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Research Article

Isolation and Amplification of the *phy* Gene Coding Phytase from *Bacillus sp.*

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ABSTRACT

Poultry are unable to metabolize phytic acid in feed due to no enzyme to digest and degrade it. Therefore, phytic acid becomes an anti-nutritional substance in poultry feed which can result in decreased feed efficiency, decreased nutrient intake which gave a negative impact on health and production. The addition of phytase in the poultry ration is necessary to degrade phytic acid. However, the availability of phytase in Indonesia is still limited because technology in Indonesia is still inadequate to produce phytase, so Indonesia cannot produce it itself and still imports phytase at quite high prices. This study aims to isolate and amplify the *phy* gene encoding phytase derived from native microbe's genus *Bacillus* from Indonesia. The method used in present study was to isolate the genome of *Bacillus* using cell lysis method, then amplify the genome using polymerase chain reaction (PCR) method. The results of this study were that genome isolation results from *Bacillus* gene sources were obtained that the purity of A_{260}/A_{280} from *B. sp* 6, *B. sp* **7,** and *B. licheniformis* was 1.86 µg/mL, 1.95 µg/mL and 1.86 µg/mL, respectively. Furthermore, the present study showed that 0.4 µM/µL primer concentration was able to amplify the *phy* gene. 200-400 ng/µL DNA template concentration can produce optimal DNA bands during amplification of the *phy* gene in *Bacillus sp***.** In conclusion, amplification was successfully carried out on *B. sp* and *B. licheniformis***.** The length of the *phy* gene in *B. sp* is 1149 bp and the *phy* gene in *B. licheniformis* is 1146 bp. The genus *Bacillus* native to Indonesia has the potential to be continued as a source of phytase producing genes.

Keywords: Amplification, *Bacillus*, Isolation, PCR, Phytase

Introduction

In general, poultry feed is formulated from cereals, grains, wheat, and legumes [1]. One of the important elements contained in these plants is phytic acid. Phytic acid *(myo-inositol 1, 2, 3, 4, 5, 6-hexakis dihydrogen phosphate)* is the dominant phosphorus storage compound in plants [2]. Some animal feed raw materials such as rice bran, soybean meal and pollard contain lots of phytic acid. It is known that poultry is unable to metabolize phytic acid due to the lack of phytate hydrolytic enzymes inside digestive system [3]. Therefore, phytic acid is an anti-nutritional agent in poultry because it can chelate mineral elements needed by poultry such as calcium, magnesium, copper and zinc [4].

Anti-nutritional substances contained in animal feed raw materials can result in a decrease in feed efficiency due to a decrease in nutrient absorption that affects digestion, health and livestock production. Phosphorus is an important mineral for the growth and development of poultry bones [5]. Phosphorus deficient in poultry lead to detrimental effects such as bone deformities and disruption of metabolic processes as well as low performances [5]. In order for poultry phosphorus needs to be met, it is necessary to add inorganic phosphate to poultry feed. However, phosphorus excreted through feces is the main cause of environmental pollution problems [6].

Phytase has been used as feed additive to help degrade phytic acid in feed. Phytase *(mio-inositol hexaxisphosphate phosphohydrolase)* is an enzyme capable of hydrolysis the reaction of a phosphodiester bond in phytic acid and produces inorganic phosphate and phosphate esters

from lower mio-inositol [4]. Phytase is generally used as an enzyme in monogastric animal feed to increase the bioavailability of phosphorus phytate and other nutrients [7].

The availability of phytase enzymes in Indonesia is limited. Indonesia is still importing phytase at quite high prices because technology and human resources in Indonesia are still inadequate to produce phytase its own phytase. Therefore, it is important to carry out various efforts to overcome and fulfill these needs. One of the efforts made is to over-express through genetic engineering which can be sourced from various sources. Phytase can be sourced from plants or microorganisms [1]. Indonesia is rich in biological natural resources that have the potential source to produce phytase like native microbial gene sources in Indonesia. A previous study showed that there was a prominent bacteria like *Bacillus* genus [4]. In this study the phytase-type *β-propeller phytases* (BPPs) were characterized, mainly from *Bacillus sp*. considered as potential candidate based on its characteristics [1]. The BPP have good thermostability and usually maintain their maximum activity at neutral or alkaline conditions [3]. BPP phytase is a potential candidate as a feed additive in the aquaculture industry and environmental applications because it has a neutral to alkaline pH profile and higher thermal stability [1].

Considering that there are many benefits from phytase, such as increasing phosphorus absorption, reducing dependence on phosphorus supplements, increasing feed efficiency, and reducing water and soil pollution [8], production of the phytase enzyme is important, especially in Indonesia, which currently cannot produce its own. Before producing phytase, the *phy* gene is needed, which is obtained by isolating the gene in a microbe to obtain DNA from the microbe. Then it is amplified to produce many copies of the DNA sequence using the polymerase chain reaction (PCR) method [9]. We hypothesize that native Indonesian *Bacillus* sp. has significant potential as a commercial bacterial phytase because of its biological characteristics, including substrate selectivity, thermophilicity, resistance to proteolysis, and catalytic efficiency. Therefore, the objective of this study is to isolate and amplify the *phy* gene encoding phytase derived from microbes native to Indonesia from the genus *Bacillus*.

Material and Methods

Ethical Statement

All experimental procedures were approved by the Research Ethics Committee Universitas Padjadjaran.

Materials

The study was using bacteria from the genus *Bacillus* as a gene source isolate. The bacteria were obtained from the National Research and Innovation Agency (BRIN) InaCC collection, Indonesia. There were 3 isolates from different *Bacillus* species according to the code and origin. *Bacillus* sp. 6 (InaCC B348) originating from tauco, Satonda Island in West Nusa Tenggara, Indonesia, *Bacillus* sp. 7 (InaCC B694) originating from stone, Solok in West Sumatra, Indonesia and *Bacillus licheniformis* (InaCC B1088) originating from marine sediments, in Rambut Island, Seribu, Indonesia. The bacteria were rejuvenated in liquid nutrient broth (NB) media and incubated for 16 h (overnight) in a shaker incubator at 37°C until the growth was homogeneous.

Genome Isolation

The bacterial genome was isolated using a modified cell lysis method based on the kit protocol used for genome isolation, namely the *Presto™ Mini gDNA Bacteria Kit* (Geneaid). Genome isolation methods include pre-lysis, cell lysis, DNA binding, washing, and DNA elution.

After the genome has been successfully isolated, the results of the genome isolation are checked using a DNA electrophoresis gel agarose 1%. DNA concentration and purity were measured using NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific).

Primer Design

Gene sequence data of *Bacillus* sp. and *B. licheniformis* was obtained from the gene bank, namely the National Center for Biotechnology Information (NCBI) *(https://www.ncbi.nlm.nih.gov/)* which was then used for primary design. Specific primers for the *phy* gene were used to amplify the phytase encoding gene from isolated genomic DNA from *Bacillus* sp. and *B. licheniformis* designed manually. Primer design was carried out using several software, namely *Oligo Calculator*, *Fast PCR*, and *SnapGene*. Each primer consists of a forward primer (F) and a reverse primer (R).

Amplification *phy* Gene

The PCR parameters in this study consisted of 5 stages, these are pre-denaturation, denaturation, annealing, elongation, and final elongation [10]. Amplification of the *phy* gene using the *My Taq™ HS Red Mix PCR Kit* (Meridian Bioscience, Bioline) was carried out according to the protocol in the kit. The PCR program consisted of (1) initial denaturation for 3 min at 95°C, followed by 35 cycles of (2) denaturation for 15 sec at 95°C, (3) annealing for 15 sec with different temperatures, (4) elongation for 1 min at 72°C, and (5) final elongation for 5 min at 72°C. The third stage of PCR, which is a crucial stage to determine amplification result. Gradient annealing temperature (Ta) for *B. licheniformis* varies in the range 53-61°C. Ta in *Bacillus* sp. *6* and *Bacillus* sp. 7 varies in the range 53-59°C. In the end, the PCR results were checked by DNA electrophoresis.

Results

We obtained that the concentration and absorbance ratio of DNA in the genome of each *Bacillus* species varied. It was found that the concentration of *Bacillus* sp. 6 was 69.1 ng/µL with an absorbance ratio of 1.86 µg/mL (A_{260}/A_{280}). The concentration of *Bacillus* sp. 7 was 184.1 ng/µL with an absorbance ratio of 1.95 µg/mL (A_{260}/A_{280}). The concentration of *B. licheniformis* was 208.2 ng/µL with an absorbance ratio of 1.86 µg/mL (A_{260}/A_{280}). In this study, the genome isolation results were examined. Muzuni [11] reported that the value of the genomic DNA concentration of *Bacillus* sp. *Rh* is 1,460 µg/µL. DNA concentration is categorized as high concentration if it has a concentration value of 1000 µg/µL. A good DNA concentration can vary depending on the purpose and type of analysis to be performed. In molecular applications, generally the DNA concentration that is considered good ranges from 50 to 500 ng/µL. Olmedo [12] reported that the DNA concentration of *Bacillus* spp. of 40 ng/µL. The study in line with Sambrook & Russell [13], the purity of a sample can be declared pure if it has an A_{260}/A_{280}-A_{260}/A_{230} ratio of around 1.8-2.0 µg/mL. Even though the DNA concentration is slightly low, this does not affect its purity.

Based on gene bank data, *Bacillus* sp. have the *phy* gene encoding phytase. The number of sequences found was 19 data with the length of the *phy* gene being 1149 base pairs (bp) and 7 *phy* gene sequences from *B. licheniformis* with length 1146 bp. Specific primers for the *phy* gene were used to amplify the phytase encoding gene from isolated genomic DNA from *Bacillus* sp. designed manually. Each primer consists of a forward primer (F) and a reverse primer (R). The length of the *Bacillus* sp. primer bases for the forward primer is 30 bases (5'-ATGAAGGTTTCAAAAACAATGCTGCTAAGC-3') with melting temperature (Tm) 57.5°C and 37% GC content and the reverse is 18 bases (5'-CTAGCCGTC CAGAACGGTC 3') with Tm 52.6°C and 61% GC content. The length of the *B. licheniformis* primer bases for the forward primer is 23 bases (5'-ATGAACTTT TACAAAACGCTCGC-3') with Tm 51.7°C and 39% GC content and the reverse is 28 bases (5'-TTATTT GGCTCGTTTTTTCAGTTTTCGG-3') with Tm 55.5°C and 36% GC content. The primers design results have been tested in silico using *Fast PCR* and *SnapGene* applications. *Fast PCR* results show that the primer can attach 100% to the template and was able to amplify the gene as a whole (100%). PCR simulations using the *SnapGene* application showed that primers can amplify the *phy* gene.

Amplification was measured using the PCR method and examined using DNA electrophoresis. It was found that the *phy* genes of *Bacillus* sp. 6, *Bacillus* sp. 7, and *B. licheniformis* had been successfully amplified. We obtained that a primer concentration of 0.4 µM/µL was able to amplify the *phy* gene and varying DNA template concentrations (200-400 ng/µL) in each species could produce optimal DNA bands for use in amplifying the *phy* gene in *Bacillus sp*.

An important step during the PCR process is determining variations in annealing temperature (Ta gradient). In *Bacillus* sp. 6 and *Bacillus* sp. 7, the annealing temperature varied over a Ta gradient from 53-59°C and all showed positive results. The best annealing temperature for *Bacillus* sp. 6 is 54.98°C and for *Bacillus* sp. 7 is 56.42°C. The *B. licheniformis phy* gene was successfully amplified at an annealing temperature of 53.54; 53.72; 55.64; 54.98 and 56.42°C.

Discussion

Considering the limited availability of the phytase enzyme in Indonesia, we are trying to utilize biological resources by isolating and amplifying the *phy* gene encoding phytase originating from *Bacillus* native to Indonesia as the first step in genetic engineering for phytase production. According to Saadi et al.[4], phytase derived from *Bacillus* genes has been widely studied and is a type of phytase that has unique characteristics, as well as the feasibility of mass production for application in animal nutrition. This study was conducted to test the potential of native Indonesian *Bacillus* as a *phy* gene encoding phytase.

The principle of genome isolation is to destroy the cell wall without damaging the target DNA [3]. In the present study, 1 kb DNA ladder marker was used as a marker indicating that the genome of *Bacillus* sp. *B. licheniformis* and has a size above 10000 bp. These results showed positive results, which means that DNA has been successfully isolated. Genome isolation aims to obtain pure DNA [14]. The quality of DNA in genome isolation is very important because it affects the success and accuracy of various analyzes and experiments involving genomic DNA. In this study, all *Bacillus* genome isolation results were obtained with good DNA quality. We obtained that the purity of A_{260}/A_{280} from *Bacillus* sp. 6, *Bacillus* sp. 7, and *B. licheniformis* was 1.86 µg/mL, 1.95 µg/mL and 1.86 µg/mL, respectively. DNA can be declared pure since it has an absorbance ratio A_{260}/A_{280} in the range of 1.8-2.0 µg/mL. This study in line with research conducted by Ni'mah [15] namely The Quantity and Quality Comparison of *Bacillus sp*. DNA between Heat Treatment and Filter Based Kit and in line with research conducted by Bonis et al.[16], who isolated *Bacillus thuringiensis* DNA using the DNeasy Blood and Tissue Kit (Qiagen) with an absorbance ratio of A_{260}/A_{280} and A_{260}/A_{230} between 1.5 and 2.5. If the purity A_{260}/A_{280} value is below 1.8 then the DNA resulting from the isolation carried out is likely to be contaminated with protein, and if the purity value is above 2.1 then it can be suspected

that the DNA resulting from the isolation carried out is contaminated with RNA [17,18].

The design of the primers aims to obtain primers that will be used when amplifying the DNA of the *phy* gene by the PCR method. The length of the primer base of *Bacillus sp*. for forward (F), that was 30 bases and 18 bases for reverse (R). The length of the primer base of *B. licheniformis* is 23 (F) and 28 (R) bases. The primer pair serves to limit the target DNA fragment to be amplified [3]. The ideal primary length range was between 18-30 bp [1]. Moreover, Saadi et al.[4]'s research used *Bacillus subtilis* to design primers for the *phyC* gene, in lenght 28 (F) and 31 (R) bases.

The optimal Tm for primers ranges from 52 to 58°C [2]. Tm *Bacillus sp*. for the forward primer at 57.5°C and for the reverse primer at 52.6°C. The temperature difference is one of the considerations in the primary design. Primer pairs that have a difference in Tm exceeding 5°C can reduce the amplification process, and may even have the potential to prevent the amplification process [19]. The difference in Tm in the primer *Bacillus* sp. and *B. licheniformis* was 4.9°C and 3.8°C, respectively.

The GC contents in the primer *Bacillus* sp. were (F) 37% and (R) 61%. The GC content in the primer *B. licheniformis* were 39% (F) and 36% (R). The base content of GC was detected to range between 40% and 60% [19]. The study in line with [28] that the optimum G+C base composition is in the range of 35-60% with the difference in GC content between the two primers being in the range of 5%. The higher the GC content in the primer, the better it will be because the adhesion will be much stronger. Guanine and Cytosine have 3 hydrogen bonds which means the bond is stronger when compared to Adenine and Thymine which only have 2 hydrogen bonds. The GC content is sought to be at the 3' end sequence therefore the primary attachment bond is not easily separated [20]. The high GC content will affect the Tm value [21].

Amplification is the process of taking genes from chromosomal DNA in vitro using the polymerase chain reaction (PCR) method. PCR is a method used to amplify millions of DNA segments in a short time [20]. The *phy* gene amplification process using the PCR method needs to pay attention to the composition, concentration, and parameters of the PCR. PCR composition includes DNA templates, gene-specific primers, dNTPs, PCR and $MgCl_2$ buffers, as well as DNA polymerase enzymes [20].

The DNA template concentration used was (Sp 6.1) 276.4 ng/µL, (Sp 7.1) 368.2 ng/µL, and (L1) 416.4 ng/µL with as much volume 2 µL for all sample. The primary concentration used was 0.4 µM/µL. There are 5 parameters observed in the PCR process [22]. These parameters include initial denaturation, denaturation, annealing, elongation, and final elongation [22]. The PCR method has a working principle that mimics DNA replication in cells *in vitro* therefore it can be used in this study to amplify DNA in the *phy* gene. The PCR process carried out lasts up to 35 cycles.

The annealing temperature will vary within a predetermined range when designing the primer. Ta on *Bacillus* sp. varies in the range 53-59°C. Ta optimum for *Bacillus* sp.6 is 54.98°C, for *Bacillus* sp. 7 is 56.42°C and for *B. licheniformis* is 54-56°C. According to Yusuf [23], the annealing temperature ranges between 36-72°C, but the temperature generally used is between 50-60°C. Moreover, Sugiarti et al.[24] in their research the PCR process using an annealing temperature of 45-50°C from *B. subtilis* and 65°C from *B. licheniformis* [24]. This difference is because the longer the primary size, the higher the temperature [23].

The amplification results were checked by DNA electrophoresis gel agarose 1%. *Bacillus* sp. was successfully amplified with a base length of 1149 bp. The results of our study are in line with Rao [25] that the length of the *phy* gene from *Bacillus sp* (DECSR1) is 1149 bp. In accordance with data at NCBI that *Bacillus sp*. has a length of 1149 bp which can translate 383 amino acids. *B. licheniformis* was successfully amplified with a base length of 1146 bp. The results are in line with Li [26] dan Liu [27] that the length of the *phy* gene from *B. licheniformis* is 1146 bp.

In conclusion, the results of genome isolation of native *Bacillus* sp. 6, *Bacillus* sp. 7 and *B. licheniformis* from Indonesia were pure and can be continued for amplification. The annealing temperature that can be used in PCR for *Bacillus* sp. 6 and *Bacillus* sp. 7 varies with the optimum temperature of 54.98°C and 56.42°C respectively. The optimum Ta for *Bacillus licheniformis* is in the temperature range of 54-56°C. Amplification was successfully carried out on *Bacillus* sp. 6, *Bacillus* sp. 7 and *B. licheniformis*. Therefore, the three *Bacillus* species native to Indonesia has the potential to be continued as a source of phytase producing genes.

Availability of Data and Materials

The data supporting this study's findings are available from the corresponding author (N. Mayasari) upon reasonable request.

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Ethical Statement

All experimental procedures were approved by the Research Ethics

Committee Universitas Padjadjaran.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributıons

DAS, YW, NH, AH, ASHAI, LT, and NM compiled and planned the experiment. DAS, LT, and NH conducted data collection and analysis. DAS, LT, and NM contributed to the interpretation of the results. DAS led the script writing. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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