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A Comprehensive Review of *Pfu* DNA Polymerase: Extraction, Production and Applications

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Abstract

Pfu DNA Polymerase, a thermostable enzyme derived from the hyperthermophilic archaeon *Pyrococcus furiosus*, is widely recognized for its high fidelity and strong processivity. Its 3'-5' exonuclease activity makes it indispensable for the correct amplification of both short and complex DNA strands. These biochemical properties of *Pfu* DNA Polymerase have encouraged considerable advancements in its extraction and production methods. This review covers some of the traditional approaches for purification, including protein purification and affinity chromatography, and updates on recent advances in recombinant gene expression, automated systems of production, and membrane-based technologies. New ways of engineering enzymes have recently been developed, such as CRISPR-Cas9-mediated gene optimization, which raised the bar on extraction efficiency to meet emerging demand. Once challenging, *Pfu* DNA Polymerase production has been significantly streamlined through recombinant expression in *E. coli* both at a laboratory and commercial scale. The optimization techniques that are involved with IPTG concentration and Response Surface Methodology have increased the yield by up to 30%. Auto-induction means even higher biomass outputs are allowed. Today, applications of *Pfu* DNA Polymerase span from standard PCR up to advanced clinical diagnostics in the fields of molecular biology, forensic analysis, clinical microbiology, and biotechnology.

Keywords: *Pfu* DNA Polymerase; *Pyrococcus furiosus*; Thermostable enzyme; Recombinant expression; Enzyme engineering; Biotechnology

1 Introduction

DNA Polymerases are crucial enzymes that are fundamental to the process of DNA synthesis and replication [1]. These enzymes are essential for the Polymerase Chain Reaction (PCR), a commonly employed method for amplifying DNA from small template samples [2]. The high temperatures required for the denaturation of DNA strands in PCR reactions require the use of heat-resistant DNA Polymerases that should also maintain efficiency and accuracy [3-5]. DNA Polymerases found in living organisms have built-in error-checking mechanisms, characterized by their ability to remove nucleotides from the 3' end of a DNA strand. This 3' to 5' exonuclease activity allows these enzymes to detect and correct mistakes in newly formed DNA strands [6]. *Pfu* polymerase, for instance, is a doughnut-shaped molecule with approximate dimensions of 50 Å × 80 Å × 100 Å. As shown in Fig.1 [7].

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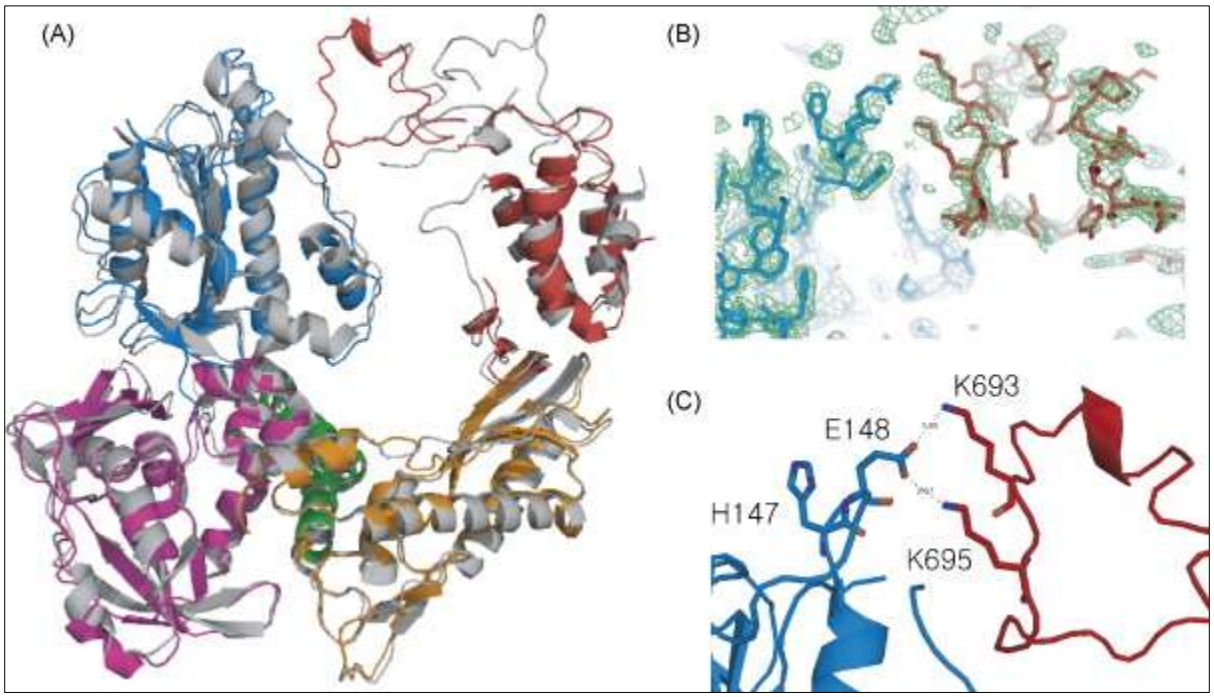

Figure 1 Overall architecture of *Pfu* DNA Polymerase and comparison with KOD1. A) *Pfu* folds into a five-domain protein that includes an exonuclease domain, colored in blue; an N-terminal domain, purple; a palm domain, orange; a finger domain, green; and a thumb domain, red

At the outset, *Taq* DNA Polymerase was the preferred enzyme for DNA amplification methods in vitro, especially in PCR (Polymerase Chain Reaction). Nevertheless, a major drawback of *Taq* DNA Polymerase is its inability to proofread, as it lacks the 3' to 5' exonuclease function [4] [8]. Consequently, *Taq* DNA Polymerase had limited uses, highlighting the necessity for a DNA Polymerase with built-in 3' to 5' exonuclease functionality to enhance accuracy. One such example was *Pfu* DNA Polymerase, extracted from the extremely heat-tolerant organism *Pyrococcus furiosus*, which can thrive at exceptionally high temperatures. This organism, along with its very high-temperature preferences, such as 80 degrees centigrade, made it particularly suitable [9]. The low error rates exhibited by *Pfu* DNA Polymerase during DNA synthesis make it valuable for high-fidelity PCR applications, including cloning and DNA sequencing [10]. [11]. Additionally, *Pfu* DNA Polymerase demonstrates higher thermostability as compared to *Taq* DNA Polymerase. Furthermore, the former possesses greater processivity, enabling efficient amplification of lengthy DNA templates in a shorter timeframe while maintaining exceptional fidelity and accuracy [12].

Table 1 Comparison between the Key Characteristics of *Taq* and *Pfu* DNA Polymerase

Polymerase Type	Thermostability (°C)	Exonuclease Activity	Fidelity	Primary Applications
Pfu DNA Polymerase	100	Yes, 3′ to 5′	High	Cloning, sequencing, PCR
Taq DNA Polymerase	95	No	Moderate	PCR, basic DNA amplification

Pfu DNA Polymerases also have the 3′ to 5′ exonuclease proofreading ability that removes any misincorporated base or base pair. *Pfu* DNA Polymerase has a 7-10 times lower error rate as compared to non-proof reading *Taq* DNA Polymerase and with a mutation frequency of 1.3 x 10^-6 per base pair duplication [13] [14] [15].These novel *Pfu* polymerases with improved performances have been developed for further enhancements in speed and amplification without compromising much fidelity, which can be extended for any long-fragment PCR and high GC-template [10].

Researchers have thoroughly investigated the inherent characteristics of DNA Polymerases, leading to significant progress in biotechnological applications [16]. The special features of *Pfu* DNA Polymerase dictate that effective protocols should be developed for their extraction and production. Considering the hyperthermophilic nature, it becomes difficult to grow *Pyrococcus furiosus* in the laboratory. Again, various kinds of extraction methods exist, such as Heat-Mediated Purification [17], microwave-assisted extraction, ammonium sulfate precipitation, and phenol-

chloroform extraction, which are simple and low-cost processes. While these techniques are being trendy, and applied in laboratories nowadays to extract DNA Polymerases, they might not be that effective and accurate. One critical issue is that DNA Polymerases used in vitro usually have lower processivity compared to their in vivo counterparts [18]. Thus, many approaches have been given to maintain processive and efficient enzyme extraction and production techniques for DNA Polymerases. Codon optimization is one of the most common methods adopted for increased protein yield by correctly aligning codon usage in accordance with the host organism's preference for translation efficiency. In the case of DNA Polymerases, recombinant protein production has been extensively improved by optimizing the codon usage in *E. coli* and thus yielding high titters with other refined expression and purification techniques, including affinity chromatography. The design of engineered or chimeric enzymes forms one powerful strategy. Most of the engineered enzymes possess strengths combined from different DNA Polymerase domains. Advanced purification methodologies, such as ethanol and acetone precipitation, benefit high-fidelity variants. Indeed, ethanol and acetone precipitation methods have been widely used in the purification of DNA Polymerases, which enables the realization of an extremely high success rate, especially when applied to *Pfu*-Sso7d. [19]. A cost-effective laboratory protocol has been developed to purify *Pfu*-Sso7d. Large-scale production is required because research and diagnostic applications have put increasing demands on the development of DNA Polymerases. Special conditions of growth, besides large-scale methodologies like cloning and fermentation, are involved in the process [20]. Affinity and ion-exchange chromatography can be practical ways of purification, while further optimization of production variables has been done, supported essentially by the use of statistical methods to refine conditions in bioreactors.

The following review is designed to give extensive information on *Pfu* DNA Polymerase, comprising the methods for its extraction and production, and what challenges are involved to increase its yield. Also, this review explains different applications of *Pfu* DNA Polymerase and how this enzyme will be a useful tool among researchers and plays a crucial role in biological techniques and experiments like polymerase chain reaction (PCR), cloning, sequencing, and so on.

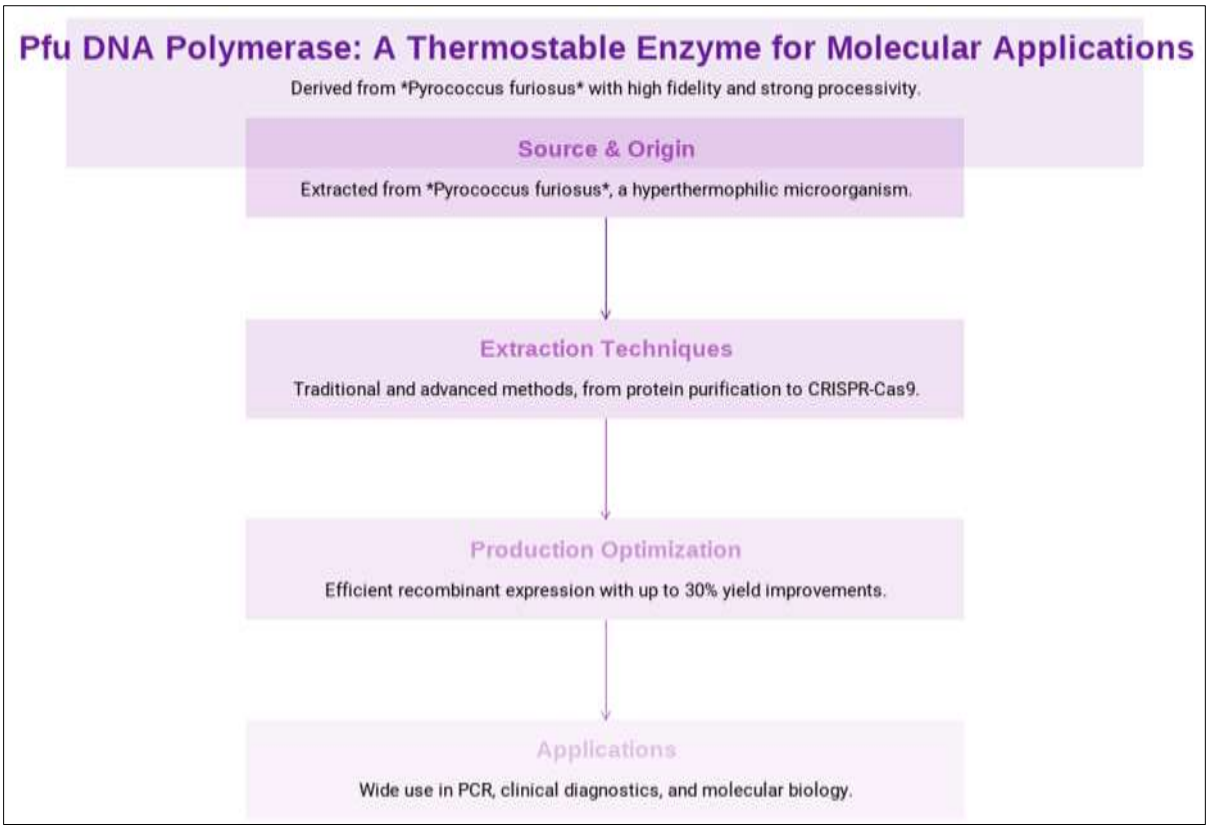

Figure 2 General Overview

2 *Pfu* DNA Polymerase Extraction

2.1 Source: *Pyrococcus furiosus*

Pyrococcus furiosus is an archaeon that thrives in extreme heat, typically found in underwater volcanic vents. This organism can survive in temperatures approaching 100°C, making it an ideal source for heat-resistant enzymes like *Pfu* DNA Polymerase. *P. furiosus*' excellent heat resistance leads to the production of enzymes that keep their stability and functionality despite the severe conditions [21]. Enzymes gained from *P. furiosus* have a rigid structure because of the increased ionic bonding and the solutions of inner hydrophobic forces which thereby make it possible for them to operate at higher temperatures. This trait is the most important one for proper DNA amplification via PCR. The other advantage, which is not so obvious at first sight, is the reverse 3'-5' exonuclease activity of *Pfu* DNA Polymerase, which allows for high-fidelity DNA synthesis. Thus the latter is crucial for ensuring accurate DNA [21].

Table 2 Key Characteristics of *Pyrococcus furiosus*

Characteristic	Description	Reference
Optimal Growth Temperature	100°C	Davletgildeeva et al., 2024
Metabolic Process	Elemental sulfur reduction	Haja, 2021
Key Enzyme	*Pfu* DNA Polymerase	Akram et al., 2023
Exonuclease Activity	3′ to 5′ proofreading, ensuring accuracy	Robb et al., 2001

This table highlights *P. furiosus* characteristics relevant to its application in high-fidelity DNA Polymerase production.

As a hyperthermophile *P. furiosus* can use elemental sulfur as its substrate and turn it into hydrogen sulfide at high temperatures. This kind of unique characteristic indicates the environmental temperature adaptability as a general approach that mostly suits the majority of biological systems [22]. Despite that, the archaeon in question can also produce various other enzymes that are effective at increasing temperatures. The DNA of *Pfu* polymerase is one of those which are specifically the most important concerning the use of this enzyme in DNA replication and also in the PCR-based biotechnology applications [23].

The thermal stability of *Pfu* DNA Polymerase certainly benefits from the evolutionary experience of *P. furiosus* [24]. The protein compositions of this organism include ionic bonds that are increased, hydrophobic cores that are tightly packed, with compactness being characteristic of tertiary structures that are the most stable and resistant to denaturation at high temperatures [23]. Moreover, as a part of a cellular response to heat shock, the HSPs are transported to the heat damaged site of a cell and function as molecular chaperones by forming complexes with unfolded polypeptides potentially correctly folding or preventing the aggregation of proteins [21].

The presence of *Pfu* DNA Polymerase of high stability in the cells of *P. furiosus* allows the cells to process the total genome by DNA to go from 3' to 5' only in the synthetic direction (powerful proofreading) [7]. This is a feature that is not found in other heat-stable DNA Polymerases like *Taq* polymerase, which are bereft of the proofreading mechanism. Thus, *Pfu* DNA Polymerase due to its superior accuracy of DNA Polymerase replication is more efficient for the creation of correct DNA constructs such as cloning, sequencing, and site-directed mutagenesis [25].

Moreover, *Pfu* DNA Polymerase acts as a valuable component in the field of pharmaceuticals, bioengineering, and environmental biotechnology apart from its use in PCR tests. The feature of stability at elevated temperatures makes it desirable for the process of enzyme stability, DNA cloning, and the others that require fluctuation of temperatures and long reactions [26].

2.2 Traditional Methods of Extracting *Pfu* DNA Polymerase

Traditional extraction strategies mainly deal with simplicity and cost-efficiency to capture *Pfu* DNA Polymerase, even though there are some cases when other factors might lower the enzyme's efficiency and accuracy. Here we illustrate frequent techniques:

Extracting and purifying *Pfu* DNA Polymerase from *P. furiosus* begins with anaerobic cultivation of the archaeon at its optimal growth temperature of about 100 °C. That's to say, the lava-like temperature adapted organism (*P. furiosus*) grows anaerobically by using the optimum growth temperature of 100 °C and its *Pfu* DNA Polymerase is then extracted and purified. On completion of this process, the cultures are said to be lysed [27]. It is recommended to be careful in the measurement of enzyme activity during cell lysis, nonetheless excess mechanical or chemical manipulation can cause *Pfu* polymerase to denature [28].

After lysis, the crude extract undergoes purification steps to concentrate *Pfu* DNA Polymerase activity. Among common classical methods, ammonium sulfate precipitation exploits enzyme solubility characteristics. This approach concentrates *Pfu* polymerase by altering its solubility with ammonium sulfate. While straightforward and economical, it demands precise pH and temperature control to avoid enzyme inactivation or purity loss [29] [30].

Subsequent purification typically involves ion-exchange chromatography and gel filtration. Ion-exchange chromatography differentiates proteins based on charge, potentially yielding highly pure *Pfu* polymerase [31], but its time-consuming nature makes it more suitable for small-scale laboratory extractions. Gel filtration, a size exclusion method, removes impurities by filtering molecules according to size [32]. Although effective in eliminating unwanted proteins, gel filtration may result in lower enzyme yields if not optimized [27]. Both techniques are crucial for removing contaminants and ensuring the isolated *Pfu* polymerase is highly pure and active.

Protein Purification Using Affinity Chromatography employs a His-tag attached to *Pfu* polymerase, enabling selective binding to a nickel or cobalt resin in a column. The enzyme is then eluted using imidazole. This method offers high specificity and purity but requires precise buffer conditions to maintain enzyme activity [33].

Affinity chromatography is a widely practiced method for the separation of recombinant proteins like *Pfu* DNA Polymerase. As a rule, the protein for engineering is tagged with His-tag for purification purposes. Thus, His-tagged polymerase is attached only to metal ions such as nickel or cobalt immobilized on column-based resins. This process successfully separates other types of proteins since only the His-tagged polymerase stays in the column [34].

The column releases the enzyme through the buffer with an imidazole that is introduced to the buffer. This compound takes the place of the His-tag and becomes the main binder to the metal ions, causing the polymerase to get off the resin [35]. During affinity chromatography, it's crucial to optimize buffer conditions such as pH and salt concentrations, as unfavourable conditions can result in enzyme inactivation or non-specific binding or disintegration [36]. Throughout the purification process, temperatures should be maintained at levels compatible with *Pfu* polymerase's thermostability to preserve its integrity.

Table 3 Summary of *Pfu* DNA Polymerase Extraction Methods

Method	**Description**	**Advantages**	**Challenges**
Ammonium Sulfate Precipitation	Precipitates enzyme by adjusting solubility	Simple, cost-effective	Requires careful pH and temperature control
Ion-Exchange Chromatography	Separates proteins based on charge	High purity	Time-consuming
Gel Filtration	Size-exclusion purification	Removes impurities	Low yield if not optimized
Affinity Chromatography (His-tag)	Binds enzyme to resin, eluted with imidazole	High specificity, purity	Requires optimized buffer conditions

This table summarizes traditional methods used to extract *Pfu* DNA Polymerase, highlighting the advantages and challenges of each technique.

2.3 Advanced Methods of Extraction

2.3.1 Recombinant Expression Systems

In the past few years, the *Pfu* polymerase gene is used for expression cloning in *E. coli* or other heterologous hosts, the researchers can save the time and hassle of the culture of *P. furiosus*, and the enzyme does not have to be extracted from the archaeon [37]. Such methods simplify the production process, improve the scalability, and guarantee more consistent yields of the enzyme, and therefore, more mass is produced.

To achieve a proper folding and functioning of the proteins, one must design the expression conditions correctly [38]. Such suitable conditions include temperature, and choice of the host strain and induction strategy. These expression conditions, if not common, can be quite deterrent to activity or lead to clustering traits that generally accompany protein misfolding [39].

2.3.2 Automated Purification Systems and Membrane-Based Technologies

The development of high-throughput protein production has primarily driven the advancement of automated protein purification systems. Special automated systems for purification have been very effective in the purification of one of the highly functional enzymes such as *Pfu* polymerase. These systems use various steps, including affinity chromatography and buffer exchange, into a single automated workflow. This method minimizes human intervention and improves reproducibility [40]. Automated purification systems have proven particularly valuable for isolating highly functional enzymes like *Pfu* polymerase.

Another advancement is supplied by membrane technologies, which enable rapid protein isolation in a more efficient manner than the conventional methods of chromatography. Membrane adsorbers can separate proteins using extremely large surfaces for binding and elution, halting protein processing at lower temperature to avoid potential denaturing condition [41]. These systems are highly beneficial for maintaining the structural integrity of heat-sensitive enzymes such as *Pfu* polymerase, ensuring their activity throughout the process.

2.3.3 CRISPR-Cas9 and Protein Engineering

With the recent strides in biotechnology, development horizons for *Pfu* DNA Polymerase are now limitless [42]. This tool allows for the creation of *Pfu* specific mutations integrated in the genome of *P. furiosus*. Such mutations have been successfully exploited to increase the industrial stability and activity of *Pfu* polymerase. There has also been success in protein engineering which has improved stability as well as catalytic efficiency enabling enzyme activity even at harsh temperature extremes, especially for those functions that are thermophilic in nature [43]. The CRISPR-Cas9 tool allows us to identify the precise genome editing, through which expression levels, folding efficacy, or thermostability can be enhanced. This achievement is of paramount importance to industrial processes that rely on high-performance enzymes [44].

Table 4 Comparison of Traditional vs. Advanced Methods of Extraction

Method	**Efficiency**	**Scalability**	**Enzyme Integrity Preservation**
Traditional methods	Moderate	Labour-intensive	Good if carefully controlled
Recombinant expression systems	High	Highly scalable	Excellent due to controlled conditions
Automated systems	Very high	Highly scalable and automated	Excellent with minimal exposure to stress
Membrane-based purification	High	Scalable for industrial use	Excellent due to rapid processing

2.4 Importance of Enzyme Integrity During Isolation

Pfu DNA Polymerase has to be kept pure and without contamination throughout the entire extraction process otherwise the polymerase's functionality may be compromised, mostly its 3′ to 5′ exonuclease activity. On the other hand, the full proofreading ability augments the level of accuracy of DNA synthesis, particularly critical for techniques such as PCR, cloning, sequencing and other related accurate techniques. Every extraction and purification step that follows thereafter is the most optimal targeting the detailed structure of enzyme protein [25].

Careful handling is required during lysis of cells if denaturation of enzyme has to be avoided. Buffered sonication is more effective than vigorous mechanical cell disruption methods in protecting the structural and functional integrity of enzymes [28]. Likewise, it is also important to maintain a proper pH and temperature during ammonium sulfate precipitation to prevent the loss of activity or unwanted protein precipitation [30].

E. coli lysates are readily available materials about cloning enzymes, flipping proteins, and they make only one or two steps of purification between lysate and resin, but during this step, the synthesis of new proteins must be prevented.

Moderate buffer conditions, such as imidazole concentration and temperature, during purification help prevent polymerase denaturation and promote active enzymes [36]. Recombinant expression and membrane-based purification also showed an effectiveness of minimal loss of the integrity of enzyme by reducing its amount to rough conditions [45].

Preserving the enzyme's proofreading activity is one of the most challenging and difficult task of purification, as any loss or damage would compromise or low down its role in high-fidelity DNA replication [46]. Purification protocols must be meticulously optimized to ensure the enzyme retains both its polymerase and exonuclease functions.

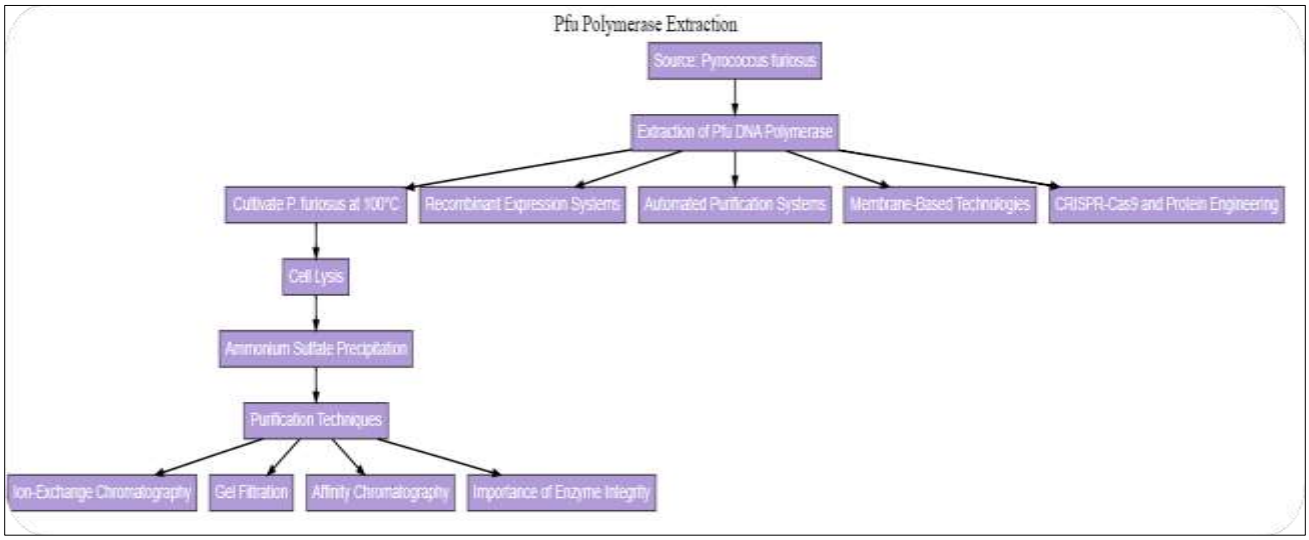

Figure 3 Flow diagram of *Pfu* DNA Polymerase Extraction

3 *Pfu* DNA Polymerase production

Pfu DNA Polymerase, derived from *Pyrococcus furiosus*, is known for its remarkable and unique high-fidelity DNA replication, making it a valuable asset in molecular biology [42]. Initially, *Pfu* DNA Polymerase was directly purified from P. furiosus, yet achieving high-yield production proved challenging. While small-scale laboratory production typically involves affinity purification, industrial production requires more advanced and up-to-date instruments [47]. Companies use large-scale fermentation technologies, addressing both upstream and downstream processes to maintain enzyme activity and purity. The creation of codon-optimized variants and advancements in fermentation techniques have greatly increased production levels and reduced costs, making the enzyme widely available in the market [48].

3.1 Research Laboratory/Institute Production

3.1.1 Recombinant DNA Technology

The production of *Pfu* DNA Polymerase has transformed due to the existence of recombinant expression systems. Rather than cultivating the *Pyrococcus furiosus*, the *Pfu* polymerase encoding gene is now cloned and expressed within the *Pfu* host organism, mostly *E. coli* [49]. As a result, enzyme production is now easily scalable and uniform owing to the presence of optimal conditions that ensures maximum activity and yield of the enzyme [50]. Key parameters such as temperature, host strain selection, and induction methods determine the proper enzyme folding and delivery. The use of these optimal conditions avoids other possible problems such as protein misfolding or loss of activity therefore recombinant expression can be said to be an innovation that plays a major role in promoting the efficient production of *Pfu* polymerase [51]. *E. coli* was then employed by the researchers as a host organism, amplified and overexpressed the *Pfu* gene using recombinant technologies. Due to its fast reproduction cycle, *E. coli* is a favorable host in recombinant DNA technology for fast protein expression. [52]. Moreover, *E. coli* is a favorable target for recombination due to its well-characterized genetics as a result of numerous studies [53]. Consequently, this led to the development of pLysS His6 tagged *Pfu*-pET28a recombinant plasmid. Upon further optimization of the tissue culture protocols, the His-tagged *Pfu* protein was introduced into competent *E. coli* BL21 (DE3) cells and harvested using a simplified single-step purification that employs Ni^{2+} chelating affinity chromatography with AKTAprime plus. *Pfu*'s protein samples were authenticated through peptide mass fingerprinting. The focus was on a bioassay that tested the performance of the modified enzyme in a PCR reaction versus the commercially available *Pfu*. It was determined that the range in the pI

value for *Pfu* DNA Polymerase was between 6.85 and 7.35. Such results certainly emphasize an efficient use of the enzyme, and prove that the methodology introduced is effective in the progress of biotechnology [27].

3.1.2 IPTG Induction and Optimization

The production of recombinant proteins is known to vary with IPTG induction concentration [54]. It is well established that the production of recombinant proteins is influenced by the concentration of the IPTG transcriptional inducer [55]. In a new study, a dual restriction method for the use of glucose and IPTG was proposed. With this method, *E. coli* fed-batch cultures exhibited enhanced biomass production while maintaining low levels of acetic acid secretion. Initially, IPTG was added only once, at 2 hours at concentrations of 0.1 to 2.0 mM. Then, during hours 2 to 6, 0.1mM IPTG was supplied every hour four times. Samples were taken every 12 hours, and the samples were also taken to analyze the concentration of IPTG in the liquid medium and inside the cells. Optical density at 600 nm was used for following the growth of cell cultures. It was 13.5% more efficient to use this new strategy as it gave *Pfu* activity in shake flasks than the traditional use of 1mM IPTG for induction. IPTG usage could also be reduced by 50% [56].

3.1.3 Codon Optimization

Obtaining high expression levels and purity of recombinant proteins can be challenging, but codon optimization offers a reliable solution [57]. Achievements of high concentration and purity of recombinant proteins are oftentimes difficult to obtain, but codon optimization presents a practical solution to this challenge [58]. The overexpression of functional recombinant *Pfu* DNA Polymerase can be made possible, while preserving the amino acid sequence, by utilizing the codons of the enhanced green fluorescent protein (eGFP). The pET28a plasmid, which is common in the expression of proteins in bacteria, was then used to clone the optimized r*Pfu* gene, making possible the purification of His-tagged proteins easily [59].

A different approach to enhance the yield of *Pfu* DNA Polymerase in *E. coli* is through a synthetic gene that has been optimized for codons. The r*Pfu* gene was expressed in four *E. coli* strains. *E. coli* strains with the expressed r*Pfu* protein then underwent purification through Ni-NTA His•Bind® resin with the aid of the His tag of the recombinant protein. Histidine-containing peptides, often referred to as His-tags, allow for simple and highly efficient purification methods via affinity chromatography. For this purification strategy to achieve optimal outcomes, up to 95 % of the protein results achieved are often impure. The His-tag allows easy and effective purification of target proteins using affinity chromatography and consists of short stretches of histidine residues. Research has indicated that this purification system can get contaminants to as low as 5% for up to 95 percent of the purified soluble recombinant protein. The enzyme produced had a bioactivity of 12,987 U/mg, was able to amplify DNA fragments of length about 5.5 kb without loss in function or efficiency and is likely to be important for molecular biology applications. It is important to note that 88,311 U r*Pfu* were obtained from 50mL of *E. coli* culture which is worth noting considering the advantages of this expression system as there may be feasibility of scaling up for practical use [60].

To further enhance codon optimization, a synthetic gene was engineered to increase *Pfu* DNA Polymerase yield in *E. coli*. This heterologous expression utilized an IPTG-inducible expression system; ATUM US synthesized the plasmid pD451SR containing a codon-optimized *Pfu* DNA Polymerase gene, pD451-SR-*Pfu*pol. *E. coli* BL21 Star DE3 transformants were cultivated in LB-kanamycin medium supplemented with 0.4% glucose, as it engineering protein lost might be possible during cultivation so anti-biotic maintenance is important for stability [61]. A two-step chromatographic purification process was employed to separate the crude extract from the supernatant. The dual-step production and purification of recombinant *Pfu* DNA Polymerase, involving nickel affinity chromatography followed by anion exchange chromatography, resulted in a high yield of 26.8 mg/L. This substantial yield demonstrates the efficiency of the purification techniques employed [62].

3.1.4 Development of Chimeric Enzymes

Chimeric enzymes are fusion proteins combining distinct domains from different polymerases, which enhance enzymatic properties such as processivity. Multiple fusions are created, and the most effective chimeric proteins are chosen for further use. One such chimera, *Pfu*-Sso7d, was produced by fusing the DNA Polymerase of *Pfu* with a small DNA-binding protein derived from *Sulfolobus solfataricus*, Sso7d [63 64]. This new chimera improved the DNA Polymerase's processivity. Recently, cost-effective laboratory methods for expressing and purifying *Pfu*-Sso7d have been reported [65]. The process begins with preparing heat-cleared lysates from the *Pfu*-Sso7d source, which helps denature proteins and remove cellular debris, enhancing subsequent purification steps. After overnight induction, bacterial cells are harvested by centrifugation at 4000 rpm for 15 minutes at 4 °C. *Pfu*-Sso7d is then precipitated from the heat-cleared and DNase I-treated lysates using ethanol and acetone. Large-scale extraction and purification of *Pfu*-

Sso7d starts with inoculating 100 mL of LB medium containing appropriate antibiotics with 1 mL starter culture, maintained under constant shaking at 37 °C [19 66].

3.1.5 Commercial Level Production

Despite its numerous advantageous characteristics, large-scale production of *Pfu* DNA Polymerase faces several obstacles [67]. Mass production necessitates efficient and economical processes, including the use of cost-effective media and reagents, along with optimized fermentation and purification techniques [68]. As a complex protein, *Pfu* DNA Polymerase expression poses challenges in host organisms. The purified enzyme is formulated into a stable solution containing suitable buffers, salts, and stabilizers. This solution optimization is crucial for maintaining enzyme activity and stability. Stringent quality control during fermentation is essential to ensure the enzyme's purity, efficiency, and stability are not compromised [69].

3.1.6 Industrial Fermentation and Upstream Processing

As previously noted, *E. coli* is the preferred host organism for cloning and producing the desired protein. Scaling up *E. coli* fermentation from small to pilot scale involves several critical factors that affect the efficiency and yield of recombinant protein production [70]. Comprehending these factors is vital for optimizing bioprocesses and ensuring successful scale transitions. Consistent metabolic responses in microbial cells can be achieved by maintaining constant parameters such as kLa (volumetric mass transfer coefficient). As bioreactors increase in size, the surface-to-volume ratio decreases, making oxygen transfer more challenging. Maintaining a constant kLa ensures consistent oxygen availability [71].

3.1.7 Response Surface Methodology (RSM) for Optimization

The production of recombinant *Pfu* DNA Polymerase in *E. coli* BL21 (DE3) can be significantly improved by fine-tuning induction variables with techniques such as Response Surface Methodology (RSM). This statistical approach is widely employed to optimize biological processes by developing empirical models [72]. For industrial-scale production, stirred-tank bioreactors are commonly utilized due to their prevalence in laboratory settings. Modifying growth media components, specifically asparagine and glucose concentrations, has been shown to boost biomass yield, which is crucial for recombinant protein production [73]. By optimizing induction parameters (IPTG concentration, optical cell density before induction, post-induction duration, and temperature), researchers successfully enhanced DNA *Pfu* polymerase expression levels and increased biomass. RSM was employed to examine the effects of these four independent variables on *E. coli* BL21(DE3) biomass, with all possible combinations tested in duplicate.

The optimal induction conditions determined through RSM in shake flask experiments were effectively replicated in larger 3L and 10L bioreactor trials. Under the recommended conditions (initial OD600nm of 0.4, induction at 32 °C for 7.7 hours, and 0.6 mM IPTG), the highest biomass yield of 14.1 g/L was achieved in shake flasks. When scaling up the experiments using these optimized conditions, biomass production increased by 22% in the 3L bioreactor and 70% in the 10L bioreactor compared to previous unoptimized conditions. Additionally, the optimization process resulted in a 30% increase in *Pfu* DNA Polymerase production. The quality of the produced *Pfu* DNA Polymerase was evaluated using PCR amplification, revealing a polymerase activity of 2.9 U/µl, when compared to commercial *Pfu* DNA Polymerase. This study's findings indicate that the proposed fermentation conditions could contribute to future research aimed at enhancing biomass production for various recombinant proteins [74].

3.1.8 Autoinduction with Defined Media

Using an innovative medium, it has been possible to optimize the autoinduction mechanism for expression of *Pfu* DNA Polymerase in a recent investigation. This sort of optimization is important to meet the increasing demand for use of this enzyme in the diverse areas of biotechnology. In recombinant expression of *Pfu* DNA Polymerase in *E. coli* systems with T7 induced promoter expression is practiced regularly, this practice, however, might become costly. Conventional induction techniques pose their own constraints when performed in large scale owing to the excessive costs of IPTG, and the tediousness of LB type media preparation [75].

Instead of IPTG, lactase is used in the auto-induction technique which integrates lactose, glycerol and glucose. Using this auto-induction into a chemically defined medium brings about tremendous benefits in enhancing the production process and meeting the demands of industrialization. It has been established that using the pD451-SR-*Pfu*pol plasmid, altering the supply of carbon in a controlled medium was most effective to produce recombinant *Pfu* polymerase. The use of glycerol, glucose and lactose as carbon sources produced good results. Adjustments to the ratios of these components including their effects on the yield of recombinant *Pfu* DNA Polymerase made progress. At first, glycerol 0.9%, lactose 0.5%, glucose 0.05% were present in the medium. However, the highest yield was achieved with optimized

concentrations of 0.05% glucose, 1% lactose, and 0.6% glycerol. A 5L bioreactor was utilized to optimize aeration, inoculant, and agitation parameters. The autoinduction method in a defined medium resulted in the successful production of approximately 71 mg of active *Pfu* DNA Polymerase per liter of culture. The use of the optimized defined medium and parameters in a 5L bioreactor increased *Pfu* DNA Polymerase production by up to 2.6 times [76].

3.2 Challenges in production

The optimal agitation rate for maximizing biomass production and achieving higher yields is 900 rpm. It was noted that *Pfu* DNA Polymerase yield at 1200 rpm was lower compared to that at 900 rpm [77]. Proper mixing of the fermentation media is vital for uniform distribution of temperature and nutrients throughout the bioreactor. Maintaining an appropriate agitation rate is crucial, as excessive agitation can lead to increased microbial death due to impeller tip speed [78]. This can negatively impact biomass production [79]. Ensuring the product is free from contaminants, such as endotoxins, is essential for its use in sensitive applications. Another challenge in large-scale biomass production is cost sensitivity, as it requires substantial investment in equipment, labor, and raw materials. Consequently, cost optimization is critical [79] [76].

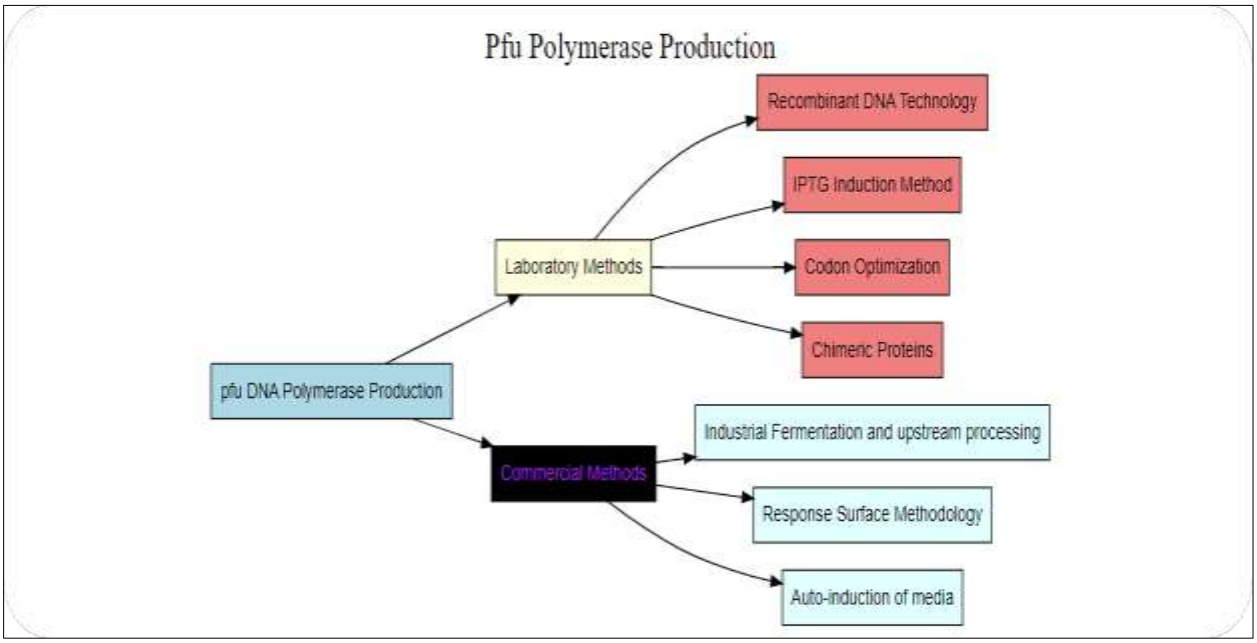

Figure 4 Flow Diagram of *Pfu* DNA Polymerase Production

4 Applications of *Pfu* DNA Polymerase in Scientific Research

Pfu DNA Polymerase derived from *Pyrococcus furiosus*, is the most common enzyme in molecular biology because of its great accuracy and stability of the enzyme to heat. It is one of the original ones of its type. The enzyme itself is important in many different polymerase chain reaction-based applications, such as gene cloning [68], microbial genomic research, metagenomics, and diagnostic microbiology, which are just some of the fields where its usefulness can be easily seen. The major advantage of these enzyme-related fields is that they can get the accuracy and durability of the enzyme, which makes *Pfu* DNA Polymerase an absolutely necessary tool in research [80].

4.1 PCR and Molecular Cloning

In Polymerase Chain Reaction, the *Pfu* DNA Polymerase is especially appreciated for achieving high-fidelity amplification and therefore has been using more and more often [81]. The *Pfu*'s 3andprime;->5andprime; exonuclease activity provides marvelous precision to the enzyme through its substantial contribution to error reduction in DNA synthesis [82]. This specific attribute is vitally important in molecular cloning where precise DNA replication is mandatory for creating valid genetic construction. The enzyme performs its main function by oxidizing flavin and transferring the electrons to M2. In both gene cloning and mutation analysis experiments, *Pfu* is a very important element, as the sequence fidelity is the main factor that affects the functionality of the cloned genes [83].

It allows for easy amplification of sequences with an error rate that is significantly lower; therefore, this enzyme increases the success rate of cloning operations in recombinant DNA technology. It gives a clue of assurance that mutations introduced accurately represent the original gene sequence [84] . Where accurate genetic sequences are needed for the study of protein function and structure, *Pfu* polymerase is often more favorably used compared to *Taq* polymerase because it is more accurate [85].

4.2 Microbial Genomics

In the field of microbial genomics, particularly for extremophiles, *Pfu* DNA Polymerase has amazing applications. Its basic advantage lies in its remarkable ability to amplify DNA from thermophilic microorganisms at high temperatures without introducing errors [27]. For instance, DNA Polymerase from *P. furiosus* can support research that is aimed at elucidating gene adaptations for survival at high temperatures to gain deeper insights into the survival mechanisms of thermophiles [86].

In pathogenic microbiology, *Pfu* helps in sequencing of genomes of the pathogen in order to identify the regulating patterns of genes and resistance marker [80]. The enzyme's high fidelity reduces the sequencing errors, making it a awesome tool for genomic analysis and pathogen characterization [87].

4.3 Applications in Metagenomics

Pfu DNA Polymerase shows significant strength in metagenomic research, especially when studying microbial communities in extreme environments. This enzyme enables the amplification of mixed DNA from environmental samples such as soil and water, facilitating the identification and examine of microbial diversity within these communities [88]. This capability is important for studying ecosystems like hydrothermal vents, where organisms have acquired unique adaptations to extreme conditions. The resilience nature of *Pfu* makes it well-suited for remarkable handling the low concentrations and degraded DNA often studied in metagenomic samples. This distinguished characteristic allows for the identification and analysis of genes crucial for microbial survival in these challenging and harsh habitats [42].

Pfu's capability in processing complex metagenomic samples with low DNA concentrations and degradation enables the detection and analyzing the genes required and important for microbial survival in these environments [89]. Moreover, *Pfu*'s ability to amplify such complex samples promises to deliver right and accurate sequencing results, supporting studies on gene diversity and ecosystem dynamics [90].

4.4 Role in Diagnostic Microbiology

The high fidelity of *Pfu* DNA Polymerase is particularly valuable in diagnostic microbiology. Accurate amplification is critical for detecting mutations and resistance genes in pathogens. The enzyme's fidelity ensures precise amplification of microbial DNA, reducing the likelihood of false positive or negative results [91-93]. In antimicrobial resistance studies, *Pfu* aids in identifying mutations within pathogens associated with resistance, a capability of growing importance in managing infectious diseases [94].

Diagnostic assays utilizing *Pfu* excel in detecting rare pathogens, with the enzyme's high specificity ensuring accuracy even for low-abundance species. *Pfu*'s precise microbial DNA identification enables early detection of pathogenic organisms, contributing to swift disease diagnosis and management [95].

5 Discussion

5.1 Advantages Over Other Polymerases

Pfu DNA Polymerase offers distinct benefits compared to other commonly used polymerases in molecular biology. A key advantage of *Pfu* is its high fidelity, attributed to an inherent 3′→5′ exonuclease activity that enables proofreading during DNA synthesis [96]. This proofreading capability distinguishes it from enzymes like *Taq* polymerase, which lacks this feature and consequently has higher error rates during DNA amplification [97]. In applications involving setView™ clones, mutation analysis, and sequencing, where even minor sequence errors can lead to significant misinterpretation, *Pfu*'s accuracy becomes indispensable [98].

Pfu polymerase's thermal stability makes it particularly well-suited for amplifying DNA from extreme or thermophilic organisms. Originating from *Pyrococcus furiosus*, an archaeon that thrives in high-temperature environments, it can endure prolonged exposure to elevated temperatures without losing functionality. This characteristic makes it ideal for

procedures involving numerous denaturation steps, such as PCR [99]. Its ability to withstand high temperatures allows scientists to investigate the most extreme microbial genomes with minimal enzyme degradation, which is valuable in thermophile and extremophile research[100]. Consequently, *Pfu* is often the preferred choice for studies requiring precision and durability. Additionally, *Pfu* DNA Polymerase shows promise in advancing probiotic research and furthering developments in biotechnology and pharmaceutical innovations [93 101]

5.2 Limitations

Despite its advantages, *Pfu* DNA Polymerase has notable drawbacks. Its primary disadvantage is a significantly slower reaction rate compared to *Taq* polymerase. Due to its reduced extension rate, *Pfu*-based reactions typically necessitate longer incubation periods, potentially extending overall experimental duration [4] [102].

Additional limitations include the substantial costs associated with large-scale *Pfu* production, especially when compared to *Taq* polymerase, which is manufactured in much larger quantities. Producing *P. furiosus* requires specific host strains and optimized conditions, driving up product costs beyond the reach of many small laboratories operating with limited budgets [103]. Furthermore, *Pfu* can be challenging to work with when dealing with severely damaged or highly complex templates. In such cases, other polymerases that are more tolerant of inhibitors may be more suitable. Nevertheless, *Pfu*'s exceptional accuracy and stability make it indispensable for numerous applications [104]. Thus, while *Pfu* excels in applications needing high accuracy and stability, these limitations may restrict its applicability in certain experimental settings.

6 Future Perspectives

6.1 Advances in Enzyme Engineering

Continued improvement in the technology of enzyme engineering offers the potential to tailor *Pfu* DNA Polymerase for the continuing and changing needs of molecular biology. Using genetic alterations and protein engineering, scientists attempt to increase *Pfu*'s fidelity. Processivity can be improved; this refers to the rate at which the DNA synthesizing enzyme produces its copy without falling off [105]. It is assumed that such modifications could allow *Pfu* to rival *Taq* polymerase in speed without compromising its high fidelity, a cocktail that would have made it highly useful in applications involving speed, such as diagnostic PCR and high-throughput sequencing. Innovations are being directed towards engineering *Pfu* mutants with desired features in view for applications in synthetic biology, including the competence of taking up non-canonical nucleotides or generating artificial DNA constructs with a high degree of accuracy. [99]. These changes indicate a greater movement for the redesign of polymerases with specific functionalities, placing *Pfu* in a prime position for easy manipulation both in gene editing and in therapeutic gene synthesis and may mark a conceptual change in genetic analysis and synthetic biology shortly [106]. These achievements shows a clear trend toward engineering polymerases with customized functionalities, positioning *Pfu* as a valuable tool in gene editing and therapeutic gene synthesis, potentially transforming approaches to genetic analysis and synthetic biology in near future [107].

6.2 Expanding Applications in Microbiology

Especially with excellent fidelity and temperature stability, the enzyme *Pfu* DNA Polymerase shows huge potential for new microbiological applications, including synthetic biology, microbial ecology, and CRISPR-Cas systems. *Pfu* has immense possibilities in synthetic biology: making high-precision gene constructs, inducing site-specific mutations at very low error rates, and the very important process of designing reliable synthetic genes and pathways [108]. It can provide microbial ecology with a better understanding of the microbial genomes and metabolic pathways from different environmental samples. The latter may allow detailed exploration, giving novel insights into microbial interaction and ecosystem dynamics, with *Pfu* accuracy [109]. More importantly, *Pfu* could provide the high-fidelity amplification of DNA to the editing of microbial genomes using CRISPR-Cas systems or even insertion of targeted genetic modification with minimum rate of off-target effects, thereby increasing the precision in gene-editing applications throughout the prokaryotic models [110]. The expanded use of *Pfu* for such purposes may bring about new opportunities regarding microbial research and, on a broader scale, have a significant ripple effect toward environmental sciences and biomedical sciences.

6.3 Environmental and Industrial Applications

In fact, the future might also see *Pfu* DNA Polymerase applied to an extended use in environmental DNA analysis and industrial microbiology. High fidelity and resistance to environmental inhibitors-features that make this enzyme very suitable for microbial community studies in complex environments such as soil, marine sediments, and wastewater

systems-demand accuracy in the detection of microbial DNA [111]. In studies of environmental DNA, *Pfu* could enable the investigation of microbial biodiversity and genetic features present within environmental samples in support of conservation biology, ecosystem monitoring, and environment remediation projects [112]. The stability of *Pfu* polymerase also makes this enzyme a promising candidate for industrial applications, such as enzyme production and biosynthesis processes, where it may enhance microbial strain resistance to extreme industrial conditions. Further development of its application in these two fields can make *Pfu* an instrument of the singular kind for both environmental and industrial biotechnology, connecting scientific study with sustainable and practical results.

6.4 Overall Future Perspective

Advancements in enzyme engineering offer exciting possibilities for enhancing the functionality and applications of *Pfu* DNA Polymerase. Genetic editing tools like CRISPR-Cas9 and protein engineering techniques can be used to further improve the enzyme's thermal stability, fidelity, and catalytic efficiency. [105] By modifying specific residues or domains, researchers can develop variants of *Pfu* that perform optimally under extreme industrial conditions or with unique substrates [111]. Additionally, integrating *Pfu* into automated, high-throughput systems could streamline large-scale production, making it more accessible for research and diagnostic use. Expanding its utility in synthetic biology, microbial genomics, and biotechnological applications will be pivotal as demand grows for high-performance enzymes capable of supporting innovative scientific and industrial processes [112] [110]

7 Conclusion

Pfu DNA Polymerase is a very important enzyme in molecular biology, with some characteristics that make it extremely useful, such as its very high fidelity and thermal stability, and the wide range of its applications from PCR and cloning to diagnostics and environmental microbiology. This enzyme's 3'-5' exonuclease activity ensures high fidelity in DNA synthesis and, therefore, is crucial for high-fidelity applications. Continuing improvements in its extraction and production techniques have led us to achieve increased biomass and prevail in its processivity and fidelity. Different methods have developed to get yields of high quantities practically. These methods enable us to perform our desirable technique according to our research specifications and economic demands to extract or produce *Pfu* DNA Polymerase. Continuing improvements in production and enzyme engineering of *Pfu* DNA Polymerase will be in a good position to sustain the ever-growing demand at research and industrial levels. *Pfu*'s role will expand further in the future as it takes part in new discoveries in genetic studies, biotechnology, and environmental science. This is an enzyme whose task puts it squarely at the intersection of precision and toughness, placing it at the leading edge of scientific progress.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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