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Catalytically active inclusion bodies of cold-adapted lipase: production and its industrial potential

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Abstract

Heterologous overexpression of certain recombinant proteins in *E. coli* often triggers the formation of inclusion bodies (IBs). Traditionally viewed as inactive aggregates, IBs are now known to sometimes retain native-like folding and catalytic activity, termed **catalytically active inclusion bodies (CatIBs)**. In this work, we investigated the CatIBs formed by recombinant LipAMS8, a cold-adapted lipase from an Antarctic *Pseudomonas* sp., expressed in *E. coli i*BL21(DE3)/pET32b. Overexpression yielded abundant insoluble aggregates which were gently isolated using 50 mM Tris–HCl, 50 mM NaCl, 1% Triton X-100 (pH 8.0), and subsequently solubilized in Tris–HCl (pH 8.0) without denaturants. Scanning electron microscopy revealed rod-like protein particles ranging from ~ 100 nm up to 1 µm. Biochemical characterization demonstrated that these LipAMS8 aggregates indeed function as CatIBs, exhibiting enzymatic activity with a defined optimum temperature, broad pH stability, specific metal ion responses, and remarkable tolerance to organic solvents. The LipAMS8 CatIBs retained significant residual activity (≥ 50%) across a wide pH range and various temperatures and showed only modest activity loss after prolonged storage (over 13 weeks at 4 °C and 8 weeks at 25 °C, maintaining > 50% activity). Notably, these aggregates displayed higher stability in extreme conditions (pH and organic media) compared to typical soluble enzymes. This study is the first to characterize a naturally formed lipase CatIB, highlighting that *LipAMS8* CatIBs are produced in vivo without any artificial tags. The LipAMS8 CatIBs combine high catalytic activity with exceptional stability and solvent tolerance, underscoring an alternative strategy to obtain cold-active lipases in immobilized form. Such robust CatIB biocatalysts are highly sought after in industries for diverse biotransformations in challenging conditions.

Keywords Catalytically active inclusion bodies, Cold-adapted, Lipase, Industrial, Potential

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Introduction

Bacterial inclusion bodies (IBs) are dense protein aggregates that commonly form during the production of recombinant proteins in microbes. Since the advent of recombinant DNA technology (Belkova et al. 2022). IBs have been frequently observed in *Escherichia coli and* were long considered an undesired by-product or "waste" of overexpression (De Marco et al. 2019). Early on, numerous strategies were developed to avoid IB formation because recovering functional proteins from these aggregates often required laborious denaturation and refolding protocols (Sørensen and Mortensen 2005). Most enzymes are only active when properly folded, and IBs were traditionally seen as improperly folded deposits (Gifre-Renom et al. 2020).

However, accumulating evidence over the past two decades has changed this perception (Peternel and Bele 2006). A growing body of literature shows that IBs can retain a considerable degree of molecular organization and often contain a fraction of correctly folded, biologically active protein (Helleckes et al. 2024). The earliest reports of enzymatically active IBs date back to the 1980 s, when inclusion bodies of β-galactosidase and endoglucanase were found to retain activity (Tokatlidis et al. 1991). These discoveries sparked the concept of **catalytically active inclusion bodies (CatIBs)**, which continue to gain attention as a low-cost, carrier-free form of enzyme immobilization (Nahálka et al. 2008) & (Jäger et al. 2020).

Recent advances have demonstrated that whether IBs are "active" (CatIBs or non-classical IBs) or "inactive" (classical IBs) depending on factors such as the intrinsic structure of the target protein, the folding environment, expression conditions (e.g., temperature and induction rate), and host strain physiology (Upadhyay et al. 2014). Moreover, synthetic biology and protein engineering techniques now allow the intentional design of CatIBs. Fusions with aggregation-inducing peptides (e.g., ELK16, coiled-coil domains, 3HAMP, or TDoT motifs) can trigger IB formation while preserving catalytic activity (Helleckes et al. 2024); (Krauss et al. 2017). These designed CatIBs have been reported for diverse enzymes, including lipases, esterases, alcohol dehydrogenases, and lyases (Nahálka et al. 2008);(Hrabarova et al. 2022).

Structurally, IBs tend to be submicron particles (50–1000 nm), highly enriched in β-sheet content, and mechanically stable (Juenemann et al. 2018). Protein molecules within IBs may adopt amyloid-like configurations, but some maintain native-like folds that are sufficient for activity (Wang et al. 2008). This has led to the development of **mild solubilization protocols**, in which active enzymes are extracted from IBs using non-denaturing buffers (Baltà-Foix et al. 2024). These strategies take advantage of residual secondary structure present in CatIBs and allow partial recovery of soluble protein without the need for harsh chaotropes such as urea or guanidine hydrochloride (Upadhyay et al. 2016).

Beyond this, CatIBs offer unique industrial advantages: they are produced at high purity and yield, require minimal downstream processing, and are inherently recyclable (Kloss et al. 2018). Their rigid aggregate structure improves thermal, pH, and solvent stability relative to soluble enzymes, making them particularly suitable for non-aqueous biocatalysis and extreme reaction environments (Bello et al. 2024; Belkova et al. 2022).

Cold-adapted (psychrophilic) enzymes are particularly interesting candidates for the development CatIB. These enzymes function efficiently at low temperatures, making them desirable for processes that require refrigeration, energy conservation, or handling of heat-sensitive substrates (Liu et al. 2023). However, their inherent flexibility also leads to instability, especially under moderate temperatures or in organic solvents (Ali et al. 2013; de Andrade Silva et al. 2022). Immobilizing psychrophilic enzymes in CatIB form may offer a solution: the CatIB matrix restricts molecular motion, increasing enzyme resilience without compromising cold activity (Ali et al. 2020).

In this study, we explored the formation and characterization of CatIBs from **LipAMS8**, a cold-adapted lipase cloned from the Antarctic *Pseudomonas* sp. strain AMS8. LipAMS8 is a member of the RTX (repeat-in-toxin) lipase family and has previously been reported to function in cold and solvent-rich environments (Mohamad Ali et al., 2013). We demonstrate that overexpression of LipAMS8 in *E. coli* leads to the spontaneous formation of CatIBs, even in the absence of fusion tags. These outstanding properties of *E. coli* resemble those reported in *Pichia pastoris*, which have wider application in recombinant technology (Ergün et al. 2022).We investigate their morphology, biochemical properties, and industrially relevant stabilities. This work is the first to report a naturally formed cold-active lipase in CatIB form and presents LipAMS8 CatIBs as a powerful biocatalyst for low-temperature and harsh-condition applications.

Materials and methods

Chemical reagents and equipment

Methanol, isooctane, toluene, hexane, benzene, ethanol, dimethyl sulfoxide (DMSO), isopropanol, acetonitrile, *n*-heptane, and octanol (each 95% purity) were purchased from Merck Millipore (Germany). Arabic gum, ammonium persulfate, and β-mercaptoethanol were from Sigma (USA). Dipotassium hydrogen phosphate, hydrochloric acid, and calcium chloride were obtained from R&M Chemicals (Malaysia). An unstained protein marker was from Fermentas (Germany). UV–visible absorbance was measured with a Pharmacia UV spectrophotometer

(USA). Cell disruption was performed using a Branson Sonifier 250 Ultrasonic Cell Disruptor (Germany), and inclusion bodies were harvested with a Beckman Coulter Avanti J-26XPI centrifuge (USA). Scanning electron microscopy (SEM) was carried out on a Zeiss SUPRA 35 VP field-emission SEM.

Bacterial strain and plasmid

Escherichia coli BL21(DE3) was used as the expression host with plasmid pET32b as the expression vector. The LipAMS8 lipase gene (from *Pseudomonas* sp. AMS8) was cloned into pET32b; the LipAMS8 protein sequence is available in NCBI GenBank (Accession HQ162821, protein ID ADM87309). Expression of recombinant LipAMS8 was induced with 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Isolation of LipAMS8 CatIBs

After IPTG induction (0.3 mM final concentration) in a 500 mL *E. coli* culture, cells producing LipAMS8 were harvested by centrifugation. Inclusion bodies were isolated using a mild cell lysis and washing procedure to avoid denaturation. The cell pellet was resuspended in lysis buffer (50 mM Tris–HCl, 50 mM NaCl, 1% Triton X-100, pH 8.0). Cells were disrupted by sonication (Branson Sonifier 250, 6 cycles of 30 s with 30 s intervals, at 40% amplitude). The lysate was centrifuged at 12,000 × *g* for 20 min at 4 °C to pellet the inclusion bodies. The pellet was washed once by resuspending in fresh Tris–HCl/Triton X-100 buffer (pH 8.0) and repeating the sonication and centrifugation. The resulting pellet (crude inclusion bodies) was then dissolved gently in plain Tris–HCl buffer (pH 8.0) and mixed on a rotary mixer for 4 h at 4 °C. A final centrifugation at 12,000 × *g* for 20 min at 4 °C yielded a clear supernatant. This mild solubilization step allowed some protein to dissolve without strong chaotropes, indicating that LipAMS8 CatIBs are partially folded (non-classical IBs).

Determination of protein content

Protein concentrations for both soluble (LipAMS8 lipase) and insoluble (CatIB) fractions were determined by the Bradford assay. Absorbance at 595 nm was measured for samples against a standard curve prepared with bovine serum albumin. For SDS-PAGE analysis, 25–30 µg of protein from each sample (total cell lysate, soluble fraction, insoluble CatIB fraction) was loaded.

Enzyme activity assay

Lipase activity was measured using an olive oil emulsion as a substrate by a colorimetric free fatty acid (FFA) quantification method (Kwon and Rhee 1986). The reaction mixture (5 mL total) contained 2.5 mL of 50 mM Tris–HCl buffer (pH 8.0) with 0.2 mL of 20 mM $CaCl_2$, 2.5 mL of olive oil, and an appropriate amount of enzyme sample (LipAMS8 CatIBs or, for comparison, soluble enzyme). Reactions were incubated at 30 °C for 30 min with agitation (150 rpm). The reaction was stopped by adding 1 mL of 6 N HCl. Released FFAs were extracted with 5 mL of isooctane, followed by the addition of 1 mL of 5% (w/v) copper (II) acetate in pyridine (pH 6.1) to form a copper-FFA complex. After phase separation, the blue complex in the organic phase was measured at 715 nm. One unit of lipase activity was defined as the amount of enzyme releasing 1 µmol of fatty acid per minute under assay conditions.

Characterization of LipAMS8 CatIBs

The optimal temperature for the activity was determined by conducting the lipase assay at temperatures ranging from 0 °C to 60 °C, in 5 °C increments. The optimal pH was determined by performing the assay in buffers of pH 5.0–10.0 (50 mM citrate, phosphate, Tris, or glycine-NaOH as appropriate) at the optimal temperature. pH stability was evaluated by initially incubating the enzyme for 1 h at various pH levels (5.0–10.0) at 4 °C, and the residual activity was measured under standard conditions. Thermal stability was assessed by incubating the enzyme at 4 °C, 25 °C, 40 °C, and 50 °C for 1 h (or longer periods for storage stability tests), then measuring residual activity at the optimal temperature. The effect of metal ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, etc., at 5 mM) on enzyme activity was tested by adding the ions to the assay mixture and incubated at 15 °C for 30 min. before it was subjected to enzyme assay. Organic solvent tolerance was examined by pre-incubating LipAMS8 CatIBs in the presence of 25% (v/v) of various solvents (methanol, ethanol, isopropanol, acetonitrile, hexane, toluene, etc.) at 15 °C for 30 min, and the residual activity was assayed. For storage stability, aliquots of LipAMS8 CatIBs were stored at 4 °C and 25 °C, and lipase activity was measured periodically over several weeks.

Statistical analysis

All the results generated are expressed in Mean ± Standard Error Mean (S.E.M). Experiments carried out were in triplicate ($n = 3$). A paired t-test and one-way analysis of variance (ANOVA) were used between different treatments using Microsoft Excel Analyze It 2016. The significance level was set at $P < 0.05$.

Results

Isolation and solubilisation of LipAMS8 CatIBs

LipAMS8 lipase was expressed in *E. coli* BL21(De3)/pET32b using 0.3 mM isopropyl β D-1 thiogalacto pyranose (IPTG). The sample was separated using centrifugation and solubilised using a mild solubilising agent. LipAMS8 CatIBs were solubilized in the absence of any

denaturant agent which shows it is non-classical nature. Centrifugation of the sample at 12,000 g resulted in the formation of a cleared supernatant devoid of any form of turbidity. The clear supernatant was used to carry out the refolding of LipAMS8 CatIBs in a ratio of 1:9 in which 1 mL of the supernatant was dissolved into 9 mL of Tris-HCl buffer (pH8.0.).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12% polyacrylamide resolving gel and 4% stacking gel. The unstained marker was used as a reference to estimate the molecular weight of LipAMS8 CatIBs. Proteins were stained using Brilliant Blue R250 and were destained using a destaining solution.

Scanning electron microscopy (SEM)

Scanning electron microscopy was carried out to ascertain the structure of LipAMS8 CatIBs. Field scanning electron microscopy of the LipAMS8 CatIBs at 20,000x. 50,000x magnification, which revealed its morphology as a spherical rod-like structure which resembles that of non-classical inclusion bodies (Active). The magnification at 20,000x indicated the size of inclusion at around 1 µm whereas that of 50,000x presented the size of 100 nm.

Effect of temperature on activity and stability of LipAMS8 CatIBs

The temperature effect on LipAMS8 CatIBs activity was evaluated at 10–80 °C. The effect of temperature on activity was carried out by preincubating the LipAMS8 CatIBs at a temperature range of 10–80 °C after which it was analysed using the Kwon and Rhee method to determine the activity. The thermal stability was carried out by preincubating the LipAMS8 CatIBs at a temperature range of 10–80 °C after which it was analysed using the Kwon and Rhee method to determine the activity. The half-life of the LipAMS8 CatIBs was investigated by preincubating the enzymes at 20 °C, 30 °C and 40 °C at a time interval of (0–10 h).

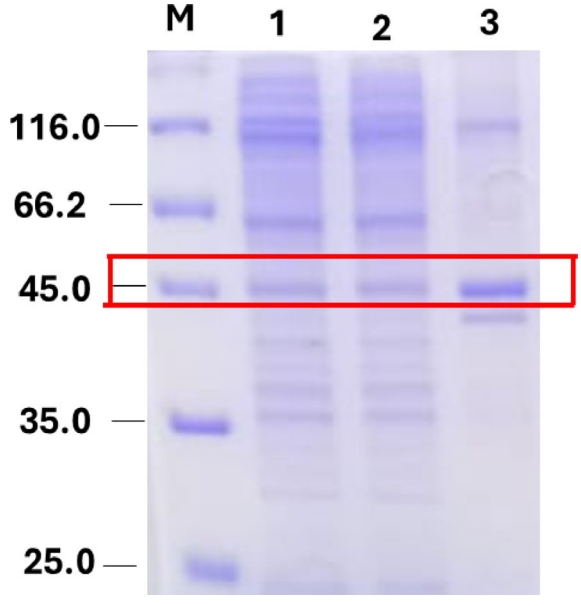

Fig. 1 SDS-PAGE analysis of the recombinant LipAMS8 CatIBs. Lane 1 is total protein Lane 2 is crude soluble, Lane 3 is insoluble (CatIBs), whereas Lane M stands for unstained protein marker

Effect of pH on activity and stability of LipAMS8 CatIBs

The effect of pH on the activity of LipAMS8 CatIBs was conducted using buffers of varying pH values (4–12.0). The effect of pH on the stability of LipAMS8 CatIBs was evaluated by initially incubating LipAMS8 CatIBs with an equal amount of buffer of different pH (4–12.0) in a ratio of 1:1 at 15 °C for 30 min. followed by the determination of its activity using the Kwon and Rhee method. As shown in Fig. 1 LipAMS8 CatIBs exhibited high stability compared to soluble enzymes.

Effect of organic solvent

It was carried out by preincubating LipAMS8 CatIBs with 25%(v/v) of various organic solvents. The organic solvents were arranged based on their log p-value and the stability of the LipAMS8 CatIBs was evaluated by assaying the initially incubated enzyme using the Kwon and Rhee method.

Effect of metal ion on the stability of LipAMS8 CatIBs

The metal ion interaction of LipAMS8 CatIBs was evaluated by preincubating the LipAMS8 CatIBs with 30%(v/v) 1 mM and 5 mM of different metal ions at 15 °C for 30 min. The incubated enzyme activity was then assayed using the Kwon and Rhee method.

Substrate specificity

The substrate specificity of LipAMs8 CatIBs was determined using **various substrates such as** Lauric acid p-Para nitrophenyl (C_{12}) followed by butyric (C_4), (C_{10}), (C_8) and (C_{16}) respectively. 0.1 M phosphate buffer (pH 7.4) and pNp-substrate with 200 rpm agitation were used to determine the activity of LipAMS8 CatIBs measured at 410 nm.

Storage stability

Storage stability of LipAMS8 CatIBs was conducted by storing the enzyme at 4 and 25 °C followed by assaying the enzyme activity. It was incubated for thirteen and eight weeks, respectively. The initial activity at zero was used as a control in the experiment.

Discussion

Isolation and solubilization of LipAMS8 CatIBs

Overexpression of LipAMS8 in *E. coli* BL21(DE3) resulted in a high-yield production of insoluble protein, visible as inclusion bodies after cell lysis. We employed a mild solubilization approach to isolate and partially dissolve these inclusion bodies (Singh et al. 2015). The use of a non-denaturing buffer with a low concentration of detergent (1% Triton X-100) allowed us to remove cell debris and loosely bound contaminants without unfolding the aggregated enzyme (Ma et al. 2020). Maintaining a low temperature (4 °C) and avoiding harsh chaotropes were crucial for preserving the secondary structure of the IB protein. Mild solubilization is recommended to prevent further aggregation during IB handling, and it exploits the fact that CatIBs already contain correctly folded regions (Klausser et al. 2023). In our case, simply resuspending the washed LipAMS8 inclusion bodies in Tris buffer (pH 8.0) and stirring at 4 °C released a fraction of the protein into solution (clear supernatant) without any denaturant. This behaviour confirms the non-classical nature of LipAMS8 IBs – some of the enzyme was natively folded enough to dissolve on its own, unlike classical IBs, which require strong denaturants to solubilize (Ban et al. 2020).

We obtained a clear supernatant after centrifugation, indicating successful separation of soluble (refolded) enzyme from the still-aggregated core. The gentle refolding (by dilution of the supernatant into fresh buffer) provided active enzyme for further assays. It is known that many factors can affect the refolding yield from IBs(Jürgen et al. 2010).In our protocol, the avoidance of any urea or guanidine may have helped preserve native-like conformations, leading to spontaneous refolding of some LipAMS8. Overall, the isolation procedure yielded a preparation of LipAMS8 CatIBs that could either be used directly as a catalytic aggregate or could be partially refolded to a soluble form as needed.

The protein content and purity of the LipAMS8 CatIB preparation Were analyzed by SDS-PAGE (Fig. 2). A single major band corresponding to a molecular weight of approximately 45 kDa was observed for the insoluble fraction (Lane 3). This is slightly lower than the ~50 kDa band reported for the soluble LipAMS8 enzyme(Mohamad Ali et al. 2013). A similar shift in apparent molecular weight upon aggregation has been previously noted for other enzymes; for example, Tokatlidis et al. (1991) observed that an endoglucanase appeared slightly altered in size after forming IBs (Toatlidis et al., 1991). The minor difference here may be due to a tag truncation or subtle conformational effects on SDS binding. The IB fraction is largely composed of the target LipAMS8 protein, with very few contaminant bands, confirming that the inclusion bodies are a relatively pure source of the recombinant enzyme. The high purity and yield of the target protein in IBs underscores the key advantage of using IBs for bioprocessing.

Morphology of LipAMS8 CatIBs by electron microscopy

The morphology of the LipAMS8 inclusion bodies is determined by field emission SEM. At 20,000× and 50,000× magnifications, the LipAMS8 CatIBs appeared as spherical to rod-like nanoparticles (Fig. 3). The aggregates had a somewhat elongated rod-shaped geometry,

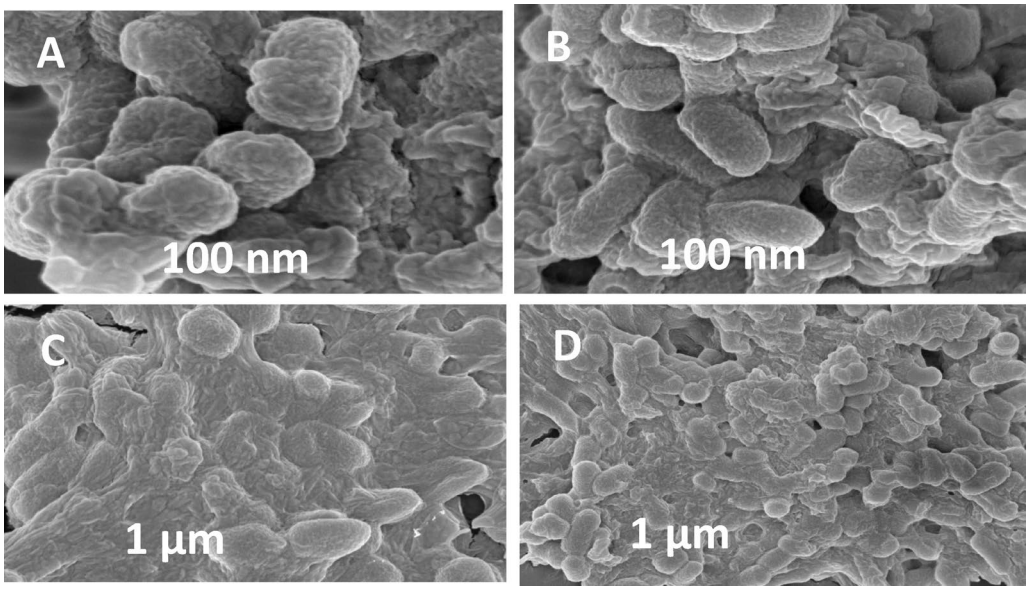

Fig. 2 Field scanning electron microscopy showing the morphology of LipAMS8 CatIBs expressed in *E. coli*. BL21(De3)/pET32b LipAMS8 lipase with 0.3 mM IPTG and viewed (A and B) at 50,000x magnification whereas (C&D) at 20,000x magnification

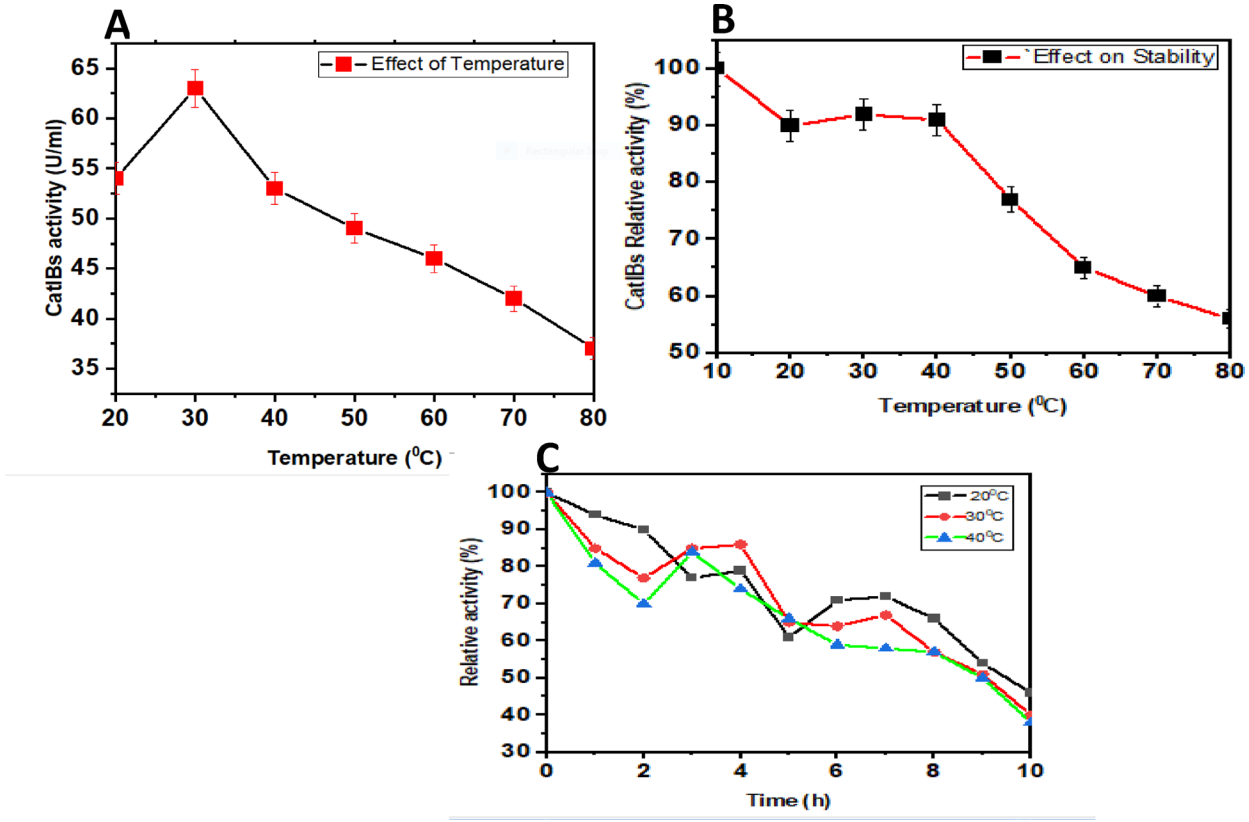

Fig. 3 Effect of temperature on activity and stability of LipAMS8 CatIBs (**A**) Presented effect of temperature on LipAMS8 CatIBs activity. The measurement was carried out in triplicate ($n = 3$) and expressed in mean ± S.E.M. (**B**) Effect of temperature on Thermal stability of LipAMS8 CatIBs measurement was in triplicate($n = 3$). (**c**) Half-life of LipAMS8 CatIBs which measurement was carried out in triplicate ($n = 3$) after preincubating LipAMS8 CatIBs (0–10 h)

with lengths in the order of 0.1–1.0 µm and widths of ~ 100–200 nm. They often formed clusters, but individual particles were discernible as curved rods or short filaments. This shape is consistent with that of many protein IBs, which can be spherical or ellipsoidal but sometimes appear as short fibrils, likely reflecting the underlying β-sheet amyloid structure (Sabaté et al. 2009).

The observed size range (hundreds of nanometers) falls within the size range of typical bacterial IBs (Rinas et al. 2017). At this resolution, the surface of the LipAMS8 CatIBs was rough and lacked any obvious regular substructure. Such a texture is common for IBs, which are aggregates of partially folded proteins. The compact, particulate nature of LipAMS8 CatIBs reveals that they could be handled like nanoparticle biocatalysts. Similar rod-like IB morphologies have been reported, for instance, for IBs in lactobacilli that were ~ 250 nm smooth spheres and for engineered enzyme CatIBs, which can form porous or fibrillar aggregates (Ponnusamy et al. 2011).

The structural integrity of LipAMS8 CatIBs under SEM (no melting or collapse during preparation) attests to their mechanical stability. This physical robustness is one reason IBs are attractive as biomaterials – they can withstand shear forces and dehydration better than soluble proteins (Hikmawati et al. 2019). In summary, microscopy confirms that LipAMS8 CatIBs are discrete, submicron protein particles with morphology typical of amyloid-like inclusion bodies.

Catalytic activity and optimal conditions of LipAMS8 CatIBs

Despite being in aggregated form, LipAMS8 CatIBs exhibited clear lipase activity in our assays, confirming their status as catalytically active inclusion bodies. The CatIB suspension hydrolyzed olive oil emulsions, producing free fatty acids (FFAs) at a rate comparable to (in fact slightly higher than) an equivalent amount of refolded soluble LipAMS8. The optimal temperature for LipAMS8 CatIB activity was determined to be around 30 °C. This aligns with the enzyme's psychrophilic nature—it operates efficiently at ambient or lower temperatures, unlike typical mesophilic lipases that often prefer 40–60 °C (Nowak and Otzen 2024).

Interestingly, the activity of CatIBs decreased above 40 °C, which is consistent with observations for other cold-active *Pseudomonas* lipases (Abdella et al. 2023). Prior studies on soluble LipAMS8 and related Antarctic lipases reported optimal activity in the 30–45 °C range (Ganasen et al. 2016). Our CatIB form maintains this

profile, revealing that the temperature-activity characteristics are an intrinsic property of the enzyme's structure and are retained even in aggregated form. While some immobilized enzymes show upward temperature optima shifts due to diffusional or structural constraints (Khan 2021).LipAMS8 CatIBs did not exhibit such a shift, reinforcing the notion that the cold-adapted nature of the enzyme persists in the aggregated form.

The optimal pH for the CatIBs was found to be approximately 8.0, which is consistent with the alkaliphilic behaviour of many microbial lipases (Khan 2021). Our experiments revealed that LipAMS8 CatIBs retained high activity in the pH range of 7 to 9, with a peak at pH 8.0. Activity declined significantly below pH 6 and above pH 10, but remained above 50% at both pH 5 and 10, demonstrating broad pH tolerance (Riyadi et al. 2024). This behaviour is advantageous for industrial applications, particularly in processes that involve extreme pH environments.

The improved pH tolerance of the CatIBs may result from the aggregated state, which can protect internal catalytic domains from external fluctuations. Similar behaviour has been reported for CatIBs of other enzymes, such as (R)-hydroxynitrile lyase, which showed enhanced activity in acidic conditions compared to the soluble form (Pei et al. 2022). The CatIB matrix may provide a stabilizing microenvironment that buffers pH and restricts conformational changes that would otherwise reduce enzyme activity. The effects of temperature and pH are presented in Figs. 4 and 1.

Stability of LipAMS8 catibs: thermal, pH, solvent tolerance, substrate specificity and storage stability

One of the most significant findings of this study is the exceptional stability of LipAMS8 in the form of CatIB. Stability was assessed in terms of thermal resilience, pH tolerance, solvent resistance, and long-term storage. In all aspects, LipAMS8 CatIBs significantly outperformed expectations for a typical cold-active enzyme.

Thermal stability

At 40 °C, LipAMS8 CatIBs retained approximately 60% of their original activity after 1 h—remarkable for a psychrophilic enzyme whose soluble form is typically unstable above 35 °C. Even at 50 °C, the CatIBs maintained detectable activity (~ 30%) for a short period. This thermal tolerance likely arises from aggregation-induced immobilization, which stabilizes partially folded structures and prevents unfolding pathways. Similar enhancements in thermostability have been reported for other CatIBs and cross-linked enzyme aggregates (CLEAs), such as those of *Candida antarctica* lipase B (CALB) (Han et al. 2021).

pH stability

The CatIBs maintained more than 50% of their original activity after 1 h across a broad pH range (5.0 to 10.0),

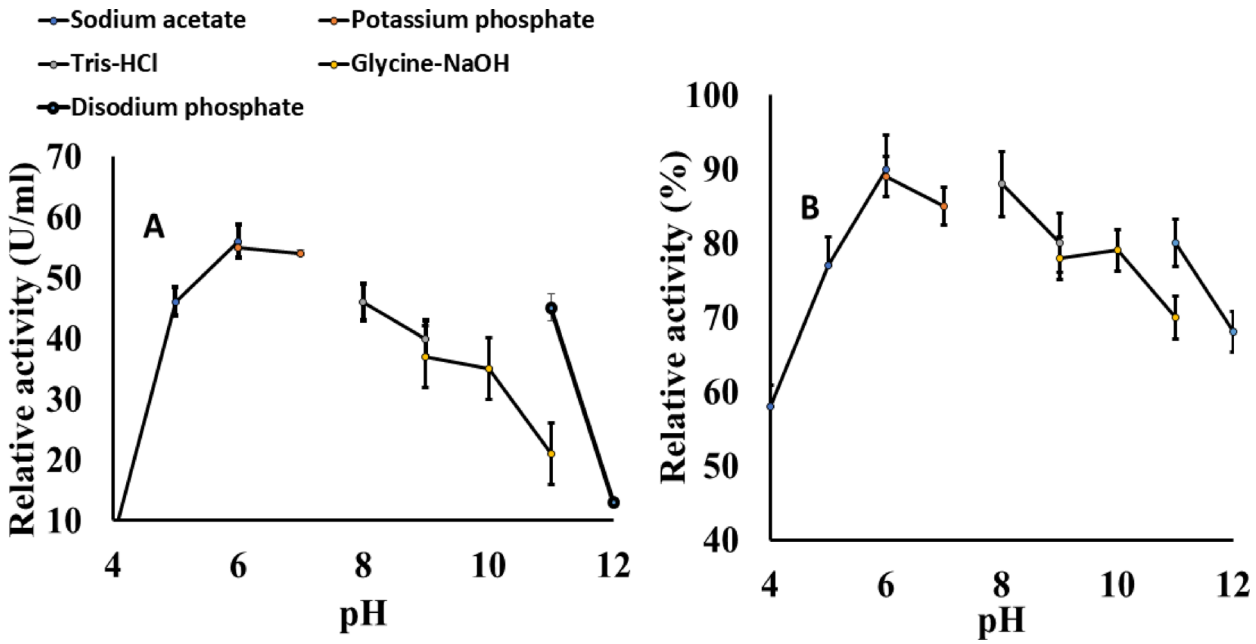

Fig. 4 Shows the effect of pH on the activity and stability of LipAMS8 CatIBs (**A**) represents the effect of pH on the activity of LipAMS8 CatIBs evaluated using buffers with values (4-12). All measurement was carried out in triplicate (n=3). (**B**) Shows the effect of pH on the stability of LipAMS8 CatIBs investigated by preincubating LipAMS8 CatIBs with buffers of pH (4-12.0). All measurement was carried out in triplicate (n=3). The value was expressed in Mean±S.E.M

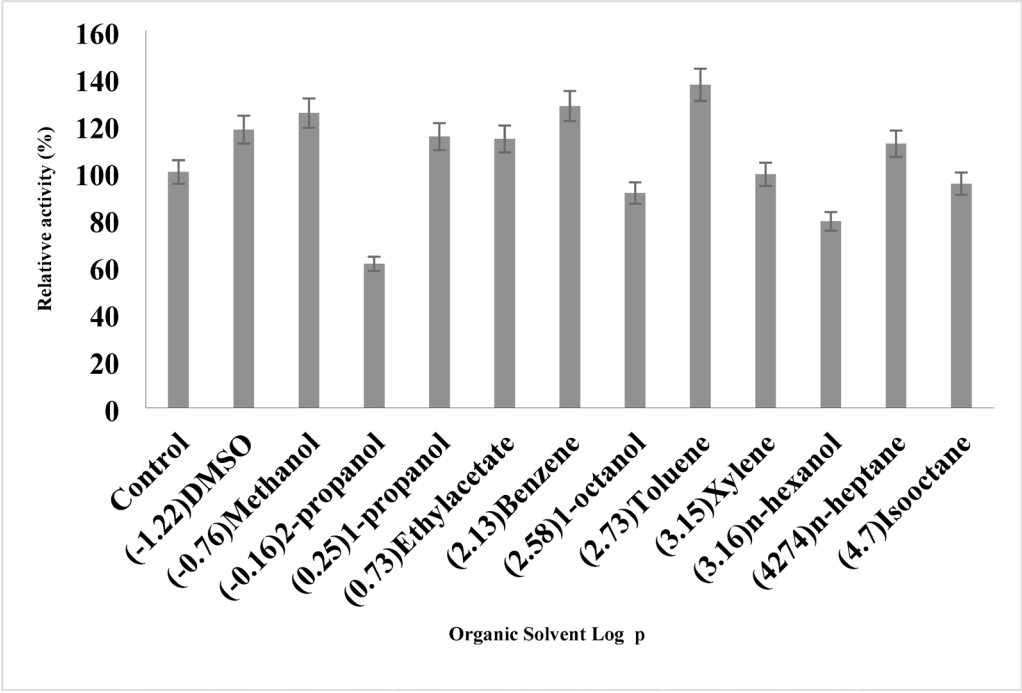

Fig. 5 Shows the effect of various organic solvents on the stability of LipAMS8 CatIBs. It was preincubated with 25%(v/v) of the different organic solvents at 15^0C for 30 min. followed by measurement of LipAMS8 CatIBs activity using the Kwon and Rhee method. LipAMS8 CatIBs in the absence of any organic solvent was used as a control with 100% activity. All measurement was carried out in triplicate (n=3). The Log P values of organic solvents were listed in parentheses

with maximal stability near pH 7–8. This range exceeds that of many soluble lipases, which often exhibit irreversible denaturation outside a narrow pH optimum (Sukohidayat et al. 2018). The aggregated matrix likely shelters catalytic residues from harsh pH environments, either by creating a buffering microenvironment or restricting structural flexibility. This trait is especially beneficial for industrial processes such as detergent formulation or ester synthesis in acidic or alkaline media.

Solvent tolerance

LipAMS8 CatIBs demonstrated remarkable tolerance to organic solvents. In the presence of 25% (v/v) methanol and ethanol, CatIBs retained over 80% of activity; with hexane, isooctane, and toluene, retention exceeded 90%. Even in highly denaturing solvents like DMSO and acetonitrile, CatIBs preserved 50–70% of their activity. This solvent resilience is a key advantage of CatIB-based immobilization and supports their use in non-aqueous biocatalysis, such as transesterification or biodiesel production (Bello et al. 2024).

Substrate specificity

LipAMS8 lipase CatIBs show the highest activity with Lauric acid p-Para nitrophenyl (C_{12}) followed by butyric (C_4), (C_{10}), (C_8) and (C_{16}) respectively, as shown in Fig. From the result, it can be deduced that LipAMS8 CatIBs can catalyse the hydrolysis of different chains of fatty acids. Many reports indicated that lipase can act on short and medium-chain fatty acids (Rozi et al., 2022). The wild type of soluble enzyme also manifests a preference for (C_{12}). The lipase isolated from *Staphylococcus caprae* NCU S6 also shows similar properties toward short and medium-chain fatty acids (Zhao et al 2021). Therefore, LipAMS8 CatIBs have broad substrate specificity across short and medium as well as long-chain fatty acids. The study conducted on *Pseudomonas aeruginosa* lipase revealed its preference for trilaurin and tripalmitin (Borkar et al., 2009). LipAMS8 CatIBs is a good candidate for short ester and biodieselsynthesis.

Storage stability

CatIBs also exhibited excellent long-term storage stability. When stored at 4 °C, they retained > 50% of their initial activity even after 13 weeks. At room temperature (25 °C), CatIBs remained > 50% active after 8 weeks. In contrast, psychrophilic enzymes typically lose significant activity over time due to their structural flexibility(Miri et al. 2024). The IB format effectively "freezes" the enzyme in a partially rigid conformation, preventing time-dependent inactivation.

These findings collectively demonstrate that immobilization via CatIB formation significantly extends the usable range of LipAMS8 in terms of thermal, pH, and

solvent stability, and shelf-life. This makes LipAMS8 CatIBs ideal candidates for industrial settings where harsh conditions or extended storage are required; the results of solvent tolerance, metal ions, and storage stability are presented in Figs. 4, 1, 6, 7 and 8, respectively.

Comparison with other CatIB systems

The successful characterization of LipAMS8 CatIBs enables comparison with other known CatIB systems. A distinguishing feature of LipAMS8 CatIBs is that they form **naturally**, without requiring any artificial aggregation tags or fusion domains. Many previously studied CatIBs rely on engineered tags (e.g., ELK16, coiled coils like TDoT or 3HAMP) to induce ordered aggregation and preserve activity (Jäger et al. 2019). In contrast, LipAMS8 spontaneously forms catalytically active IBs upon overexpression in *E. coli*, highlighting a rare and desirable property—**natural self-assembly into an active immobilized form**.

This spontaneous CatIB formation is likely due to the intrinsic folding and oligomerization tendencies of LipAMS8, a cold-active RTX lipase. Naturally forming CatIBs have been observed in only a limited number of enzymes(Gil-Garcia et al. 2020), making LipAMS8 a valuable model for studying non-engineered CatIB formation and function.

In terms of **biocatalytic performance**, LipAMS8 CatIBs showed high residual activity—comparable to or even exceeding the activity of the soluble, refolded enzyme. While some CatIB systems suffer from reduced catalytic efficiency due to misfolding within aggregates, others, like LipAMS8, retain native-like conformations. Jäger et al. (2020) reported that CatIBs formed with coiled-coil tags had residual activities ranging from 30 to 150%, depending on enzyme and tag used (Jäger et al. 2020). Our findings place LipAMS8 at the high end of this spectrum.

LipAMS8 CatIBs also performed well relative to **immobilized enzyme systems**. Unlike conventional immobilization strategies, which often require multi-step processes and expensive carriers, CatIBs are formed directly during expression. They bypass purification, support binding, and chemical cross-linking, simplifying production and lowering costs. In fact, some studies (e.g., Mestrom et al. 2020) have shown that CatIBs outperformed traditional immobilized enzymes in productivity, despite slightly lower specific activity(Mestrom et al. 2020).

From an industrial standpoint, LipAMS8 CatIBs offer **key advantages**:

- **High yield and purity** from direct expression.
- **Enhanced robustness** under thermal, pH, and solvent stress.
- **Recyclability and ease of use** akin to solid catalysts.
- **Long shelf-life** even without refrigeration.

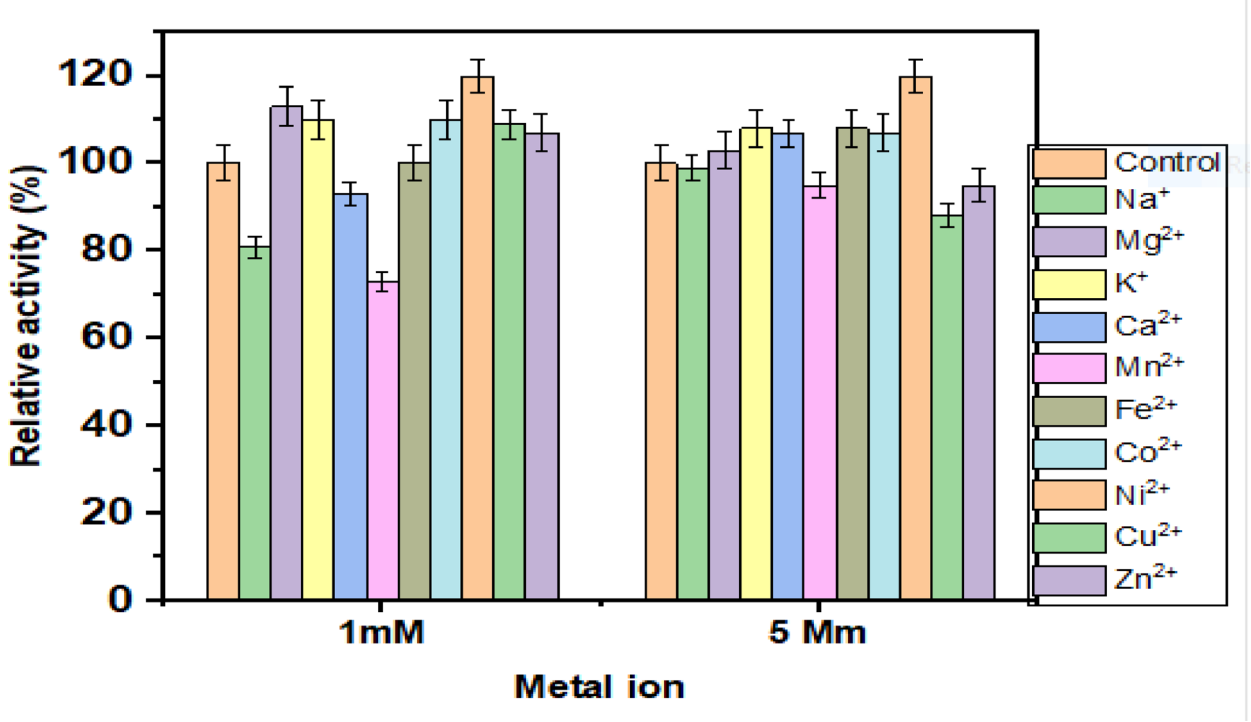

Fig. 6 Effect of metal ion on stability of LipAMS8 CatIBs. LipAMS8 CatIBs was preincubated with 30%(v/v) 1 Mm and 5 Mm metal ion at 15^{0}C for 30 minutes followed by LipAMS8 CatIBs activity determination. All measurement was performed in triplicate (n=3)

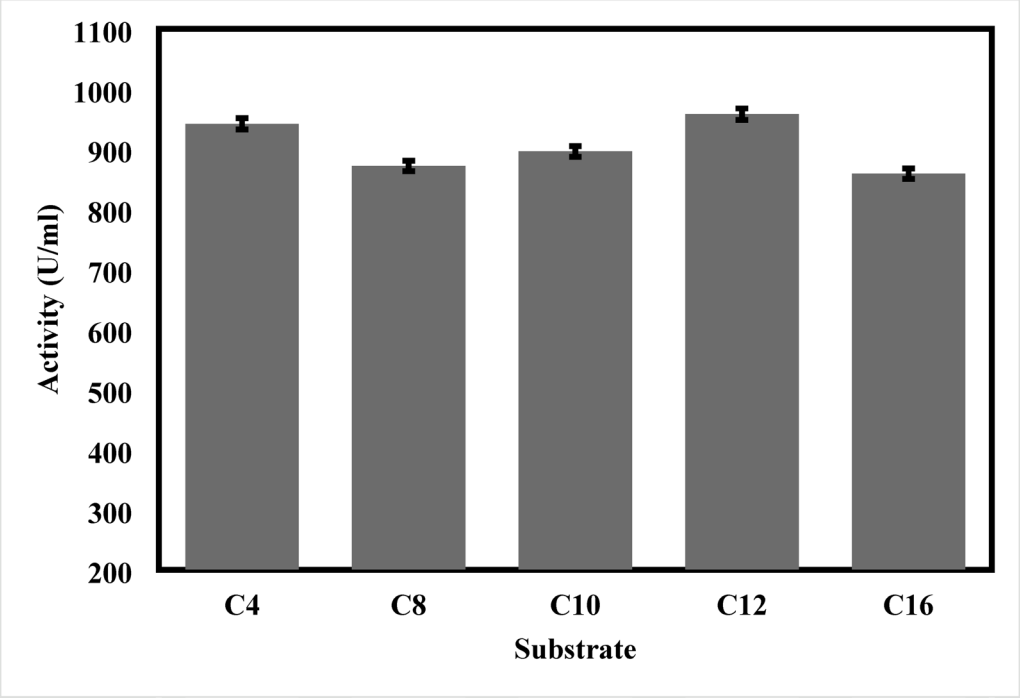

Fig. 7 Substrate specificity of LipAMS8 CatIBs. LipAMS8 CatIBs were evaluated using different p- Para nitrophenyl substrates (C_4-C_{16}). 220 µl of 0.1 M phosphate buffer (pH 7.4) and 20 µl of various substrates were incubated with 5 µl of LipAMS8 CatIBs with agitation of 200 rpm for 1o min. to produce a yellow colour which was read at 410 nm in a microplate reader

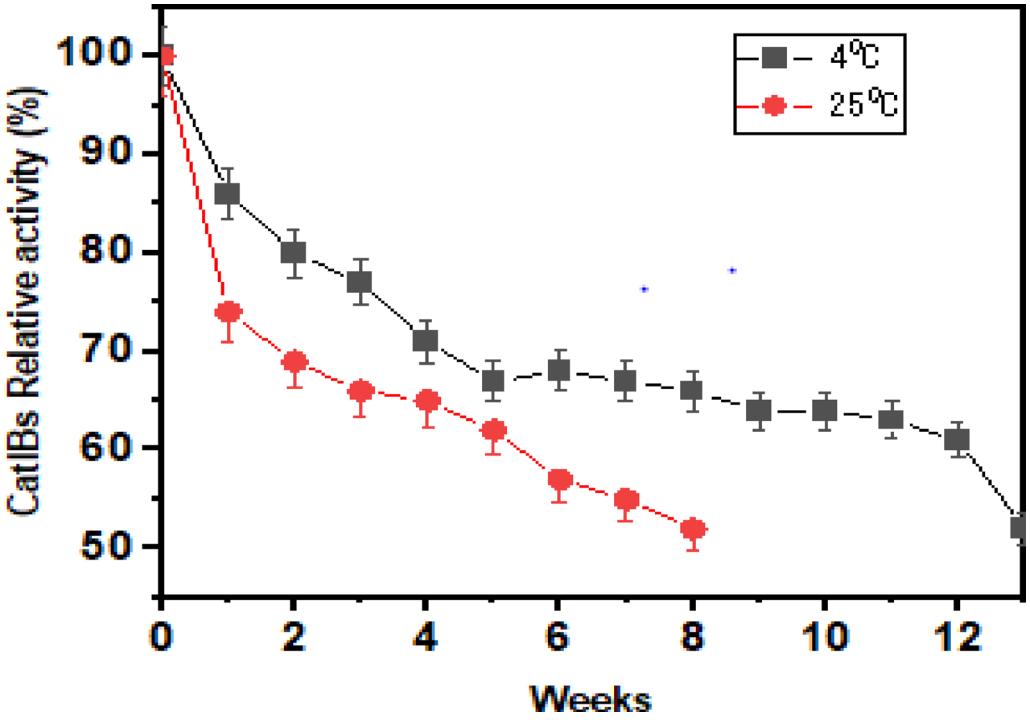

Fig. 8 Storage stability of LipAMS8 CatIBs stored at 4^0C and 25^0C for 13 and 8 weeks respectively. LipAMS8 CatIBs activity measured at zero week was considered as 100%. All measurements were conducted in triplicate (n=3)

Furthermore, their **cold-activity profile** makes them uniquely suited for energy-efficient biocatalysis, low-temperature esterification, or enzymatic processing of volatile compounds. This is particularly important for food, pharmaceutical, and green chemistry sectors where temperature control is critical.

In summary, LipAMS8 CatIBs match or exceed the performance of engineered CatIBs and immobilized enzymes across several metrics. Their natural formation, combined with exceptional stability and solvent tolerance, suggests a new class of CatIBs—**self-forming, cold-active nanobiocatalysts**—that could be harnessed for scalable industrial processes.

Conclusion

In this study, we successfully produced and characterized **catalytically active inclusion bodies (CatIBs)** of the cold-adapted lipase LipAMS8 from Antarctic *Pseudomonas* sp. AMS8. These CatIBs were obtained naturally during overexpression in *E. coli* without the use of aggregation-inducing tags and were purified under mild, non-denaturing conditions. SEM analysis revealed nano- to microscale aggregates with typical IB morphology. Biochemical assays confirmed that these CatIBs retained substantial enzymatic activity and demonstrated optimal activity at ~30 °C and pH 8.0.

Notably, LipAMS8 CatIBs exhibited enhanced thermal and pH stability, exceptional solvent tolerance, and long-term storage resilience. These properties distinguish LipAMS8 CatIBs from traditional soluble enzymes and position them as robust, carrier-free immobilized biocatalysts. Compared to engineered or tagged CatIB systems, LipAMS8 offers a streamlined production route with high yield, activity, and simplicity.

This work marks the first detailed study of a naturally occurring cold-active lipase in CatIB form and expands the potential for using psychrophilic enzymes in industrial processes. LipAMS8 CatIBs are especially promising for applications in non-aqueous biotransformations, biodiesel production, low-temperature catalysis, and eco-friendly industrial biotechnology. Future work may focus on reactor-scale performance and recyclability under process conditions.

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

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Author contributions
Conceptualization, methodology and writing original draft, Muhammad. N.B, Conceptualization, methodology, resources and writing reviewing Mohd Shukuri. M. A., validation and writing reviewing, Normi M.Y, validation and resources Suriana S, formal analysis, and resources, Fairolniza M.S. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval
The research does not involve humans or animals.

Consent to participate
The research does not use human subjects.

Consent for publication
The manuscript does not contain any images that are from other authors.

Competing interests
The authors declare no competing interests.

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