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Research Paper

Optimization of PCR Annealing Temperature in Xylanase-Producing Bacteria

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ABSTRACT: *This research studied the optimization of annealing temperature in the PCR process by varying the annealing temperature (49ºC, 55ºC, and 58ºC). The samples obtained from compost isolation taken in the mesophilic area at 45ºC. There were 5 bacterial isolates obtained at this mesophilic temperature. The stages carried out in this study were screened for xylanase enzymes, DNA isolation from selected isolates (F2E), and PCR. The optimum temperature obtained was 58ºC where the band appeared quite clear and thick at 1500 bp.*

KEYWORDS: *Annealing, Bacteria, PCR, Xylanase*

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I. INTRODUCTION

Xylanase is an enzyme of the hydrolase class (EC 3.2.1.8) that plays a role in degrading the linear polysaccharide β-1,4-xylan to xylose and breaking down hemicellulose which is one of the main components of plant cell walls [1]. Xylanase can be isolated from bacteria [2], and fungi [3]. The product of hydrolysis from xylanase is xylose which is widely used for pharmaceutical applications, the food industry, the paper industry, and others. Xylose is becoming a new preference for consumers looking for healthier and natural products, increasing the market for xylose. The forms of xylose that are widely marketed are L-Xylose, D-Xylose, and DL-Xylose.

Bacteria that can produce xylanase enzymes are also called cellulolytic bacteria because they have the ability to degrade cellulases so they can hydrolyze cellulose in plants. Many bacteria from the *Bacillus* genus are known to produce xylanase enzymes including *Bacillus amyloliquefaciens* [4], *Bacillus subtilis* [5] *Bacillus pumilus* [6].

Previous research has carried out the isolation of xylanase-producing bacteria at thermophilic temperature [7] and temperature 37 [8]. In this study, the isolation of xylanase-producing bacteria was carried out from compost samples taken at mesophilic temperature, and further testing was carried out using the 16S ribotyping method. Ribotyping is a technique used to identify and characterize various organisms from various species, such as 16S ribotyping for bacteria and 23S ribotyping for fungi [9]. This technique facilitates the recognition of microbes. In bacteria, the ribosomal operon is a gene that encodes different ribosomal RNA structures such as 23S, 5S, and 16S where these three structures are conserved. However, 16S is the most conserved so it is used for identification of bacterial phylogeny and taxonomy. The 16S rRNA is a conserved and variable region with a length of 1550 bp.

To understand the PCR-based ribotyping process, it is necessary to learn about the success of the PCR process. PCR is useful for generating millions of copies of a particular DNA sequence with a piece of amplified DNA. The PCR technique involves three main steps, namely DNA denaturation, annealing, and DNA elongation. Annealing is the primary attachment stage to a single DNA chain, the annealing time is generally 30-45 seconds [10]. The annealing temperature must be within the range of 50°C and 68°C, depending on the length, GC concentration, and specificity of the primer. It must be between 5 and 7 ºC colder than the melting point. The optimal PCR product is obtained from the right PCR conditions. The resulting PCR products were then viewed by electrophoresis and visually analyzed for bands. The expected result is a thick band and the right size [11]. The aim of this research is to optimize the annealing temperature in PCR from DNA of xylanase-producing bacteria in order to obtain optimal PCR products.

II. RESEARCH METHODS

The steps carried out in this study were xylanase screening, DNA isolation, PCR amplification and electrophoresis to see the size of the DNA and the results of PCR amplification.

Bacterial Isolate

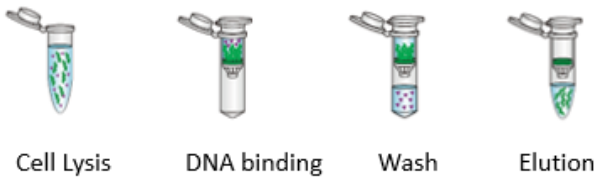
The bacterial sample in this study was a collection of bacteria obtained from compost isolation taken in the mesophilic area. There were 5 bacterial isolates at this mesophilic temperature. The bacterial isolates used were F1C, F2E, F4D, F4E and F5D.

Xylanase Screening

Xylanase screening was carried out to determine whether the sample bacteria could produce xylananse enzymes. Materials used for selection of xylanase-producing bacteria were K2HPO4 1.5%, MgSO4.7H2O 0.025%, NaCl 0.25%, NH4Cl 0.5%, Na2HPO4 0.5%, Bakto agar 2%, yeast extract 0.2 % (w/v) and 0.5% xylan. Xylan was obtained from Sigma Aldrich. The cultivation of bacteria on Agar media was carried out by streak the culture from the collected bacterial stock. Selection of bacterial colonies was carried out based on the clear zone that was produced around the colonies after the addition of congo red solution.

DNA isolation

Chromosomal DNA was isolated using a DNA extraction kit from Genaid. The reaction volume used was adjusted according to the Genaid protocol. The results of the isolation were seen using electrophoresis. The steps for isolating bacterial DNA in outline can be seen in scheme 1 [12].



Scheme 1 : DNA isolation step

PCR amplification

PCR amplification was carried out with 30x cycles. The reagent used at this PCR stage is MyTaq Extract-PCR Kit BIOLINE. The primers used are universal primers 27f and 1492r. The total volume used was 25 µL. The kit mixture was then reacted with pre-denaturation at 95ºC for 3 minutes, denaturation at 95ºC for 30 seconds, annealing for 30 seconds and elongation at 72ºC for 1 minute. This condition was repeated 30 times.At this stage optimization of the annealing temperature is carried out, namely 49ºC, 55ºC and 58ºC. This stage aims to amplify DNA.

Electrophoresis

This stage is carried out to see the size of the band resulting from the results of PCR and DNA isolation. The results of DNA amplification can be seen through agarose gel electrophoresis. As much as 0.3 grams of agarose was added to 30 ml of TAE buffer 1x. The solution was then heated until the agarose completely dissolved and the color of the solution becomes clear. As much as 1 µl EtBr was added to the agarose solution, then the solution was poured into the tray until it solidified. The tray containing the agarose gel was put into the electrophoresis device and then immersed with 1x TAE buffer until the entire surface of the agar was covered with the solution. As much as 5 µl of PCR results were put into the agarose well by first mixing it with 1 µl of loading buffer so that the total volume that was put into the well was 6 µl. The electrophoresis process lasted for 45 minutes with a voltage of 70 volts. Electrophoresis results can be seen by placing the agarose gel under UV light (360 nm).The band is expected to be thick and clear.

III. RESULTS AND DISCUSSION

There were 5 bacterial isolates sampled from mesophilic compost isolation. These bacteria are isolates F1C, F2E, F4D, F4E and F5D. From these five isolates, xylanase screening was carried out and it was found that there were several isolates that could produce xylanase (Figure 1). Bacteria that positively produce xylanase enzymes were F1C, F2E, and F4E bacteria. This screening is qualitative so at this stage it only provides information about whether or not the sample bacteria can produce xylanase enzymes. The clear zone produced on solid LB media indicated that the sample bacteria could hydrolyze xylan polysaccharides into simpler compounds, namely xylooligosaccharides, and xylose. Quantitative testing was not carried out in this article.

Congo red, a water-soluble dye that is carcinogenic, has been utilized as a standard contrast dye for cellulose, amyloid fibrils, and agricultural starch products. Only alkaline proteins are bound by congo red [13].

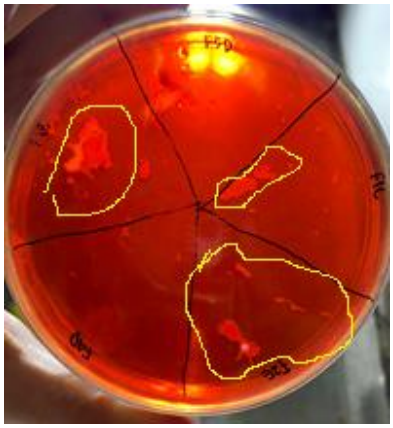


Figure 1: xylanase screening

The positive isolates were then isolated from the DNA. The main principles of DNA isolation are lysis (destruction), extraction or separation of DNA from solid materials such as protein (binding), and DNA purification (washing and elution). The results of DNA isolation can be seen in Figure 2 below.

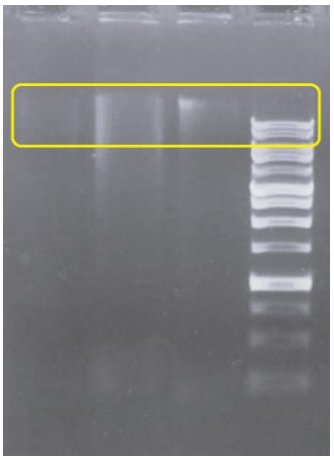


Figure 2: DNA isolation

Prokaryotic nucleic acid isolation requires much less effort than plants and animals. Bacteria cell walls can readily be broken or maybe lysed to separate their DNA and RNA. The ideal source of the material is pure culture bacteria. As long as there is no contamination throughout the process, either agar or liquid cultures can produce identical results depending on the kind of organism employed for the genetic material. The majority of airborne bacteria may grow in nutrient-rich media, so extra caution must be exercised when utilizing them [14]. Each buffer contained in the kit has a different function. Proteinase K was used during DNA extraction to digest the many contaminating proteins present. It also degrades nucleases that may be present in DNA extraction and protects the nucleic acids from nuclease attacks. The DNA binding step is the process of binding DNA/RNA so that it can be separated from proteins and other impurities. The washing process step aims to remove contaminants while the DNA remains bound to the membrane. The genomic DNA elution step is a resuspension step by adding a buffer solution as a solvent for the DNA/RNA material. Elution of pure genomic DNA is ready for subsequent reaction [12]. F2E isolates were selected for further testing of DNA amplification by PCR.

In this research, optimization of the annealing temperature of PCR was carried out by varying the temperature at 49ºC, 55ºC, and 58ºC. The primers used are universal primers 27f and 1492r. The primer sequence of 27f is 5'-AGAGTTTTGATCCTGGTCCAG-3'. Meanwhile, the primer sequence of 1492r is 5'-GGTTACCTTGTTACGACTT-3'. At annealing temperature conditions of 49ºC, a non-specific band was obtained (Figure 3). Non-specific bands are bands that do not correlate with the expected mutant, transgene, or wild-type bands. They are the result of unspecified primary annealing. The presence of a non-specific band is highly undesirable. Non-specific bands are the result of mispriming at unwanted and off-target sites on the target genome or contaminating DNA. If the annealing temperature is too low, the primer may bind nonspecifically to the template. Non-specific bands can also be caused by too large a primary volume [15],[16]. During the PCR annealing phase, the reaction temperature must be low enough to allow forward and backward primers to bond with the template, but not too low to allow the formation of non-specific duplexes or unwanted intramolecular hairpins, both of which reduce reaction efficiency. One of the factors that influence the success of amplification is annealing temperature because the process of attaching primers to DNA strands that are already open requires an optimal temperature. It is important to find the optimum temperature so that it is hoped that the maximum amount of DNA in the target area can be obtained so that it is quite easy for DNA analysis [17].

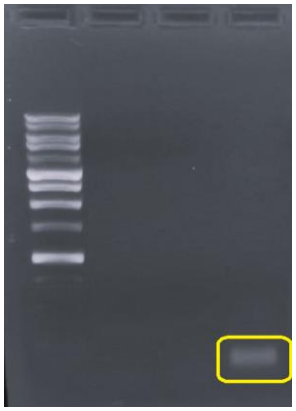


Figure 3: PCR at 49ºC

The second optimization was carried out to obtain a better band. The annealing temperature was increased to 55ºC. From the results of this second optimization, PCR visualization was obtained as shown in Figure 4 below. The non-appearance of the band can be caused by an inappropriate attachment temperature. . For this reason, further PCR was carried out by changing the annealing temperature to 58ºC (figure 5).

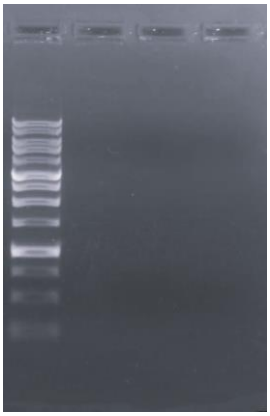


Figure 4: PCR at 55ºC

From the third PCR result, a fairly good band was obtained. The expected band is a band that is firm enough and at the target size. In this study, the band was expected to appear at ±1500 bp. The annealing temperature in this third stage was 58ºC (figure 5). It can be said that the optimum annealing temperature in the PCR process of this study was 58 ºC. In the image below it can be seen that the band appears around 1500 bp.

Positive control was used to ensure the experiment was carried out correctly and produce a positive effect on the dependent variable. Short, single-stranded nucleic acids known as oligonucleotides or oligos are employed as primers to start the synthesis of DNA. They anneal to the plus and minus strands of the template DNA during PCR operations, flanking the target sequence. The region of interest in the template DNA must be considered while designing PCR primers, as well as the parameters of the reaction.

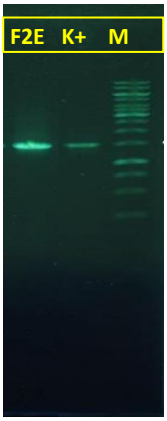


Figure 5: PCR at 58ºC

IV. CONCLUSION

In this study, optimization of the PCR annealing temperature of xylanase-producing bacteria has been carried out. The optimal temperature obtained was 58ºC. From these PCR results, the optimal PCR product was indicated by a thick band.

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