveness against different plastic polymers (Pérez-García et al., 2021). In this study an *in-silico* screening was performed to identify genes involved in polyester plastic degradation within the genomes of *Psychrobacter* spp. strains ASPA161_6 and ASPA161_9, with a

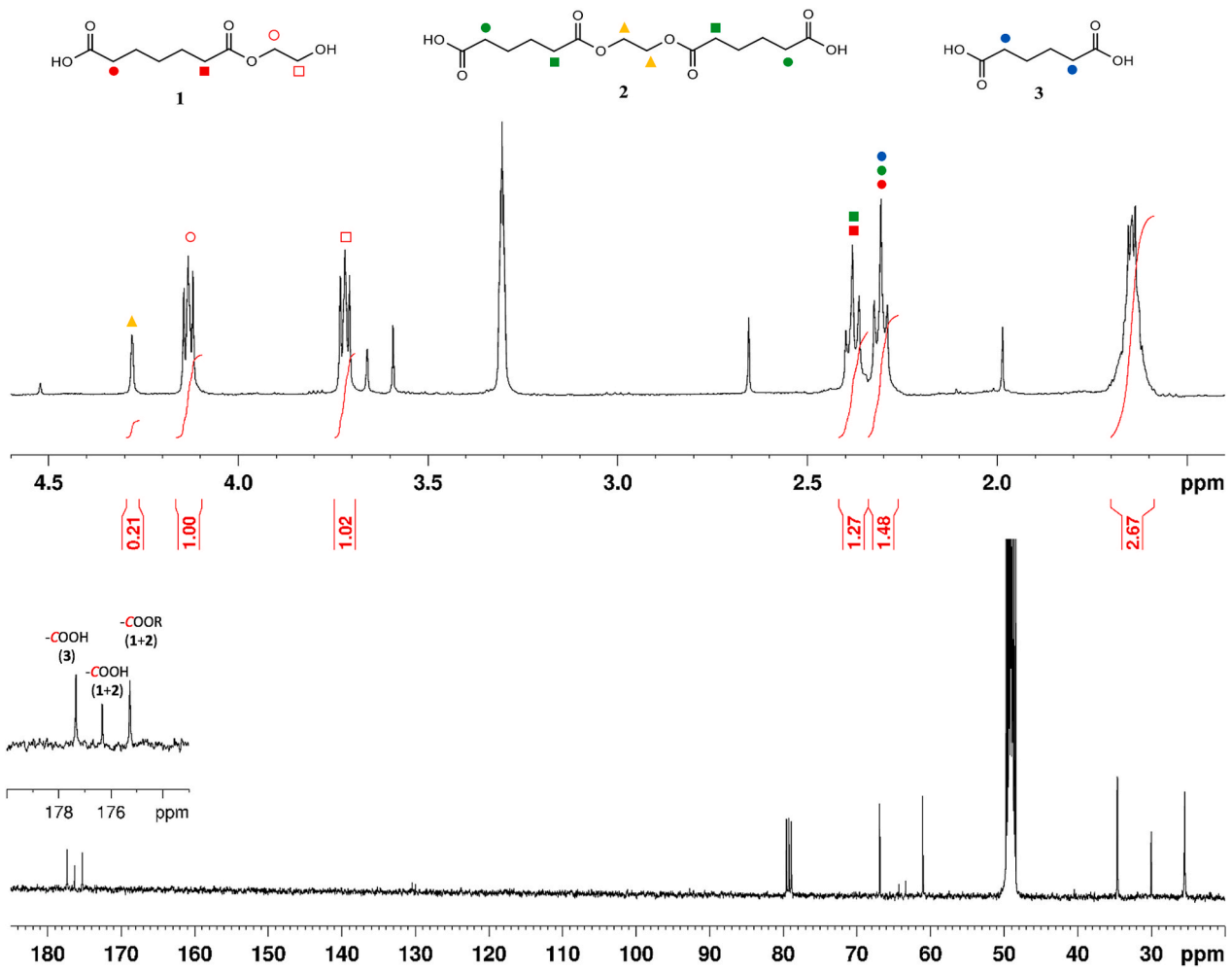

Fig. 5. ^{1}H- and ^{13}C NMR spectra of the EtOAc/MeOH extract of the hydrolysis reaction (12 days) biocatalyzed by crude extract from ASPA161_6 and assignment of resonances to compounds 1–3.

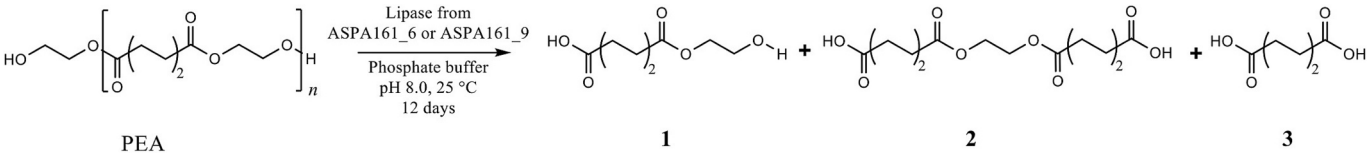

Fig. 6. Main products obtained in the biocatalyzed degradation of PEA polymer.

focus on esterases and lipases whose presence was confirmed by the following *in-vitro* experiments. The genes corresponding to lipase and esterase activities were firstly identified in the annotated genomes elaborated with RAST server, in addition with other genes involved in the degradation of different plastic polymers and they were presented in Table 4.

Moreover, an additional annotation was performed using PlasticDB, a specialized database for enzymes involved in plastic degradation and related microorganisms. The database currently describes 219 proteins known to break down plastics, and it includes 753 species of microorganisms documented to have plastic-degrading capabilities (update 05/13/24) (Gambarini et al., 2022). All matching results were presented in a Krona pie chart tool (Fig. 1), and the complete list of the hits with the corresponding E-values were provided (see supplementary materials).

Psychrobacter strain ASPA161_6 exhibited a wide enzymatic profile, starting with two hits for laccase enzymes both identified with an E-value of 0.00E+00, indicating a strong potential for degrading polyethylene (PE). This is a significant finding, especially considering the pervasive use of PE in everyday products (Pérez-García et al., 2021). Additionally, different hits for generic polyester hydrolases were identified (E-value comprised between 4.2E-186 and 4.7E-64). These enzymes may prove effective against a diverse range of plastics, including polyethylene terephthalate (PET), polycaprolactone (PCL), polyurethane (PU), polybutylene succinate (PBS), polylactic acid (PLA), and polyhydroxyalkanoates (PHA). PETase were identified with five hits and an E-values as low as 1.30E-78, exhibiting a high affinity for degrading PET, one of the most prevalent polymers used in bottles and packaging. Furthermore, cutinase enzymes were present with seven hits (E-values between 1.3E-64 and 9.6E-65), suggesting an activity against PET and PLA. For polyethylene glycol (PEG) dehydrogenase and PEG aldehyde dehydrogenase combined, there were eleven hits with different ranges of E-values, two for alkane-1 monooxygenase (E-value 1.5E-38 and 3.4E-66) and one for PLA depolymerase (4.2E-52). Lipase and esterase were also noted with a significant presence. On the other hand, strain

ASPA161_9 showed some overlap in terms of enzyme activity with ASPA161_6, but it additionally demonstrated its own unique potential for plastic degradation. There were identified seven genes matching with PEG dehydrogenase and PEG aldehyde dehydrogenase with varying E-values (from 1.0E-70 to 6.0E-08), which could have roles in the degradation of PEG. Regarding polyhydroxybutyrate (PHB) depolymerase, there were five hits (E-values from 1.2E-85 to 4.4E-11). Moreover, one hit was for a laccase (E-value 0.0E+00), one for PETase (6.0E-8), one for PLA depolymerase (6.9E-53), and one for alkane-1 monooxygenase (7.9E-60). For hydrolase and lipase there were three hits each, with potential applicability against various polymers.

All these combined results indicated that both strains possess the capacity to degrade both traditional and biodegradable plastics, which are commonly used in various industries. The distinct enzymatic profiles of the two strains highlighted their potential complementary roles in plastic degradation: they showed potential effectiveness against a range of plastic polymers, yet each possesseed unique enzymatic capabilities that could be leveraged in tailored approaches to plastic waste management. Further research is required to optimize these strains and to gain a deeper understating of their ecological implications, to fully realize their potential for sustainable waste management practices.

3.2. In-vitro experiments

3.2.1. Optima growth conditions

To identify the optima growth parameters for the two strains, a series of experiments was performed, varying temperature of incubation, salinity, and starting pH value of the medium (Fig. 2). Incubation at 20 °C for 24 h in TSB resulted in a higher optical density measured at λ540 nm for both strains, with a slight decrease observed at incubation temperatures of 15 or 30 °C. At 4 °C growth was observed, consistent with the cold-adaptation characteristics typical of the genus *Psychrobacter* (Kondakova et al., 2012). Conversely, at a temperature of 37 °C, no growth was evident (Fig. 2A). While maintaining a temperature of incubation of 20 °C, growth after 24 h of incubation was evaluated by varying the salinity of the medium, starting from 0.5 % (w/v) of NaCl and up to 15.0 % (w/v) (Fig. 2B). The trend for the two strains was analogous, with maximum growth at 0.5 and 1.0 % NaCl, followed by a constant decrease at higher concentrations, although growth persisted at 3.0 and 6.0 % NaCl. No growth was measured at 15.0 % NaCl for either strain. This indicateed a halotolerance of both strains. The genus *Psychrobacter* has been linked to the ability to low water activity, which can be obtained with exposure to subzero temperatures or high salinity. Studies have described changes in the composition of the membrane, as well as changes in the morphology and size of the cells in response to an increase in osmolarity (Rodrigues et al., 2009). Finally, initial pH of the buffered medium was adjusted between 4.0 and 11.0, while maintaining the previously defined optimal conditions for 24 h of incubation (20 °C, 0.5 % NaCl). For both strains, no growth was observed at pH values below 5.0, with optimal pH range comprised between 7.0 and 8.0. Additionally, *Psychrobacter* sp. strain ASPA161_9 maintained a low growth in a pH comprised between 9.0 and 10.0, with no growth at pH 11.0. Instead, strain ASPA161_6 showed no growth at pH higher than 9.0 (Fig. 2C). This was consistent and in line with the preference of the genus *Psychrobacter* for a neutral pH (Rodrigues et al., 2009).

3.2.2. In-plate and in-vitro assays for esterase and lipase activities

Qualitative *in-plate* assays for the hydrolysis of tributyrin and olive oil resulted positive after six days of incubation for both *Psychrobacter* sp. strain ASPA161_6 and strain ASPA161_9 (see supplementary materials). This indicated the expression of carboxylic ester hydrolase activities.

Extracellular carboxylic ester hydrolases released in the supernatant in the optimal growth conditions were partially purified to obtain crude extracts, which were then tested at different temperatures with quantitative chromogenic-spectrophotometric assays. Strain ASPA161_6 yielded 20.3 mg total proteins/g dry cells, while strain ASPA161_9 yielded 7.9 mg total proteins/g dry cells.

Plots of the amount of free *p*NP versus time are presented in Fig. 3. Hydrolysis of *p*NP-acetate indicated the presence of esterases, and for both ASPA161_6 and ASPA161_9 strains a higher release of nmol of *p*NP by mg of protein was measured at 35 °C after 30 min of incubation (0.5 and 0.8 nmol *p*NP released/mg protein, respectively). Tchigvintsev et al. (2015) discussed how the cold-adapted esterases described in their work showed higher activity from 5 to 40 °C, as a reflection of the environmental conditions where they evolved in association to marine bacteria from the Barents Sea and the Mediterranean Sea. Over the same time course, at 25 °C, 71.8 and 68.1 % of the activity were maintained onto this substrate, while at 15 °C they were 50.1 and 49.6 %, respectively, suggesting psychrotolerant characteristics of these enzymes. Assays with *p*NP-dodecanoate indicated the presence of lipase activity in the crude extracts. Similarly, for both strains the specific enzymatic activity was higher at 35 °C after 30 min of incubation (2.6 and 3.5 nmol *p*NP released/mg protein). Most of the enzymatic activity was showed at 25 °C after the same incubation period, measured at 73.4 and 66.3 % in comparison with the optimal conditions for strain ASPA161_6 and ASPA161_9, respectively. Although the release of *p*NP at 15 °C was 45.3 and 38.6 % respectively of the one measured at 35 °C, a notable increase was observed over time. This slow increase may be attributed to the reduced kinetic activity of the selected incubation temperature. This observation suggested potential use of carboxylic ester hydrolases produced by these bacterial strains to treat plastic waste at low temperatures, including bioremediation of natural environments, with an increased exposure time.

In the study by Kim et al. (2015), lipolytic enzymes from *Psychrobacter* sp. ArcL13 were assayed on a range of *p*NP-esters. This approach involved direct testing of the supernatants, without a prior purification. The results with *p*NP caprylate showed maximum activity at 40 °C, with 39 and 70 % of the activity showed at 10 and 20 °C, respectively. Those results were comparable to those of this study, despite the use of a different *p*NP ester. Furthermore, the authors conducted statistical optimization and fed-batch fermentations to improve enzymatic expression. They then correlated it with bacterial cells optical density and enzymatic activity. Despite the cell density of strain ArcL13 ranged from 0.800 and 1.500 and being lower in comparison with strains ASPA161_6 and ASPA161_9 (2.440 and 3.600 OD_{600} at end of growth phase, respectively), there was an activity improvement as result of the intended increased expression in the optimal medium. To compare all these data, an optimization should be performed for strains ASPA161_6 and ASPA161_9.

In other research papers, the quantification of the enzymatic activity onto *p*NP-esters is described, including both esterases and lipases, but in these experiments the authors studied purified enzymes obtained through heterologous expressions (Esakkiraj et al., 2022; Paun et al., 2024; Zhang et al., 2024). This resulted in optimized specific enzymatic activities, and for a complete comparison a heterologous expression of the enzymes from stains ASPA161_6 and ASPA161_9 should be performed. However, presence of both esterases and lipases in the crude extract from the supernatant of the *Psychrobacter* strain here under investigation suggested the potential for a synergistic effect against a complex matrix of plastic polymers. This could enable hydrolysis of both long and short chains connected with ester bonds, without the necessity of a prior purification of the enzymes.

3.2.3. Degradation of poly(ethyleneadipate) with partially purified extracellular fractions of bacteria

The potential of *Psychrobacter* spp. strain ASPA161_6 and strain ASPA161_9 in degrading polyester polymers was assessed on PEA, chosen as model of biodegradable polymer, using lyophilized cell-free extracts of the bacterial culture supernatants as biocatalysts (Tokiwa and Suzuki, 1977). Due to its low crystallinity, PEA degrades faster than other polyester polymers and it has been used as plasticizer in polymer

blends or in the synthesis of copolymers with enhanced biodegradability (Atanase et al., 2022; Howell and Lazar, 2019). To the best of the authors' knowledge, no other research on the bacterial degradation of pure polymer of PEA has been reported in the scientific literature.

Dihydroxy-terminated PEA was subjected to biocatalyzed hydrolysis in the presence of enzymatic preparations from ASPA161_6 and ASPA161_9 and the reaction progress was followed by ^{1}H NMR, monitoring the decrease in the signal ratio between resonances of methylene groups of the internal ethylene glycol moieties, visible as a singlet, and the triplet associated to the terminal hydroxymethyl groups (Fig. 4). This ratio is indeed related to the polymerization degree and allows to calculate the number-average molecular weight M_n of the polymer (Hichem et al., 2014). While the starting polymer gave a ratio of 4.14:1 between these signals, as degradation proceeded this ratio decreased until reaching after 12 days of reaction the values of 1.40:1 and 1.69:1 for product mixtures from the reactions catalyzed by ASPA161_6 and ASPA161_9 preparations, respectively.

Furthermore, in the ^{1}H NMR spectrum of the reaction mixture an additional resonance, attributed to a methylene in the α-position to –COOH group, became evident suggesting the occurrence of hydrolysis of ester bond(s). The presence of molecules with free –COOH group(s) was also confirmed in the ^{13}C NMR spectrum, in which resonances downfield shifted compared to that of COOR groups were visible. In a more detailed analysis, the mixtures from biocatalyzed reactions were sequentially extracted with two solvents with different polarity and it was found that the more polar extract (65 % total recovered weight) contained the monomer **1** as main product (≈80 %) together with minor amounts of compounds **2** and **3** (Figs. 5 and 6). These data, which are compatible with the degradation degree calculated from the decrease of M_n, indicate that both the tested enzymatic preparations are effective in the hydrolytic degradation of PEA (55–65 % degradation range over 12 days), which resulted in higher amount of monomer **2** produced in the reaction catalyzed by ASPA161_6 compared to ASPA161_9. The observed reaction course in the degradation of PEA seems to suggest that the hydrolysis of the polymer takes place in repeated steps, in which the terminal ester groups of the polymer are hydrolyzed yielding a polymer with a lower molecular weight and the same terminal hydroxyl groups. After a suitable number of steps, no more ester groups are present in the substrate and the monomers adipic acid and ethylene glycol are the only degradation products.

Over 12 billion tons of plastic are expected to be accumulated in the environment by 2050 (Plastics Europe, 2022). Considering that plastic waste generates microplastics (1 μm-5 mm) and nanoplastics (<1 μm) which are found in all biotic and abiotic components of the ecosystem (Booth et al., 2017), the discovery of novel carboxylic ester hydrolases biocatalysts usable in the biodegradation of plastics and bioplastics is of growing interest (Yoshida et al., 2016).

4. Conclusions

Two novel psychrophilic *Psychrobacter* strains, ASPA161_6 and ASPA161_9, were isolated from Antarctic marine sediments and deposited in the SUS-MIRRI microbial collection (https://susmirri-catalog.di.unito.it/collections/CE-ICB). Genome analyses revealed genes encoding carboxylic ester hydrolases, and enzymatic assays confirmed esterase and lipase activities with significant performances at low temperatures. Both strains degraded up to 65 % of poly(ethylene adipate) within 12 days, demonstrating their potential for cold-active bioplastic degradation. These findings contribute to the understanding of psychrotolerant microbial metabolism and highlight promising candidates for sustainable plastic biotreatment in cold environments.

5. Research support

This research received no external financial or non-financial support.

6. Relationships

There are no additional relationships to disclose.

7. Patents and Intellectual Property

There are no patents to disclose.

8. Other activities

There are no additional activities to disclose.

CRediT authorship contribution statement

Andrea Cattaneo: Writing – original draft, Visualization, Validation, Investigation, Formal analysis, Data curation. **Ilaria Finore:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Claudia Sanfilippo:** Writing – original draft, Methodology, Investigation. **Razmik Papikyan:** Software, Formal analysis, Data curation. **Luigi Leone:** Investigation, Data curation. **Angela Patti:** Writing – original draft, Methodology, Investigation. **Paola Di Donato:** Project administration, Funding acquisition. **Annarita Poli:** Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.133052.

Data availability

Data will be made available on request.

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