Optimization of annealing temperature for detection of lipase gene in *Bacillus subtilis* using polymerase chain reaction (PCR) method

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**Abstract.** Enzyme lipase (EC 3.1.1.3, triacylglycerol acylhydrolase) is a group of enzymes that hydrolyzes fat into fatty acids and glycerol. In addition, the lipase enzyme plays a role in various reactions, such as esterification, interesterification, and transesterification reactions. Lipase can be obtained from microbes, plants, and animals. The most important source of lipase is microorganisms that are produced by fermentation of different bacteria. Microbial Lipases are easy to produce and cost of production is low. Bacteria like *Bacillus* are commercially used for lipase production. Recombinant lipase enzyme can be expressed by lipase gene (*lip* gene) and secreted in the bacterial *Bacillus subtilis*. Polymerase Chain Reaction (PCR) is in vitro amplification Deoxyribonucleic acid (DNA) method. The purity and yield of the reaction products depend on several parameters, one of which is the annealing temperature. The aim of this research was to optimize an annealing temperature for lip gene amplification using PCR. The success in amplifying a gene by PCR technique using a specially designed primer is determined by the precision of the primary attachment temperature with the DNA. Two Primer pairs are designed to successfully amplify DNA sequence fragment, namely xynlip_for_41_nt_ and xynlip_rev_38_nt_. The primer combination produced the best results regarding band intensity and relative absence of unspecific bands, producing the expected amplicons of approximately 792 bp. Regarding the annealing temperatures, the best amplification was obtained at 60 °C.

**Keywords:** *Bacillus subtilis*, enzyme lipase, polymerase chain reaction (PCR)

1. Introduction
The lipases, namely triacylglycerol acylhydrolases (EC 3.1.1.3) specifically hydrolyzes the emulsified triglycerides. In organic solvents, lipases can catalyze the reverse reactions as well, e.g. esters, and transesterifications synthesis. Lipases are an industrially important subgroup of the hydrolase superfamily [1]. Over 70 lipases covering leastwise 47 dissimilar lipases from bacteria were cloned and sequenced. Lipase has an important role, especially in the field of biotechnology for many applications in the food industry, detergents, and enzymatic processes in the production of lipophilic chemicals [2]. Lipases can be obtained from microbes, plants, and animals. The most important source of lipases is microorganisms that are produced by fermentation of different bacteria. Microbial Lipases
are easy to produce and cost of production is low. Bacteria like Bacillus are commercially used for lipase [3]. Microbes are the main source of the 100 or so enzymes industrially generated for the above mentioned ground. Mostly, commercial lipase derived from microorganisms. Bacillus subtilis showed extracellular lipase activity with high growth rate at optimum conditions. Recombinant enzymes in prokaryotic systems are good for expression of enzymes extracellularly production [4].

During the technique of polymerase chain reaction (PCR), amplification of DNA is performed with in vitro through a polymerization cycles series containing the three stages of temperature-free procedure: denaturation of DNA, primer-template annealing, and synthesis of DNA with a thermostable DNA polymerase. The reaction products’ yield and purity are depended on some factors, such as the annealing temperature (Ta). At the Ta values of both sub- and super-optimal, generation of non-specific yields might be produced, the reduction of product yield is occurred. Optimization of the Tm is specifically crucial when long product is prepared for PCR [5].

One of the most effective method applied presently in molecular biology is DNA amplification by polymerase chain reaction (PCR). The PCR optimization covers the number of variables tests, namely the annealing temperature (Ta). When the Ta is significantly low, the amplification of non-specific DNA fragments takes place, leading to the multiple bands emerge on on agarose gels. If the Ta is very high, the expected product yield, and sometimes the purity is lower, in consequence of the poor annealing of primers [6]. The purpose of this research is to study the optimization annealing temperature for lip gene amplification using PCR.

2. Materials and method

2.1. Sample
The sample used was the lipase B. subtilis gene that has been cloned into the pGEMlip plasmid in E. coli DH5α as the pSKE xyn AQ1 insertion gene and vector which has been transformed into E. coli DH5α. The microorganism inserts, vectors and strains were the collections of the Non-Virus Molecular Biology Laboratory, the Agro and Biomedical Industry Technology Development Laboratory (LAPTIAB), the Agency for the Assessment and Application of Technology (BPPT).

2.2. Amplification lip gene using PCR
For amplification lip gene two primers are used. The forward matches the 5’region of the insert and has 20–25 nt. Size of reverse primer is 40–50 nt, which contains 20–25 nt sequence with reverse complementary to the region on the target plasmid and matches the 3 end of the insert. The 1 EMP PCR reaction components are 25 ng template DNA, 0.5 μM primer F1, 0.5 μM primer R1, 1× HF Phusion buffer (NEB), 200 μM of each dNTP, and 0.02 U/μL Phusion DNA Polymerase (NEB) in a volume of 50 μL. To increase the efficiency of the reaction, GC Phusion buffer was added up to 3 % dimethyl sulfoxide (DMSO). Every cycle of PCR covers stages for template denaturation, primer annealing and primer extension. The first denaturation step is carried out at the beginning of PCR. Initial Denaturation for 30 seconds at 98 °C, denaturation for 10 seconds at 98 °C. The next step was annealing at 60 °C, 63 °C and 65 °C, and extension for 15 s/1Kb at 72 °C. The final extension step for 5 minutes at 72 °C, follows completion of the last PCR cycle. OligoAnalyzer 3.1 (IDT) are used for calculated the Tm values. After the PCR, the product needs to be loaded on a gel.

3. Results and discussion
Two Primer pairs are designed to successfully amplified DNA sequence fragment, namely xynlip_for_41_nt_ and xynlip_rev_38_nt_ (table. 1). If the amplification is successful, then the known length of that DNA sequence will be appeared after running the PCR product through electrophoresis, a technique that separates DNA by length. The primary pair can amplify the lip gene and produce bands of 750 base pairs. Electrophoresis of PCR products indifferent annealing temperatures
showed that the optimal annealing temperature was 60 °C. When an annealing temperature is 63 °C, the lipase gene could not be consistently amplified. When an annealing temperature is 65 °C, no DNA bands could be detected (figure 1). This is consistent with PCR optimization studies that have been carried out in other gene primers that the annealing temperature used is usually 5 °C lower than the melting temperature (Tm) value.

Amplification of lipase gene was done by Polymerase Chain Reaction (PCR) technique. PCR is a molecular method of DNA replication in vitro using enzymes and a pair of primers that are specific to the target DNA. Certain segments of DNA can be multiplied millions of times in a relatively short time, making it easier for other techniques to use DNA. Amplification of genes by PCR requires a corresponding pair of primers [7]. The primary base arrangements (primary and reverse primers) are designed based on the amplified sequence of nucleotide genes and the sequence of recognition restriction enzymes found in multiple cloning sites (MCS) of expression vectors used. The way PCR works is very similar to the mechanism of DNA replication in cells. Each PCR cycle consists of a denaturation stage, annealing, and primary elongation or elongation [8].

The primers used for PCR reactions in this study are primary forward (Xynlip_For_41_nt_) and primary reverse (Xynlip_rev_38_nt) (table 1). The template used for the amplification of the lipase gene is the result of the isolation of the pGEMlip recombinant plasmid. The synthesis and amplification of lipase genes begins by optimizing template dilution and annealing temperatures. The primer combination and using the annealing temperatures of 60 °C produced the best results regarding band intensity and relative absence of unspecific bands, producing the expected amplicons of approximately 792 bp (figure 1). In one study, the annealing temperature of 45–50 °C was used for the detection of lipase gene from B. subtilis [9]. In another study, the DNA from Bacillus licheniformis was used for the amplification of lipase gene using the annealing temperatures of 65 °C [10].

**Table 1.** Primer and sequence for amplification lip gene using PCR.

| Primer              | Sequence                          |
|---------------------|-----------------------------------|
| xynlip_for_41_nt    | 5'- tgc cgg tgt ttg ggc gcc gga cca ata atg acc tct gaa tc -3 |
| xynlip_rev_38_nt    | 5'- cag att cat tcc atg gtc att aat tcg tat tct ggc cc -3' |

![Figure 1](image1.png)

**Figure 1.** The effect of the annealing on PCR (a) annealing temperature 60 °C, (b) 63 °C, (c) 65 °C. M: Marker (DNA ladder 1 kb) 1: DNA sample Gel agarose 1 %, 100 V, 27 minutes.
4. Conclusion
Two Primer pairs are designed to successfully amplified DNA sequence fragment, namely xynlip_for_41_nt_ and xynlip_rev_38_nt_. The primer combination produced the best results regarding band intensity and relative absence of unspecific bands, producing the expected amplicons of approximately 792 bp. Regarding the annealing temperatures, the best amplification was obtained at 60 °C.

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