Bioprocess Design of Escherichia coli SHuffle T7 Express for IsPETase Production

CHBE 221 - Founding Principles in Chemical and Biological Engineering II

Presented to: Dr. Gabriel Potvin

Date Submitted: April 8th, 2025

Group 6

Anna Dinh - 88861737

Christine Agustin - 68251602

Kaitlyn Pennefather - 75530899

Yanya Yang – 19103753

Table of Contents

Abstract

IsPETase production is achieved by the genetic modification of the *E.coli* SHuffle T7 Express by insertion of the PETase gene native to *I.sakaiensis* 201-F6 for recombinant protein production. SHuffle T7 Express was chosen for its protein folding efficiency that ensures a higher proportion of soluble IsPETase produced. It has a doubling time of 20-30 minutes and an induction time of 6 hours. The optimal growth conditions are to be aerobic at a pH of 8.0 and temperature range of 16°C to 37°C.

Cell cultivation is achieved in a fed-batch STR and Optimized Medium-I that ensure an improved biomass and product yield while being cost-effective and supportive of the induction phase. Tryptone, yeast extract, kanamycin are added in the media to aid in oxidative stress sensitivity and antibiotic selectivity. Control systems for temperature, pH, pressure, media distribution, and oxygenation are considered for growth and maintenance.

The T7 promoter was selected for its specificity and strong affinity to assure targeted transcription. A codon randomization strategy is employed to increase protein expression as well as a NEXT protein tag for improved soluble expression. A 6x His-Tag is selected to aid in purification rates. As a precaution, the 26 base-pair signal sequence is removed to prevent the misdirection of proteins intracellularly that causes misfolding.

The product obtained from the downstream processing of the SHuffle cells is a lyophilized powder of recombinant IsPETase enzyme of ≥95% (w/w) purity with a recommended concentration of 20–50 mg/mL (2–5% w/v protein) . Cells are sent through a disc stack centrifuge and lased by a bead mill. After microfiltration and ultrafiltration, affinity chromatography is done for further purification. The final product is achieved through freeze drying. These steps optimize scalability and efficiency while preventing protein denaturation.

1.0 Introduction

1.1 Bioproduct Selection

The production of IsPETase, a cutinase-like enzyme produced from genetically engineered *Escherichia coli* SHuffle T7 Express is analyzed for bioprocess design. IsPETase is the enzyme

naturally produced by *Ideonella sakaiensis* 201-F6 that breaks down PET plastic through hydrolysis [1]. IsPETase has the highest hydrolytic activity rate as opposed to other PET degrading enzymes such as PET27 and RgPETase [2]. Researchers in Japan discovered *I.sakaiensis* by isolating a polyethylene terephthalate (PET) debris sediment sample. PET is the most abundant polymer present in these landfills, making up 18% of global polyester production [2]. *I.sakaiensis* attaches to plastic waste and secretes IsPETase enzyme to the surface of the plastic. IsPETase possesses the ability to hydrolyze ester bonds of the PET plastic for depolymerization [3].

1.2 Applications and Uses

The issue of plastic waste continues to be at the forefront of the climate crisis as its everyday use plagues our ecosystems. *I.sakaiensis*' ability to metabolize PET as a carbon source allows it to convert PET waste into compounds for PET synthesis [1]. IsPETase's high activity rate and low operational temperature allows for the potential of industrial scaling [2]. These traits allow for microplastic decomposition in marine ecosystems, reducing microplastic ingestion by marine life. IsPETase shows higher activity than thermophilic cutinases on crystalline PET films in these temperatures. Additionally, it can be used in industrial textile manufacturing as an alternative to chemical lye for manipulating textile surfaces due to the enzyme's ability to degrade the PET fiber [3]. All listed applications above demonstrate IsPETase as a promising bio-catalytic enzyme for sustainable plastic recycling.

1.3 Market Information

The waste produced from plastic consumption approaches 400 million tons each year with only 20% of it being recycled. A PET bottle will take 450 years to decompose which emphasizes the importance of having a way to recycle [2]. Mechanical recycling methods compromise the structural integrity of plastic. Processes like mechanical grind and melting reduce ductility by 307% after three cycles. Chemical processes fare better as bond cleavages of the plastic will not degrade the material properties, but they require specific reaction conditions and hazardous reagents that release environmental pollutants, thus making the process redundant. In comparison, enzymatic recycling with IsPETase can regenerate monomers and convert the PET waste into viable feedstock. This feedstock can produce a virgin PET that is comparable to those synthesized from petrochemical origins [2]. A French Bio Tech company Carbios aims to scale this form of advanced PET recycling, obtaining over €71.8 million in funding with hopes to operate its first commercial plant for recycling 50,000 tonnes of PET waste per year. The demand for advanced PET recycling methods is projected to be a €200 billion market by 2050 [4]. The benefits of engineering *E. coli* to produce IsPETase can meet the demands of plastic waste management.

1.4 Production Requirements

IsPETase is naturally produced by the bacterium *I.sakaiensis* 201-F6, which secretes the enzyme extracellularly to break down PET plastic. However, for large-scale production, genetic engineering is required to enhance enzyme yield and efficiency. One promising method involves using Shuffle T7 Express, a strain of genetically modified *E. coli* to efficiently produce IsPETase. This strain uses recombinant production, where the PETase gene is inserted into a specialized DNA plasmid, allowing for efficient enzyme expression and secretion [1]. Another commonly used strain, *E. coli* BL21-Gold (DE3), follows a similar recombinant production method but lacks the optimized folding capabilities of SHuffle T7 [1]. SHuffle T7's ability to produce massive quantities of IsPETase makes it a strong candidate for large-scale production.

1.5 Physical and Chemical Properties of IsPETase

IsPETase has a wide substrate-binding cleft, allowing it to hydrolyze PET polymers into mono(2-hydroxyethyl) terephthalic acid (MHET), bis(2-hydroxyethyl) terephthalic acid (BHET), and terephthalic acid (TPA) [1]. IsPETase has a strong preference for p-nitrophenyl (p-NP)

esters, particularly p-hexanoate (p-NH) at the highest activity rate of 59%, demonstrating to be advantageous in hydrolyzing ester bonds [5]. IsPETase functions best in an optimal pH range of 7-9 and peak activity at a pH of 8. Its optimal temperature range is 25-35°C, but its activity declines rapidly at higher temperatures [1] In terms of chemical stability, IsPETase is influenced by external factors, where the presence of salts and moderate glycerol concentrations of 10-20% enhances enzymatic activity, while organic solvents and detergents tend to decrease enzyme function [5]. The catalytic mechanism of IsPETase is stabilized by anion interactions, which help maintain the catalytic center, making substrate binding and hydrolysis easier [5]. From an environmental aspect, IsPETase is promising as a bioremediation tool for PET plastic degradation, as its ability to hydrolyze polyesters presents a solution to reducing plastic waste

2.0 Cell Selection

2.1 Cell Type

E. coli, a prokaryotic host, was chosen for IsPETase production in comparison to other microorganisms due to rapid protein expansion, ease of genetic manipulation, and lower cultivation cost [1]. The specific strain chosen was *E. coli* SHuffle T7 Express, a modified strain of *E. coli* BL21-Gold (DE3) [6]. Shuffle T7 Express was chosen for its ability to correctly fold bonds which results in a simpler purification process, leading to a higher number of usable and stable enzymes [7]. SHuffle T7 can also produce about 13 times more active IsPETase than its parent bacteria as shown in Figure 1 [8]. *I.sakaiensis*, in which *Is*PETase is naturally produced, was also briefly considered. However, *I.sakaiensis*' slower growth and fermentation process and high cultivation costs showed it was unsuitable for our needs [1]. SHuffle T7's high enzymatic yield eases process scalability for manufacturing [1].

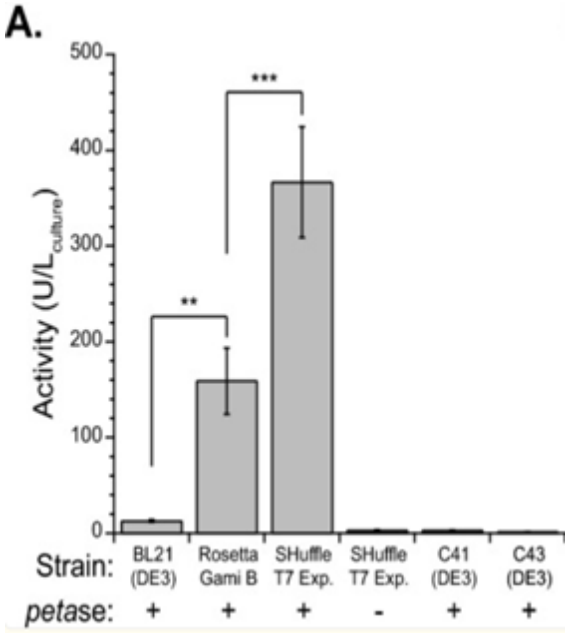


Figure 1. Petase activity compared with types of E. coli strains, including BL21-Gold (DE3) and SHuffle T7 Express [8]

2. Physical and Biological Characteristics

E. coli SHuffle T7 Express's main advantage is its protein folding efficiency. The strain has genetically modified trxB and gor genes which create an oxidizing cytoplasm, allowing disulfide bonds to form [7]. The oxidizing environment helps the IsPETase fold correctly inside the cytoplasm instead of breaking the bonds and preventing IsPETase formation inside the cell. It also contains an integrated disulfide bond C (DsbC) isomerase, which rearranges misfolded proteins [6]. Correct protein structure improves enzyme activity and solubility, in part, increasing the number of functional proteins and the overall product yield. SHuffle T7 has a doubling time of 20-30 minutes. This is the same growth time as its B strain parent, *E. coli* BL21-Gold (DE3). The induction time for SHuffle T7 is typically 6+ hours and is most effective in a temperature range of 16°C to 30°C and a pH of 7.0 [11]. Although the production rate is slower than *E. coli* BL21-Gold (DE3), the proportion of correctly folded and soluble IsPETase produced is significantly higher in SHuffle T7 Express than in any compared cell types [8].

3.0 Biological Modifications

3.1 Expression Cassette

The following components of the expression cassette (*Figure 2*) were chosen to ensure a successful expression of the IsPETase enzyme.

Figure 2: Proposed Gene Expression Cassette

1) SHuffle T7 promoter as a DNA sequence recognized by T7 RNA polymerase to initiate transcription of downstream genes.
2) Lac promoter as a regulatory system in *E. coli* to control T7 RNA polymerase production.
3) NEXT as a protein tag to enhance purification and functional yield.
4) 6x His-Tag as the affinity tag for purification through affinity chromatography.
5) T7hyb10 as an effective terminator for T7 RNA polymerase.

3.1.1 SHuffle T7 Promoter

The expression of IsPETase is driven by the T7 promoter, a bacteriophage-derived promoter known for its high strength and specificity. The T7 promoter sequence (5'-TAATACGACTCACTATAG–3') is located upstream of the IsPETase gene, ensuring rapid and efficient transcription upon activation by T7 RNA polymerase [42]. This strong promoter ensures high transcription rates, resulting in higher protein levels. However, this strength also requires tight upstream control, hence the importance of the regulatory system briefly described above. The synergy between the lac-controlled T7 RNA polymerase and the plasmid-borne T7 promoter allows for robust but precise expression.

3.1.2 Regulatory Operator

To tightly regulate the expression of IsPETase, a lac operator sequence is positioned downstream of the lac promoter in the SHuffle T7 genome. This operator sequence serves as the binding site for the LacI repressor protein, which inhibits transcription in the absence of an inducer [52]. This mechanism forms a key part of an inducible expression system, preventing unnecessary protein production during cell growth and reducing metabolic burden.

The process uses auto-induction to initiate expression. In this system, lactose (included in the medium) binds to LacI, causing a conformational change that detaches the repressor from the lac operator [42]. This enables transcription of the T7 RNA polymerase gene, which then binds to the T7 promoter on the pET21b(+)-IsPETase plasmid to drive IsPETase production.

This system offers several advantages:

- Prevents premature or leaky expression of the target gene.
- Reduces cellular stress by delaying protein production until cells are in optimal condition [43].
- Requires less intervention than IPTG-based induction [54].
- Is cost-effective, scalable, and suitable for both lab-scale and industrial processes [45].

Since IsPETase is structurally complex and can be difficult to fold, this layered regulatory control helps improve protein solubility, minimize toxicity, and increase overall yield.

3.1.3 Coding Region

The coding region for IsPETase originates from *I.sakaiensis* strain 201-F6 and spans approximately 870 base pairs, encoding a 290-amino acid enzyme. IsPETase catalyzes the hydrolysis of PET polymers into environmentally benign monomers, making it highly suitable for plastic degradation applications.

3.1.4 Modifications to the Coding Region

Modifications to the coding region are made to increase efficiency and lower production costs. For codon optimization, the strategy of codon randomization will be used. Codon randomization is a strategy that selects from multiple synonymous codons for each amino acid based on the host organism's codon usage frequencies, balancing tRNA demand to enhance translation efficiency. In comparison to the "one amino acid-one codon" method, codon randomization produces an increase in protein expression. It also produces up to 70% more of the desired gene than the native sequence [46].

Protein tags are added to facilitate later purification, one of which is NEXT, an intrinsically disordered protein (IDP) tag consisting of 53 amino acids. NEXT prevents hydrophobic interactions that occur when insoluble aggregates form during protein expression, therefore enhancing the water solubility of proteins and allowing for proper folding. Its small size allows for a minimal impact on the properties of the target protein, and by helping the proteins fold correctly, NEXT can improve protein yield. NEXT-IsPETase can have a soluble expression of more than 8 times, continuously increasing for up to 12 hours. In comparison, IsPETase's soluble expression can last for only 3 hours, due to a lack of proper folding for most of the expressed protein [47].

Affinity tags help with the purification of the enzyme by making it easier to increase protein yield [48]. A 6x His-Tag is selected as the tag rarely influences the target protein's structure and function due to its minimal size and charge. It is the most used production tag as it possesses a high throughput purification of tagged recombinant proteins. The ideal placement of the tag will be determined experimentally through protein solubility as the C and N-terminals placements are protein specific.

The 26 base-pair signal sequence will also be removed from the native gene because *E. coli* does not need the signal for intracellular expression [42]. This is because *E. coli* produces

recombinant proteins in the cytoplasm. The signal sequence is meant to direct proteins to different locations of the cell and if the signal sequence is not removed, the proteins may be misdirected and, therefore, misfolded [39].

3.1.5 Termination Region

Termination of the coding region is crucial as T7 RNA polymerase is prone to read-through transcription, making transcriptional termination difficult to enforce [48]. Through the engineering of the native terminator hairpins, T7hyb10, a modified version of the native T7 terminator (T7nat), outperforms T7nat in E. coli expression systems by 37%. T7hyb10 favorably acts at 98% for T7 RNA polymerase to ensure the optimization of protein production.

4.0 Cell Cultivation

4.1 Nutritional Requirements

SHuffle T7 requires specific nutrients and environmental conditions to support its growth and metabolic functions. As a chemoheterotroph, SHuffle T7 relies on organic carbon and nitrogen sources for growth. It obtains carbon from organic compounds such as glucose or glycerol, though it has been observed to grow poorly when glycerol is the sole carbon source in minimal media [12]. Amoni acids or peptides must be added with glycerol to support optimal growth. Nitrogen is also essential for protein synthesis and cellular functions. It is supplied in the form of peptides and amino acids. In addition to these organic nutrients, SHuffle T7 requires essential minerals, including magnesium (Mg^{2+}), potassium (K^{+}) and sodium (Na^{+}); their specific roles will be discussed in the next section. Due to SHuffle T7's genetic modifications, it has an oxidative cytoplasmic environment that promotes disulfide bond formation. This is crucial for the correct folding and stability of proteins, which other host cells struggle with. This causes it to be highly sensitive to oxidative stress, particularly from hydrogen peroxide exposure [12]. Carbon, nitrogen, minerals, and a stable oxidative environment are provided in the media through complex ingredients like tryptone or yeast extract, but it is important to maintain the right balance for the survival and efficiency of SHuffle T7.

4.2 Medium Composition

The growth medium for SHuffle T7 must provide essential nutrients for optimal cell growth and protein expression. Optimized Medium-I (OM-I) is identified as an effective formulation, significantly improving biomass yield and protein solubility compared to standard Luria-Bertani (LB) medium [13]. The composition of OM-I contains tryptone, yeast extract, kanamycin, magnesium chloride ($MgCl_2$), potassium chloride (KCl), sodium chloride (NaCl), glucose, glycerol, and phosphate buffer (Table 1).

Table 1: Composition of SHuffle T7 using Optimized Medium-I formulation.

Component	Concentration		Role
Tryptone	2.5% (w/v) *	[13]	Nitrogen source, provides peptides and amino acids for growth [14].
Kanamycin	50 µg/ml	[16]	Media selectivity to verify plasmid retention [15.1].
Yeast Extract	2.5% (w/v)	[13]	Rich in vitamins, nucleotides and supports robust growth [15.1].
Magnesium Chloride ($MgCl_2$)	10 mM	[13]	Essential for enzyme function and stabilizes ribosomes [17].
Potassium Chloride (KCl)	5 mM	[13]	Cell growth regulation [18].
Sodium Chloride (NaCl)	8.5 mM	[13]	Assists in nutrient transport.
Phosphate Buffer	0.89M	[13]	Maintains a stable pH 8, evokes elevated cell growth and delayed entrance to the death phase [13].
Lactose	0.5 (w/v)	[19]	Allows controlled auto-induction in the cells, leading to a more stable metabolic state [19].
Glucose	10mM	[13]	The initial carbon source ensuring there isn't early induction of the lac operon [19].
Glycerol	2.5% (w/v)	[13]	Used as a steady carbon source [19].

* Refers to weight/volume

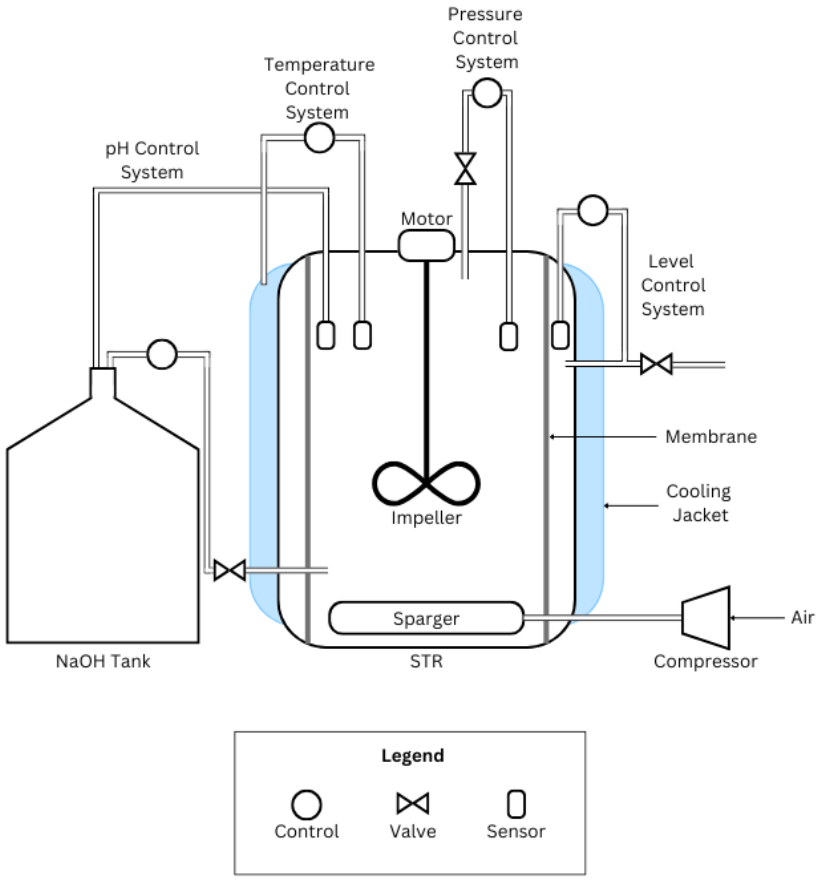
This medium formulation ensures that Shuffle T7 has adequate nutrient availability while maintaining optimal conditions for disulfide bond formation. The use of salts and buffer components helps sustain metabolic activity, while the high tryptone and yeast extract concentrations provide the necessary building blocks for protein synthesis. Kanamycin is present in the broth as the plasmid injected has kanamycin resistance. Kanamycin is widely available, cost-effective and is not naturally occurring within SHuffle cells [15]. Glucose and glycerol are used as carbon sources and lactose is added for auto induction. This composition is particularly crucial due to the oxidative stress sensitivity Shuffle T7 cells, as it helps the cell stay healthy and enhance protein yield.

4.3 Reactor Type

A stirred tank bioreactor (STR) is the preferred choice to produce SHuffle T7 Express due to its ability to maintain controlled conditions necessary for high-density bacterial growth and recombinant protein expression [20]. Other bioreactors, such as plug flow reactors (PFRs), and packed bed reactors (PBRs) are not suitable for this process. PFRs are not ideal because they lack proper aeration and mixing, leading to uneven nutrient distribution and inconsistent bacterial growth, negatively impacting IsPETase production [21]. PBRs present challenges such as difficult temperature control and channeling of gas stream, making it hard to maintain optimal conditions for microbial cultures [22]. STRs are preferred over these alternatives because they ensure uniform conditions, precises temperature regulation, and effective mixing, all of which are essential for achieving stable protein expression and high cell density.

4.4 Reactor Schematic

Figure 3: Schematic of a stirred tank bioreactor with a dialysis component.



4.5 Operating Mode and Conditions (Parameters)

To produce SHuffle T7, a fed-batch operating mode was selected because of its cost-effectiveness and high product yield [20]. Compared to the batch operating mode, the fed-batch mode has higher cell yield, better flexibility, and a longer cell culture duration. Fed-batch mode provides a longer residence time to support the induction phase of the SHuffle T7 [23]. In the case of contamination, the feed-batch system can be shut down immediately and the contaminated batch can be disposed of. If there is contamination in a continuous reactor, there is a high chance the entire system could be ruined due to the continuous inflow and outflow of the system. To avoid the risk of contamination, sterilization techniques are used [24].

The selected reactor will also have a dialysis component to counteract a build-up of inhibitory by-products typically found in fed-batch systems. The membrane that separates the inner and outer chambers allows the OM-I medium to be stored in the reactor, limiting exposure to environmental conditions that could affect temperature or pH of the media. The two chambers allow fresh medium to be supplied to the cells through the membrane, avoiding substrate

concentration fluctuations found in conventional fed-batch systems. Supplying the medium through the surface area of the membrane also avoids substrate build-up on the feed nozzle tip that would be typically used, allowing an efficient usage of the medium and minimizing material and economical waste. Through the membrane, inhibitory by-products can diffuse from the inner chamber to the outer chamber, decreasing their effect on cell concentration and yield. Compared to pumping the medium into the reactor, dialysis is a better method as pumping usually causes stress to the cells, decreasing their performance [25].

Reactor feed will consist of the OM-I medium to provide SHuffle T7 with the required nutrients to sustain biomass yield and cell health (Table 1). The reactor should be at a pH of 8.0 [26]. This pH value is ideal for growth of the cell and for IsPETase stability. During the growth phase, the reactor will operate at a temperature of 37°C, an optimal temperature for the cells to have the highest possible growth rate [19]. Once the lactose concentration is fully depleted, the temperature will then be lowered to a range of 16°C for the induction phase [27]. The temperature drop should be gradually lowered to ensure the cells are not disrupted. Since higher temperatures lead to more misfolded proteins, the lower induction temperature allows the cells to maintain more accurate folding [27]. This process ensures that the cells have a fast growth rate and high product yields.

Aeration will be used to further support cell metabolism. As the cell density increases, there is a larger demand for oxygen due to heightened metabolic activity. Using aeration to increase oxygen in the system is critical for cell growth.

4.6 System Controls

To maintain optimal conditions, several control strategies are implemented. The pH control system monitors the environment of the vessel and adds buffers accordingly to maintain a pH of 8. Acetic acid is produced as a by-product, lowering the pH of the system over time [28]. NaOH is added to the reactor to raise the pH back to 8 when necessary [28]. The temperature is adjusted from growth phase to induction phase using a cooling jacket and a density sensor. The optical density of the media is measured using light. When the OD_{600} reaches a value of around 0.8, the cells have reached their desired density, and the temperature can be decreased for the induction phase [29]. Because the cells produce heat while growing, a temperature control system will also be used to ensure that the temperature of the bioreactor is in the optimal range for the specific

growth period the media is in. Chilled water in a temperature range of 4°C - 10°C will be used for cooling. The pressure of the system can be affected by aeration and heat expansion of cells. The optimal pressure is maintained using a pressure control loop. A valve connects to a gas vent line which releases air from the system when the pressure is too high. The vent has a filter to prevent contamination. The liquid levels are controlled by level sensors and are focused on the outer chamber where the OM-I medium is added. A level sensor in the outer chamber monitors fluid volume, stopping the feed in to prevent overfilling. The inner chamber maintains a relatively constant volume because the nutrients are delivered by dialysis.

4.7 Design Considerations

For the growth and maintenance of SHuffle T7, the bioreactor design must ensure adequate temperature, pH, pressure, media distribution, and oxygenation. In Table 2, the necessary features to optimize the production of IsPETase are outlined.

Component	Function
Dished end	Stainless-steel dished end tank for energy efficiency for the industrial scaling of enzyme production [28].
Sparger	Located at the bottom of the reactor for aiding in aeration and mixing [28].
Cooling jacket	Used as a heat exchanger as it is external to the reactor to reduce sterilization and volume requirements. This will be aided by the addition of temperature sensor to monitor the temperature changes for growth and induction periods [28].
Impeller	Uses mechanical agitation to create homogeneity for the substrate and cells within a specific shear stress speed [28].

Table 2: Components of fed-batch reactor

4.8 Sterility

The bioreactor process is at risk of contamination due to the glucose-rich media, feed additions, and aeration. Keeping the system sterile is critical to maintaining cell health. To minimize contamination, all surfaces and reactor inputs will be sterilized using heat, chemicals, or filtration. The growth media will be sterilized by heating the stream to a temperature of 121°C using a heat exchanger [30]. Heat is used instead of point-of-use steam to ensure the media is not diluted. 0.2 μm pore size filters will be placed on gas input lines to prevent airborne contamination [31]. The bioreactor will be sterilized using bleach in between batches

5.0 Process Flow Diagram

5.1 Target Specifications

The product in this bioprocess is recombinant IsPETase enzyme, which has the capability to hydrolyze polyethylene (PET), an essential feature used primarily in industrial PET recycling. The target application involves depolymerizing PET plastics into their constituent monomers, terephthalic acid (TPA), mono-(2-hydroxyethyl) terephthalate (MHET), and ethylene glycol (EG), which can be re-polymerized into new PET products [40]. This enzymatic recycling is environmentally friendly, more efficient than traditional chemical recycling, and directly addresses the global challenge of plastic waste [41].

The enzyme will be delivered as a lyophilized powder, highly concentrated and ready for reconstitution and immobilization onsite. Lyophilization ensures enzyme stability, prolonged shelf-life and minimizes degradation, crucial for maintaining high enzymatic activity [42], [43]. The absence of water also prevents microbial contamination and enzyme denaturation, essential for the industrial-scale operations in PET recycling. Furthermore, the dry powder allows easy transport, precise dosing and flexible onsite immobilization [44].

The enzyme is recommended to achieve a purity of at least ≥95% (w/w) IsPETase, particularly when the final PET monomers are intended for re-polymerization into new plastics for food-grade or pharmaceutical use. This high purity level is important for immobilization, maximizing catalytic activity per unit mass of immobilized enzyme [45]. When the immobilization yield is less than 100%, the presence of impurities can complicate the process. However, in industrial applications involving post-consumer PET waste destined for less sensitive products, moderately lower enzyme purity may be acceptable. The following impurity guidelines are recommended when the enzyme's downstream products are intended for regulated or sensitive applications:

- Host cell proteins (HCP): Should be maintained at less than 5% or preferably much lower [46]. HCPs are undesired proteins made by *E. coli* that can reduce enzyme stability, cause support fouling during immobilization, and reduce catalytic efficiency.
- Residual DNA: Should be limited to ≤100 ng DNA per mg protein [47]. Genomic DNA from *E. coli* must be removed to avoid viscosity and interference in processing. In cell lysate, DNA release can hinder filtration or chromatography.

- Endotoxins (LPS): Levels must be minimized to below 100 EU/mg enzyme [48]. Endotoxins are bacterial contaminants from the cell membrane of *E. coli*, which can interact with the enzyme, potentially reducing its catalytic efficiency and overall purity. Minimizing endotoxins is particularly important when depolymerized PET monomers (TPA and EG) are intended for use in sensitive applications.

The final enzyme product must be highly concentrated, with a recommended concentration of 20–50 mg/mL (2–5% w/v protein). This allows for maximum enzyme loading onto solid supports (typically 5-20mg enzyme per gram of carrier), minimizes buffer use, and ensures manageable reactor volumes

5.2 Process Flow Diagrams and Decisions

Downstream processing of IsPETase is executed through cell separation, cell lysis and purification to achieve the desired product (see Figure 1). Biomass and fermentation broth flows from the bioreactor to the disc centrifuge continuously for large-scale production optimization of cell harvesting. A disc stack centrifuge is chosen for its ability to maintain cell quality and efficient separation in comparison to tubular centrifugation on an industrial scale [49]. To achieve cell lysis as IsPETase is intracellularly expressed, the filtered cells are pumped into a horizontal chamber filled with 80% beads to undergo mechanical disruption, producing the lysate. This method is typically used in large-scale production for its ease of scalability and high disruption efficiency rate. Other methods, such as ultrasonication and chemical lysis, pose issues of heat dissipation, time inefficiency, and further downstream removal costs which are unfavorable to the desired scale of production [50]. The lysate is pumped into a mixer with flocculants of polyionic polymers to aggregate cell debris. The precipitated cell debris and solution are pumped into a microfilter of 1 μm fibers to separate the enzyme from the lysate for further purification [51], with the filtrate as the cell debris and solution and the retentate as the lysate.

Although IsPETase naturally precipitates as inclusion bodies, using SHuffle as the carrier cell ensures that proteins are properly folded, significantly removing the risk of insoluble bodies [52]. Renaturing of insoluble bodies is not necessary, and the process can proceed to the intermediate recovery stage. Crossflow ultrafiltration is used to remove the product from unwanted small contaminants, such as salt and free amino acids. A semi-permeable

membrane, with a 10-30 kDa molecular weight cut off, forces majority of impurities through the filters while the IsPETase, proteins, and a small amount of leftover impurities flows through as retentate [53]. Crossflow ultrafiltration is a good option for the intermediate purification step as IsPETase has a molecular weight of around 35kDa, which is significantly larger in comparison to most of the salt and amino acids[54]. The process is continuous, good for industrial scaling, and removes the risk of protein denaturation.

To further purify the product, nickel affinity chromatography is used to isolate IsPETase. This is enabled by the 6x His-tag that is genetically inserted into the C-terminal of the protein [55]. The 6x His-tag binds to the nickel ions in the column and loosely bound contaminants are washed off using a standard wash of 8 column volumes (CVs) of 20 mM imidazole. To extract the purified final product and elute undesired proteins that are tightly bound, a standard elution of 1 CV of 250 mM imidazole is used [56], [57]. This washing and elution method is typically used in nickel affinity chromatography, is good for scalability and provides a high level of purification. Compared to other affinity tags such as FLAG and GST, the 6x His-tag stands out as it is relatively small and has less charge, minimizing impacts on the structure and function of target proteins. The 6x His-tag also has a minimal metabolic burden on hosts and provides nondenaturing conditions.

To dry the final product, freeze drying is used. The purified IsPETase goes into a chamber where the pressure is reduced and the IsPETase is slowly frozen. This allows the solvent to evaporate by sublimation, leaving the product as a dry powder and ready for shipment. Because IsPETase is sensitive to heat with an optimal temperature range of 25-35°C, freeze drying is the best drying option as it removes solvent at low temperatures, keeping the product from denaturing [58], [1].

5.2.1 PFD

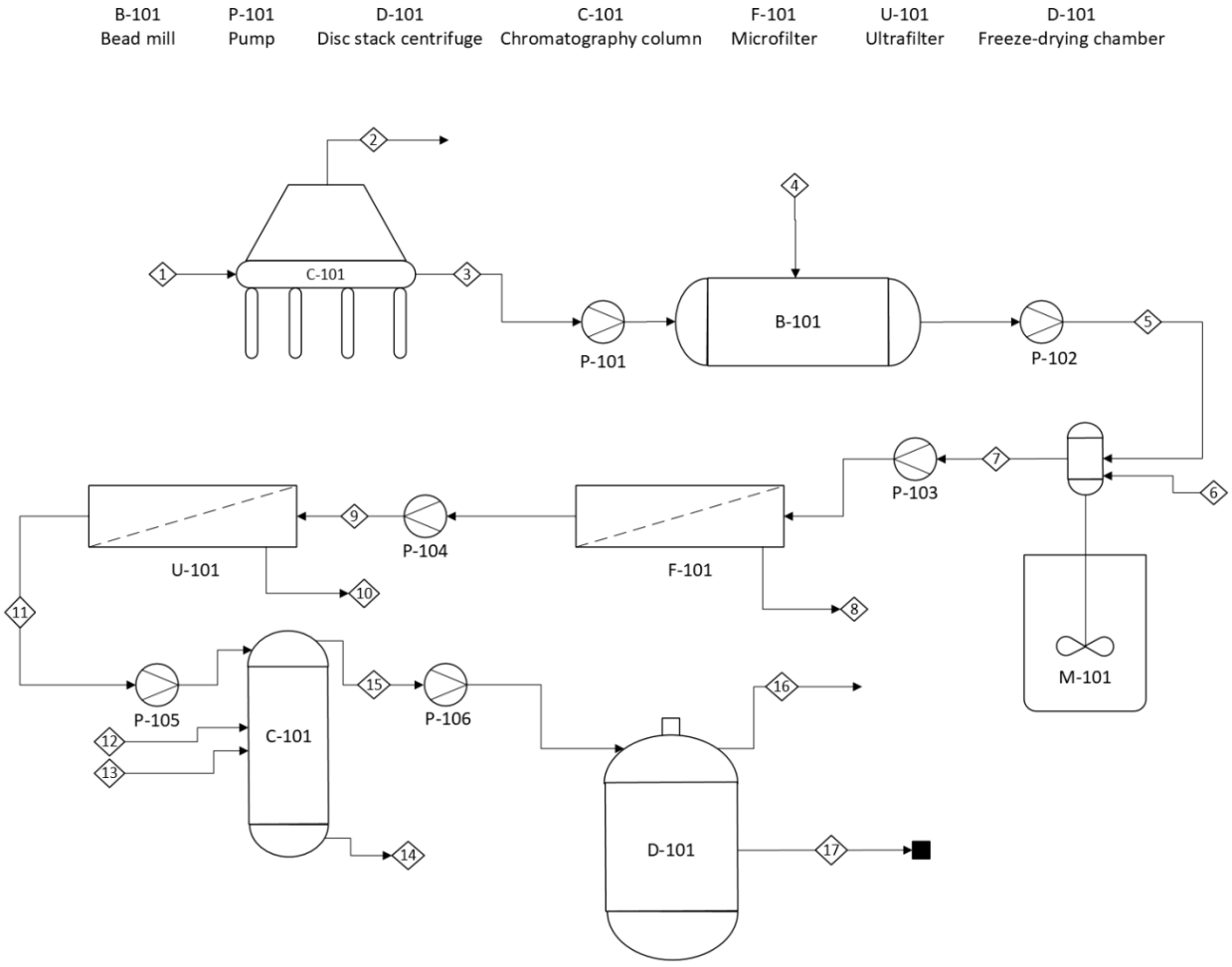


Figure 3: Process flow diagram of IsPETase product recovery

5.2.2 Stream Table

Stream	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Component	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Biomass	✓		✓														

Fermentation broth	✓	✓														
Beads				✓												
Cell debris					✓		✓	✓								
Lysate					✓		✓		✓							
Polyionic polymers						✓										
Product											✓				✓	✓
Salt										✓	✓			✓		
Amino acids										✓	✓			✓		
Wash												✓				
Elution													✓			
Vapor															✓	

5.2.3 Process Description

Downstream processing of IsPETase is executed through cell separation, cell lysis and purification to achieve the desired product (see Figure 1) [8]. Biomass and fermentation broth flows from the bioreactor to the disc centrifuge continuously for large-scale production optimization of cell harvesting. A disc stack centrifuge is chosen for its ability to maintain cell quality and efficient separation in comparison to tubular centrifugation on an industrial scale [49]. To achieve cell lysis as IsPETase is intracellularly expressed, the filtered cells are pumped into a horizontal chamber filled with 80% beads to undergo mechanical disruption, producing the lysate. This method is typically used in large-scale production for its ease of scalability and high disruption efficiency rate. Other methods, such as ultrasonication and chemical lysis, pose issues of heat dissipation, time inefficiency, and further downstream removal costs which are unfavorable to the desired scale of production [50]. The lysate is pumped into a mixer with flocculants of polyionic polymers to aggregate cell debris. The precipitated cell debris and solution are pumped into a microfilter of 1 μm fibers to separate the enzyme from the lysate for further purification [51], with the filtrate as the cell debris and solution and the retentate as the lysate.

Although IsPETase naturally precipitates as inclusion bodies, using SHuffle as the carrier cell ensures that proteins are properly folded, significantly removing the risk of insoluble bodies [52]. Renaturing insoluble bodies is not necessary, and the process can proceed to the intermediate recovery stage. Crossflow ultrafiltration is used to remove the product from unwanted small contaminants, such as salt and free amino acids. A semi-permeable membrane, with a 10-30 kDa molecular weight cut off, forces majority of impurities through the filters while the IsPETase, proteins, and a small amount of leftover impurities flows through as retentate [53]. Crossflow ultrafiltration is a good option for the intermediate purification step as IsPETase has a molecular weight of around 35kDa, which is significantly larger in comparison to most of the salt and amino acids [54]. The process is continuous, good for industrial scaling, and removes the risk of protein denaturation.

To further purify the product, nickel affinity chromatography is used to isolate IsPETase. This is enabled by the 6x His-tag that is genetically inserted into the C-terminal of the protein[55]. The 6x His-tag binds to the nickel ions in the column and loosely bound contaminants are washed off using a standard wash of 8 column volumes (CVs) of 20 mM

imidazole. To extract the purified final product and elute undesired proteins that are tightly bound, a standard elution of 1 CV of 250 mM imidazole is used [56]. This washing and elution method is typically used in nickel affinity chromatography, is good for scalability and provides a high level of purification. Compared to other affinity tags such as FLAG and GST, the 6x His-tag stands out as it is relatively small and has less charge, minimizing impacts on the structure and function of target proteins. The 6x His-tag also has a minimal metabolic burden on hosts and provides nondenaturing conditions.

To dry the final product, freeze drying is used. The purified IsPETase goes into a chamber where the pressure is reduced and the IsPETase is slowly frozen. This allows the solvent to evaporate by sublimation, leaving the product as a dry powder and ready for shipment. Because IsPETase is sensitive to heat with an optimal temperature range of 25-35°C, freeze drying is the best drying option as it removes solvent at low temperatures, keeping the product from denaturing [58], [1].

Conclusion

IsPETase is intracellularly produced through the genetic modification of SHuffle T7 Express strain of *e.coli* with the 201-F6 gene from *I.sakaiensis*. Cell cultivation is carried out in a fed-batch STR with OM-I medium where the production efficiency is monitored. Downstream processing, including affinity chromatography and freeze-drying, is applied to achieve the final powder form of IsPETase with a purity of ≥ 95%. The enzyme's ability to hydrolyze PET bonds for depolymerization lends to its diverse applications across industries such as textile and recycling. IsPETase is a powerful tool to aid in the reduction of plastic waste, providing a pathway into a future free of plastic pollution.

References

[1] X. Meng et al., “Protein engineering of stable IsPETase for PET plastic degradation by Premuse,” Int J Biol Macromol, vol. 180, pp. 667–676, Jun. 2021, doi: 10.1016/J.IJBIOMAC.2021.03.058.

[2] R. T. Guo et al., “Natural and engineered enzymes for polyester degradation: a review,” Environmental Chemistry Letters 2024 22:3, vol. 22, no. 3, pp. 1275–1296, Mar. 2024, doi: 10.1007/S10311-024-01714-6.

[3] Y. Kan, L. He, Y. Luo, and R. Bao, “IsPETase Is a Novel Biocatalyst for Poly(ethylene terephthalate) (PET) Hydrolysis,” ChemBioChem, vol. 22, no. 10, pp. 1706–1716, May 2021, doi: 10.1002/CBIC.202000767.

[4] “Plastic-eating bacteria could revolutionize recycling - HSBC Global Private Banking.” Accessed: Apr. 07, 2025. [Online]. Available: https://www.privatebanking.hsbc.com/individual-investors/frontiers-series/plastic-eating-bacteria-could-revolutionize-recycling/

[5] C. Liu, C. Shi, S. Zhu, R. Wei, and C. C. Yin, “Structural and functional characterization of polyethylene terephthalate hydrolase from Ideonella sakaiensis,” Biochem Biophys Res Commun, vol. 508, no. 1, pp. 289–294, Jan. 2019, doi: 10.1016/J.BBRC.2018.11.148.

[6] “SHuffle® T7 Express Competent E. coli | NEB.” Accessed: Jan. 30, 2025. [Online]. Available: https://www.neb.com/en/products/c3029-shuffle-t7-express-competent-e-coli

[7] J. Lobstein, C. A. Emrich, C. Jeans, M. Faulkner, P. Riggs, and M. Berkmen, “SHuffle, a novel Escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm,” Microb Cell Fact, vol. 11, no. 1, pp. 1–16, May 2012, doi: 10.1186/1475-2859-11-56/TABLES/3.

[8] L. M. Carter et al., “Increased cytoplasmic expression of PETase enzymes in E. coli,” Microb Cell Fact, vol. 23, no. 1, pp. 1–18, Dec. 2024, doi: 10.1186/S12934-024-02585-W/FIGURES/5.

[9] G. D. Metcalfe, F. Sargent, and M. Hippler, “Hydrogen production in the presence of oxygen by Escherichia coli K-12,” Microbiology (N Y), vol. 168, no. 3, p. 001167, 2022, doi: 10.1099/MIC.0.001167.

[10] "Tryptone (vegetable) for microbiology 100209-45-8." Accessed: Jan. 30, 2025. [Online]. Available: https://www.sigmaaldrich.com/CA/en/product/sial/16922

[11] A. R. Tuttle, N. D. Trahan, and M. S. Son, "Growth and Maintenance of Escherichia coli Laboratory Strains," Curr Protoc, vol. 1, no. 1, p. e20, Jan. 2021, doi: 10.1002/CPZ1.20.

[12] J. Lobstein, C. A. Emrich, C. Jeans, M. Faulkner, P. Riggs, and M. Berkmen, "SHuffle, a novel Escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm," Microb Cell Fact, vol. 11, no. 1, pp. 1–16, May 2012, doi: 10.1186/1475-2859-11-56/TABLES/3.

[13] A. B. Khalilvand, S. Aminzadeh, M. H. Sanati, and F. Mahboudi, "Media optimization for SHuffle T7 Escherichia coli expressing SUMO-Lispro proinsulin by response surface methodology," BMC Biotechnol, vol. 22, no. 1, pp. 1–13, Dec. 2022, doi: 10.1186/S12896-021-00732-4/TABLES/6.

[14] "800-155 EG-Casein digest peptone (tryptone) 100g - Wisent." Accessed: Feb. 12, 2025. [Online]. Available: https://www.wisentbioproducts.com/product/casein-digest-peptone-tryptone-800-155-eg/

[15] "Plasmids 101: Choosing an Antibiotic Resistance Gene." Accessed: Mar. 17, 2025. [Online]. Available: https://blog.addgene.org/plasmids-101-choosing-an-antibiotic-resistance-gene

[16] GoldBio, "19 Common Questions About Kanamycin," *GoldBio Articles*, [Online]. Available: https://goldbio.com/articles/article/19-Common-Questions-About-Kanamycin?srsltid=AfmBOooZl0HB6m7ZqtlaWG3-Xj51JYMDbCJfb29yThj9M43dqDltNDPF. Accessed: Apr. 8, 2025.

[17] D. yeon D. Lee *et al.*, "Magnesium Flux Modulates Ribosomes to Increase Bacterial Survival," *Cell*, vol. 177, no. 2, pp. 352-360.e13, Apr. 2019, doi: 10.1016/J.CELL.2019.01.042.

[18] M. R. Shen *et al.*, "The KCI cotransporter isoform KCC3 can play an important role in cell growth regulation," *Proc Natl Acad Sci U S A*, vol. 98, no. 25, pp. 14714–14719, Dec. 2001, doi: 10.1073/PNAS.251388798/ASSET/0791FB86-76F1-4C34-A7D9-38946CD30480/ASSETS/GRAPHIC/PQ2513887007.JPEG.

[19] F. W. Studier, "Protein production by auto-induction in high-density shaking cultures," *Protein Expression and Purification*, vol. 41, no. 1, pp. 207–234, May 2005, [Online]. Available: https://www.sciencedirect.com/science/article/pii/S1046592805000264. Accessed: Apr. 8, 2025

[20] [18.1] "Process development for PETase production and purification," 2024, doi: 10.32657/10356/181799.

[21] S. Brahma *et al.*, "Biodiesel production from mixed oils: A sustainable approach towards industrial biofuel production," *Chemical Engineering Journal Advances*, vol. 10, May 2022, doi: 10.1016/j.ceja.2022.100284.

[22] "Packed Bed (PBR) – Visual Encyclopedia of Chemical Engineering Equipment." Accessed: Feb. 13, 2025. [Online]. Available: https://encyclopedia.che.engin.umich.edu/packed-bed-reactors/

[23] J. J. Díaz-Rodríguez, I. Aguilera-Correa, J. P. Fernández-García, and J. Esteban, "IsPETase and IsMHETase: Enzymatic Synthesis of Poly(ethylene terephthalate) (PET) Degrading Enzymes," *Front. Bioeng. Biotechnol.*, vol. 8, p. 573607, Oct. 2020, doi: 10.3389/fbioe.2020.573607. [Online]. Available: https://www.frontiersin.org/journals/bioengineering-and-biotechnology/articles/10.3389/fbioe.2020.573607/full.

[24] "Bioprocess Operation Modes: Batch, Fed-batch, and Continuous Culture - Eppendorf Canada." Accessed: Feb. 13, 2025. [Online]. Available: https://www.eppendorf.com/ca-en/lab-academy/applied-industries/bioprocessing/introduction-to-bioprocessing/batch-fed-batch-and-continuous-culture/

[25] G. A. L. Gonçalves, K. L. J. Prather, G. A. Monteiro, and D. M. F. Prazeres, "Engineering of Escherichia coli strains for plasmid

[26] A. B. Khalilvand, S. Aminzadeh, M. H. Sanati, and F. Mahboudi, "Media optimization for SHuffle T7 Escherichia coli expressing SUMO-Lispro proinsulin by response surface methodology," *BMC Biotechnol*, vol. 22, no. 1, p. 1, Dec. 2022, doi: 10.1186/S12896-021-00732-4

[27] A. A. Alalwan, H. M. Alminshid, and H. A. Aljaafari, "Promising evolution of biofuel generations: Subject review," *Renewable Energy Focus*, vol. 28, pp. 127–139, Mar. 2019, doi:

10.1016/j.ref.2018.12.006. [Online]. Available: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7162232/.

[28] L. Hoshan *et al.*, "Effective bioreactor pH control using only sparging gases," *Biotechnol Prog*, vol. 35, no. 1, Jan. 2019, doi: 10.1002/BTPR.2743.

[29] European Molecular Biology Laboratory, "Small-scale expression and purification tests," *EMBL Protein Expression and Purification Services*, [Online]. Available: https://www.embl.org/groups/protein-expression-purification/services/protein-expression/small-scale-expression-and-purification-tests/?utm_source=chatgpt.com.

[30] *CABRI.* (n.d.). *Guidelines for Micro-organisms: M203Ap1.* [Online]. Available: http://www.cabri.org/guidelines/micro-organisms/M203Ap1.html. Accessed: Apr. 8, 2025.

[31] *Cytiva Life Sciences.* (n.d.). *Parts of a Stirred Tank Bioreactor and Their Function.* [Online]. Available: https://www.cytivalifesciences.com/en/us/news-center/parts-of-a-stirred-tank-bioreactor-and-their-function-10001.

[32] M. Rong, H. Biao, W. T. Mcallister, and R. K. Durbin, "Promoter specificity determinants of T7 RNA polymerase," *Proc Natl Acad Sci U S A*, vol. 95, no. 2, pp. 515–519, Jan. 1998, doi: 10.1073/PNAS.95.2.515/ASSET/C5B7D8E3-2969-4A1D-90F9-917C8515D20D/ASSETS/GRAPHIC/PQ0283805004.JPEG.

[33] M. Fathi-Roudsari, N. Maghsoudi, A. Maghsoudi, S. Niazi, and M. Soleiman, "Auto-induction for high level production of biologically active reteplase in Escherichia coli," *Protein Expr Purif*, vol. 151, pp. 18–22, Jan. 2018, doi: 10.1016/J.PEP.2018.05.008.

[34] F. W. Studier, "Protein production by auto-induction in high-density shaking cultures," 2005, doi: 10.1016/j.pep.2005.01.016.

[35] M. Lecina, E. Sarró, A. Casablancas, F. Gòdia, and J. J. Cairó, "IPTG limitation avoids metabolic burden and acetic acid accumulation in induced fed-batch cultures of Escherichia coli M15 under glucose limiting conditions," *Biochem Eng J*, vol. 70, pp. 78–83, Jan. 2013, doi: 10.1016/J.BEJ.2012.10.006.

[36] "Plasmids 101: Choosing an Antibiotic Resistance Gene." Accessed: Mar. 17, 2025. [Online]. Available: https://blog.addgene.org/plasmids-101-choosing-an-antibiotic-resistance-gene

[37] H. G. Menzella, “Comparison of two codon optimization strategies to enhance recombinant protein production in Escherichia coli,” *Microb Cell Fact*, vol. 10, no. 1, pp. 1–8, Mar. 2011, doi: 10.1186/1475-2859-10-15/FIGURES/2.

[38] D. G. Calvopina-Chavez, M. A. Gardner, and J. S. Griffitts, “Engineering efficient termination of bacteriophage T7 RNA polymerase transcription,” *G3 Genes|Genomes|Genetics*, vol. 12, no. 6, May 2022, doi: 10.1093/G3JOURNAL/JKAC070.

[39] A. Karyolaimos *et al.*, “Enhancing Recombinant Protein Yields in the E. coli Periplasm by Combining Signal Peptide and Production Rate Screening,” *Front Microbiol*, vol. 10, p. 460608, Jul. 2019, doi: 10.3389/FMICB.2019.01511/BIBTEX.

[40] Q. Yin, S. You, J. Zhang, W. Qi, and R. Su, “Enhancement of the polyethylene terephthalate and mono-(2-hydroxyethyl) terephthalate degradation activity of Ideonella sakaiensis PETase by an electrostatic interaction-based strategy,” *Bioresour Technol*, vol. 364, p. 128026, Nov. 2022, doi: 10.1016/J.BIORTECH.2022.128026.

[41] T. Muringayil Joseph *et al.*, “Polyethylene terephthalate (PET) recycling: A review,” *Case Studies in Chemical and Environmental Engineering*, vol. 9, p. 100673, Jun. 2024, doi: 10.1016/J.CSCEE.2024.100673.

[42] L. Fohler, L. Leibetseder, M. Cserjan-Puschmann, and G. Striedner, “Manufacturing of the highly active thermophile PETases PHL7 and PHL7mut3 using Escherichia coli,” *Microb Cell Fact*, vol. 23, no. 1, p. 272, Dec. 2024, doi: 10.1186/S12934-024-02551-6/TABLES/3.

[43] “Lyophilization Applications for Industries - Catachem.” Accessed: Mar. 28, 2025. [Online]. Available: https://catachem.com/lyophilization-applications-for-industries/?utm_source=chatgpt.com\

[44] T. Farr, J. L. Wissner, and B. Hauer, “A simple and efficient method for lyophilization of recombinant E. coli JM109 (DE3) whole-cells harboring active Rieske non-heme iron dioxygenases,” *MethodsX*, vol. 8, p. 101323, Jan. 2021, doi: 10.1016/J.MEX.2021.101323.

[45] J. ; Boudrant, J. M. ; Woodley, and R. Fernandez-Lafuente, “General rights Parameters necessary to define an immobilized enzyme preparation,” *Downloaded from orbit.dtu.dk on*, 2025, doi: 10.1016/j.procbio.2019.11.026.

[46] C. E. M. Hogwood, D. G. Bracewell, and C. M. Smales, “Measurement and control of host cell proteins (HCPs) in CHO cell bioprocesses,” *Curr Opin Biotechnol*, vol. 30, pp. 153–160, 2014, doi: 10.1016/J.COPBIO.2014.06.017.

[47] D. Li *et al.*, “Detection of residual E. coli host cell DNA by 23S ribosomal RNA gene-targeted quantitative polymerase chain reactions,” *J Pharm Biomed Anal*, vol. 198, p. 114000, May 2021, doi: 10.1016/J.JPBA.2021.114000.

[48] “Guidance for Industry: Pyrogen and Endotoxins Testing: Questions and Answers | FDA.” Accessed: Mar. 28, 2025. [Online]. Available: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/guidance-industry-pyrogen-and-endotoxins-testing-questions-and-answers?utm_source=chatgpt.com

[49] P. Esmaeilnejad-Ahranjani and M. Hajimoradi, “Optimization of industrial-scale centrifugal separation of biological products: comparing the performance of tubular and disc stack centrifuges,” *Biochem Eng J*, vol. 178, p. 108281, Jan. 2022, doi: 10.1016/J.BEJ.2021.108281.

[50] J. P. Pieracci, J. W. Armando, M. Westoby, and J. Thommes, “Industry Review of Cell Separation and Product Harvesting Methods,” *Biopharmaceutical Processing: Development, Design, and Implementation of Manufacturing Processes*, pp. 165–206, Jan. 2018, doi: 10.1016/B978-0-08-100623-8.00009-8.

[51] M. N. Karim, H. Graham, B. Han, and A. Cibulskas, “Flocculation enhanced microfiltration of Escherichia coli lysate,” *Biochem Eng J*, vol. 40, no. 3, pp. 512–519, Jul. 2008, doi: 10.1016/J.BEJ.2008.02.006.

[52] P. Wagner-Egea, V. Tosi, P. Wang, C. Grey, B. Zhang, and J. A. Linares-Pastén, “Assessment of IsPETase-Assisted Depolymerization of Terephthalate Aromatic Polyesters and the Effect of the Thioredoxin Fusion Domain,” *Applied Sciences 2021, Vol. 11, Page 8315*, vol. 11, no. 18, p. 8315, Sep. 2021, doi: 10.3390/APP11188315.

[53] “Protein Sample Ultrafiltration.” Accessed: Mar. 28, 2025. [Online]. Available: https://www.sigmaaldrich.com/CA/en/technical-documents/technical-article/protein-biology/protein-concentration-and-buffer-exchange/protein-sample-ultrafiltration?srsltid=AfmBOop4jb_sII1cplT1yPDqpDaZZ7ZLQhkXfYK9CxcFTZP9UQmITSdu

[54] "Part:BBa K3997000 - parts.igem.org." Accessed: Mar. 28, 2025. [Online]. Available: https://parts.igem.org/Part%3ABBa_K3997000

[55] H. Dan, A. Balachandran, and M. Lin, "A Pair of Ligation-Independent Escherichia coli Expression Vectors for Rapid Addition of a Polyhistidine Affinity Tag to the N- or C-Termini of Recombinant Proteins," *J Biomol Tech*, vol. 20, no. 5, p. 241, 2009, Accessed: Mar. 28, 2025. [Online]. Available: https://pmc.ncbi.nlm.nih.gov/articles/PMC2777343/

[56] C. Robichon, J. Luo, T. B. Causey, J. S. Benner, and J. C. Samuelson, "Engineering Escherichia coli BL21(DE3) derivative strains to minimize E. coli Protein contamination after purification by immobilized metal affinity chromatography," *Appl Environ Microbiol*, vol. 77, no. 13, pp. 4634–4646, 2011, doi: 10.1128/AEM.00119-11/SUPPL_FILE/FIGURES2.ZIP.

[57] "Basic Theory - Chemistry LibreTexts." Accessed: Apr. 07, 2025. [Online]. Available: https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumentation_and_Analysis/Affinity_Chromatography/02_Theory/01_Basic_Theory

[58] E. Z. L. Zhong-Johnson *et al.*, "Analysis of Poly(ethylene terephthalate) degradation kinetics of evolved IsPETase variants using a surface crowding model," *Journal of Biological Chemistry*, vol. 300, no. 3, pp. 105783–105784, Mar. 2024, doi: 10.1016/J.JBC.2024.105783/ATTACHMENT/6434ECC1-94F7-4110-BBFC-0E3DA77A137C/MMC1.PDF.