Cold-Active Lipases and Esterases

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One of the survival strategies adopted by microorganisms living in cold environments is their expression of cold-active enzymes that enable them to perform an efficient metabolic flux at low temperatures necessary to thrive and reproduce under those constraints. Cold-active enzymes are ideal biocatalysts that can reduce the need for heating procedures and improve industrial processes' quality, sustainability, and cost-effectiveness.

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1. Introduction

Cold-active enzymes are produced by psychrophilic microorganisms that are often heat-labile and perform a high catalytic activity at moderate to very low temperatures in contrast to their thermophilic and mesophilic orthologs [1][2]. The cold-active extremozymes generally achieved their efficient biochemical reactions by lowering both the enthalpy of activation and Gibbs free energy compared to their thermophilic and mesophilic counterparts [3]. Cold-active enzyme structures are homologous to their mesophilic counterparts. They only differ by discrete changes in their amino acid and spatial polypeptide structures, which are responsible for their distinct functions [4][5].

Cold-active enzymes have a high specific activity, low-affinity on the substrate at low temperatures, and they are structurally more flexible at their active sites; this flexible nature is due to weak intermolecular forces and increased exposure of hydrophobic residues [3][6][7]. Compared to their mesophilic and thermophilic counterparts, these features of high catalytic efficiency at low temperatures make these extremozymes highly attractive to the scientific community and provide potential applications in detergency, bioremediation, biofuels, and food industries [2][8][9]. Cold-active hydrolases such as protease, lipase, amylase, and cellulase were the most frequent enzymes characterised and used for industrial purposes compared to other cold-active enzymes [10][11][12][13]. Cold-active enzymes, in general, are ideal biocatalysts that can reduce the need for heating procedures, which improves the sustainability, cost-effectiveness, energy consumption, and quality of industrial production [2].

2. Cold-Active Lipase and Esterase Overexpression in Recombinant Heterologous Hosts

The most common strategy for obtaining large quantities of desired proteins is recombinant overexpression in a heterologous host [14][15]. Although the technique is often used in producing cold-active lipases and esterases, it is not specific to even cold-adapted enzymes but all recombinant proteins. When expressed in the cytosol,

recombinant proteins are often produced at a greater yield, but they may also be regulated to be released into the culture media [14]. The overexpression of recombinant cold-active lipase and esterase is often achieved using mesophilic expression systems such as *E. coli* [16], yeast [17], and insects [18]. The production of large quantities of such enzymes at high concentrations remains challenging. As for other cold-active enzymes, the temperatures that cold-active lipase and esterase require for proper folding is inconsistent with the optimal growth temperature of these expression hosts [2]. The typical approach to mitigate folding problems in *E. coli* is to reduce the post-induction temperature below 20 °C. However, this slows down the host growth rate and the heterologous enzyme's synthesis rate. **Table 1** summarises some recently reported overexpression of cold-active lipase and esterase in a recombinant heterologous host.

E. coli was selected as the preferred expression host, and just one of the enzymes was produced in *Saccharomyces cerevisiae* (*S. cerevisiae*). However, the *E. coli* Rosetta ™ strain was reported to be used once [19], BL21 (DE3), was the most popular. Other Gram-negative bacteria, such as *Pseudomonas* and *Burkholderia*, lack suitable promoters and require foldase (a special chaperon) and extracellular fatty acids to induce their expression, a mechanism that is primarily unclear [20][21]. The two most common yeasts used for expression systems were *S. cerevisiae* (Baker's yeast) and *Pichia pastoris* (*P. pastoris*). Its major drawback is its strong natural tendency of S. cerevisiae to ferment carbohydrates to ethanol, which is toxic at low culture density. However, *P. pastoris* lacks the problem of harmful ethanol synthesis, but it cannot express any gene of interest. While specific proteins may have no issues with being expressed, others may have problems associated with glycosylation, secretion, and folding [22][23]. A recent study on recombinant overexpression by Xue, Yao [17] found excellent expression of cold-active esterase in the *S. cerevisiae* heterologous host, which was attributed to similarities between the yeast family to which the wild gene and *S. cerevisiae* belongs. Since the carbon source was n-propanol and isobutanol and not sugars, the limitation of using *S. cerevisiae* was not mentioned. Another heterologous host for recombinant proteins is insect cell culture systems, which are well-known for their use in creating vaccines and viral insecticides [24][25]. Compared to other eukaryotic expression systems, high levels of heterologous gene expression are frequently achieved, especially for intracellular proteins [26]. In several instances, the recombinant proteins are soluble and easily collected from infected cells [26][27]. In one study, a *Yarrowia lipolytica* (LIPY8) extracellular lipase gene was expressed using a baculovirus expression system in insect cells, and it was interesting that the best pH and temperature for cold-active lipase LipY8p expressed in insect cells were very different from those for the same enzyme expressed in *P. pastoris* [18]. Moreover, it is too early to conclude how the change in heterologous host from yeast to insect increases the cold activeness of a particular enzyme. On the other hand, adaptability to a wide range of culture broths and its rapid growth and high enzyme yield were the major favourable characteristics that allowed the utilisation of *E. coli* for recombinant overexpression of heterologous proteins [28][29]. The major disadvantage of using *E. coli* host is the production of bodies [30].

Inclusion bodies are insoluble protein aggregates that lack biological function [31]; their formation often occurs when eukaryotic proteins are overexpressed in a heterologous host such as *E. coli* [32]. Inclusion bodies have been considered a significant obstacle to producing soluble and active recombinant proteins [33][34]. In **Table 1**, most of the cold-active lipase and esterase were overexpressed in soluble forms, and only five (5) were produced as insoluble or soluble but in inactive forms. It is difficult to explain why most articles examined herein reported more

soluble expression than insoluble inclusion bodies. Furthermore, there has been a great success not only in using biochemical and molecular techniques to prevent their formation or to address various challenges during their isolation, solubilisation, refolding, and purification [33], but their biological activity is also emerging [35][36] contrary to the previous notion that they lack activity [31].

Table 1. Cold Active Lipase and Esterase Overexpressed in Heterologous Host.

Organisms/Enzymes	Source	Host	Vector	Localization of Expressed Enzyme	Optimum Temp./Residual Activity	References
Alkalibacterium sp. SL3/esterase	Uncultured	*E. coli* BL21 (DE3)	pET-28a (+)	Soluble	30 °C and 68% at 0 °C	[37]
Chitinophaga pinensis-like/esterase	Uncultured	*E. coli* RosettaTM (Novagen)	pGEX-6P-2	Insoluble inclusion body	20 °C and NA	[19]
Lactobacillus plantarum/*LpLp_2631*/esterase	Microbiological Culture	*E. coli* BL21 (DE3)	pURI3TEV vector	Soluble	20 °C and 90% at 5 °C	[38]
Burkholderia pyrrocinia/BpFae esterase	Microbiological Culture	*E. coli* BL21 (DE3)	pET28a pCold-TF and pGEX-4T-1.	Insoluble/soluble non inactive form	NA	[39]
Candida parapsilosis/esterase	Cultured	*S. cerevisiae*	pYES2	Soluble	NA and at 20 °C	[17]
Monascus ruber M7/esterase	Cultured	*E. coli* BL21(DE3)	pET-30a (+)	Soluble	40 °C and 50% at 4–10 °C	[40]
Alcanivorax dieselolei/lipase	Cultured	*E. coli* BL21(DE3)	pGEX-6p-1 (GE)	Soluble	20 °C and 95% at 10 °C	[41][42]
Pseudomonas fluorescens KE38/lipase	Uncultured	*E. coli* BL21(DE3)	pET28a	Insoluble inclusion body	25 °C and NA	[43]
Aphanizomenon flos-aquae/esterase	Uncultured	*E. coli* BL21(DE3)	pET28a	Insoluble inclusion body	5–15 °C	[44]
Bacillus halodurans/lipase	Uncultured	*E. coli* BL21 (DE3)	pET-28a (+)	Soluble	30 °C	[45]
Bacillus licheniformis/*esterase*	*Cultured*	*E. coli* BL21 (DE3)	pET-28a (+)	Soluble	30 °C and 35% at 0 °C	[16]

Organisms/Enzymes	Source	Host	Vector	Localization of Expressed Enzyme	Optimum Temp./Residual Activity	References
G. antarctica PI12/esterase	Expressed sequence tag	BL21 (DE3)	pET200_GaDlh	Soluble	10 °C and 50% at 0–30 °C	[46]
Paenibacillus sp. R4/esterase	Cultured	BL21 (DE3)	pET-22b (+)	Soluble	35 °C and 45% at 10 °C	[47]
Pseudomonas sp./lipase	Uncultured	BL21(DE3)	pET32b (+)	Insoluble inclusion body	35 °C and 50% at 15–40 °C	[48]
Yarrowia lipolytica(*LIPY8*)/lipase	Cultured	Insect (Sf9)	pFastBac1	Soluble	17 °C and 70% at 8–30 °C	[18]

NA—not available.

lates the shelf life on of the [37]. For ations of [39]. The targeted in the experimental design and analytical phases [40]. One example is a high-throughput process development (HTPD) that saves time and cost while harmonising purification procedures through increased automation, miniaturisation, and practical data analysis [41]. A similar format with miniaturised columns enables a high-throughput selection of adsorbent and separation parameters during binding and elution purification experiments. Integrated robot platforms are also employed for choosing a suitable adsorbent in 96-well plates or microcolumn that is essential for determining the success or failure of the purification step [42]. In addition, functionalised microchips, combined with mass spectrometry, are used for protein solution binding, subsequent elution, and analysis. It is possible to determine the optimum binding conditions, the ionic strength for binding, and the lowest ionic strength for the elution [40][43].

Cold-active lipolytic enzymes were purified like other enzymes and proteins sequentially depending on the purity required. For instance, the recommended purity level for structural and functional studies is greater than 98% [44]. Conventional methods include ammonium sulfate precipitation, affinity chromatography, size exclusion (gel filtration), and hydrophobic interaction [37][45][46][47]. **Table 2** summarises the various methods used to purify recombinant cold-active lipolytic enzymes. In most cold-active lipase and esterase purification procedures, affinity chromatography is either employed in a one-step or a double-step purification strategy. One-step purification using affinity chromatography generally reduces the time and cost of purification. Even so, the prominent double-step procedure uses ammonium sulfate precipitation with size exclusion and hydrophobic interaction; however, this strategy is suitably employed if the enzymes are produced extracellularly. The affinity chromatography technique is highly specific, while size exclusion, hydrophobic interaction, and ammonium sulphate precipitation are less-specific methods. Sometimes the purpose of using affinity chromatography or ammonium sulphate precipitation in single or first-step purification is to concentrate the recombinant proteins, while less-specific procedures are used to polish the purification. The double-step purification strategy using ammonium sulfate precipitation and nickel affinity has not been utilised much, despite having been reported [49]. In general, obtaining high-purity recombinant enzymes in their stable and active form is expensive, time-consuming, and complex. One-step purification using ammonium sulfate is usually term as partial purification; a well-designed ammonium sulfate precipitation is regarded as a gold standard among several purification strategies [50].

Affinity chromatography is usually achieved by fusing tags at an enzyme's C or N terminal before its expression [51]. Several affinity tags have been known to facilitate the expression, solubility, detection, and purification of proteins [52][53]. Poly-histidine tagging, also known as His_6 or His-tag, is widely employed to express and purify most

recombinant proteins, including cold-active lipases and esterase [54]. Despite the high affinity, specificity, and size of His-tag, the technique possesses some disadvantages, including (1) co-purification of other histidine-rich microbial host proteins and (2) negative impact on enzyme stability, activity, binding affinity, and structure [55]. The latter is subject to much contrasting opinion and is still debated because some authors observed that its presence is mainly tolerated for enzymes such as lipase; this cannot be ignored due to its effect on reaction specificity. In a study on the thermal stability of some selected proteins conducted by Booth, Schlachter [56], cleavage of the his-tag can be neutral to some of the proteins while influencing the stability of other protein molecules. In general, the his-tag has an effect (positive or negative) or neutral on proteins.

As shown in **Table 2**, several scholars have reported a single-step purification of cold-active esterase and lipase using nickel Sepharose or agarose affinity chromatography with good fold and recovery. Furthermore, Noby, Saeed [57] have purified a cold-active esterase EstN7 from *Bacillus cohnii* strain with 94.5% yield and 5-fold, adopting Tris–HCl (pH 8.0) in the lysis buffer and potassium phosphate (pH 7.5) in the binding buffer differentiate the study from others that utilised the same buffer in both the purification processes. Kim, Park [58], and Lee, Yoo [59] have purified cold-active esterase using a double-step purification that incorporates nickel-affinity and size exclusion chromatography. Another cold-active lipase, B8W22 from *Bacillus aryabhattii,* was purified in a greater fold of 59.03 using nickel Sepharose affinity and ion-exchange chromatography [60].

Table 2. Purification of Cold-adapted Esterase and Lipase.

Enzymes	Type of Purification	Purification Steps	Buffer	Column/Resin	Fold/Yield	Molecular Mass	References
GaDlh	Complete	Single-step/Ni-affinity chromatography	Tris–HCl	Ni–NTA column	1.9/7.7%	28 kDa	[61]
AMBL-20	Partial	Single step/ammonium sulfate precipitation	Tris–HCl	NA	NA	NA	[62]
*Ha*SGNH1	Complete	Single-step/Ni2+-affinity	Tris–HCl	HisTrap HP	2.5/~5 mg/g	24 kDa	[63]
LSK25	Complete	Single-step/Ni-Sepharose affinity	Tris–HCl	Ni Sepharose® 6Fast Flow column	1.3/44%	65 kDa	[48]
AaSGNH1	Complete	Single-step/Ni-Sepharose affinity	Tris–HCl	Ni-NTA agarose	0.6–0.7 mg/mL	43.9 kDa	[64]
B8W22	Complete	Double-step/Ni-Sepharose	Tris–HCl	DEAE FF column/Octyl	59.03/20%	35 kDa	[65]

Enzymes	Type of Purification	Purification Steps	Buffer	Column/Resin	Fold/Yield	Molecular Mass	References
		affinityand ion-exchange		Sepharose FF column			
ERMR1:04	Complete	Triple-step/ammonium sulfate precipitation, Size exclusion, and hydrophobic interaction	Tris–HCl	Sephadex G-100 column, Octyl-Sepharose fast flow column	21.3/NA	250 kDa (hexameric) 39.8 kD (monomeric)	[66]
estHIJ	Complete	Single-step/Ni-affinity	Phosphate buffer	Ni-NTA affinity column.	3.5/47.5%	29 kDa	[67]
ZY124	Complete	Double step/ammonium sulfate precipitation and hydrophobic chromatography	Tris–HCl	Phenyl Sepharose FF column andmicrocolumn reversed-phase LC-1MS	1.34/NA	37.9 kDa.	[60]
AMS8	Complete	Reverse Micelle Extraction	Sodium phosphate	NA	NA/58.84%	NA	[68]
KM12	Complete	Double-step/ammonium sulfate precipitation and ion-exchange	Tris–HCl	Q-Sepharose FF column	15.63/36.0%	33 kDa	[69]
KCTC 22881	Complete	Double-step/affinity chromatography and size-exclusion chromatography	Tris–HCl	HisTrap FF, PD-10 and Sephacryl S200 HR	NA	31.0 kDa	[59]
EstN7	Complete	Single-step/Ni-affinity	Potassium Phosphate	Ni–NTA affinity column	5/94.5%	37.0 kDa	[57]
GlaEst12-like	Complete	Single-step/Ni-sepharose affinity	Sodium Phosphate	Nickel-Sepharose HP	1.7/40%	63 kDa	[70]
RSAP17	Complete	Double-step/ammonium sulfate	Tris–HCl	DEAE-cellulose anion exchanger	NA	103.8 kDa	[71]

References

Enzymes	Type of Purification	Purification Steps	Buffer	Column/Resin	Fold/Yield	Molecular Mass	References
		precipitation and ion-exchange					
PsEst3	Complete	Double-step/nickel-affinity and size-exclusion chromatography	Tris–HCl	Ni-affinity and HiLoad 16/60 Superdex 200 column	NA	29 kDa	[58]

NA—not available.

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