# Cloning of β-Lactamase Encoding Gene as the Initiation Approach in Providing High Quality Milk

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**Abstract.** In silico exploration of several recombinant plasmids by previous study, informed that recombinant plasmid pLA230 is one of the plasmids harboring potent β-lactamase encoding gene producing enzyme that possesses specific activity to degrade penicillin antibiotics and its derivatives. The known target gene sequence in the recombinant plasmid pLA230 can facilitate the DNA isolation process, as source of β-lactamase genes that are more specific than gene isolation from wildtype organisms. Based on the information related to the potential of β-lactamase gene source pLA230, it can then be used as the basis to conduct direct gene exploration through isolation of β-lactamase gene from plasmid pLA230 and validate the nucleotides sequence via sequencing method. This study aims to obtain β-lactamase encoding gene from pLA230 through PCR method; obtain *E.coli* transformant carrying pTA2 with insert gene β-lactamase from pLA230 facilitated by TA cloning method; and obtain information related to the source, structure, and group of β-lactamase protein obtained through sequencing data analysis using bioinformatics software in silico. This study succeeded in obtaining 861 bp of β-lactamase enzyme encoding gene from pLA230, which was confirmed based on the alignment data between the origin sequence of the recombinant plasmid map pLA230 with the clone sequence read by sequencing method. The 861 bp of β-lactamase coding gene amplified using TA cloning method in the pTA2 vector via heat shock transformation. Positive *E. coli* transformants were screened using blue-white screening that can grow well on selective media containing 100 mg/mL penicillin. Analysis using Blastp software showed that the gene most likely came from Klebsiella pneumoniae, *Escherichia coli*, or *Acinetobacter haemolyticus* with a percentage confidence level of 99.95%, 99.30% and 99.30%. Tertiary protein modeling using I-TASSER software showed 5 protein models with the highest C score of -0.07 in the 1st model. (c) The analysis results inform that the β-lactamase protein is a group of Extended

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Spectrum Beta Lactamase (ESBL) of class A which is a class of Serine Beta Lactamase (SBLs) with the greatest specific similarity to the SHV-1 β-lactamase type protein (Sulphydryl Variable beta lactamase) which is a type of β-lactamase enzyme that has specific activity on penicillin antibiotics, and TEM-1 β-lactamase protein (Analysis of Temoniera beta lactamase) which is a type of class A β-lactamase (SBLs) from Gram-positive and negative bacteria, with Enzyme Commission Number (EC) of 3. 2.5.6.

**Keywords:** Antibiotics, Recombinant Plasmid, pLA230, Gene Cloning, PCR.

# 1 Introduction

Cow's milk is one of the livestock products with high nutritional content that is useful to help meet daily nutritional needs in the community. A common problem in cow's milk production is its low quantity and poor quality due to dairy cows suffering from mastitis. Mastitis is an inflammatory reaction in the mammary glands of cows caused by bacterial infections including *Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma, Enterobacter aerogones* and *Klebsiela pneumonia* [1].

A common way to overcome the problem of mastitis in livestock is with the use of antibiotics. The most commonly used antibiotics are β-lactam antibiotics such as benzyl penicillin and ampicillin, but this has other implications, namely the presence of antibiotic residues that are still detected in fresh milk products on the market. On the other hand, the continuous use of antibiotics without regard to withdrawal time will cause health problems including hypersensitivity reactions, resistance, and the possibility of poisoning as well as problems with failures in the processing process [2]. One treatment that can be applied to overcome the problem of antibiotic residues in milk is to use degraders of these antibiotics. An approach that has been developed as a solution is the use of β-lactamase enzyme. The β-lactamase enzyme is one of the hydrolase enzymes that has a good ability to degrade β-lactam antibiotics by breaking the β-lactam ring in the antibiotic structure [3]. Therefore, the main objective of this research is as a solution to create antibiotic contaminant free milk so that it is classified as Aman, Sehat, Utut, dan Halal also known as ASUH (Safe, Healthy, Whole, Halal) food.

Shinkai & Loeb (2001) in his research has successfullyconstructed isolates with genes encoding β-lactamase coded as pLA230 [4] . Gene or DNA insert in the recombinant plasmid pLA230 has a Some alternative names include beta-lactamase, ampicillinresistant, and carbenicillin resistant [5]. The full sequence size of the recombinant plasmid pLA230 is 3,683 pb nucleotides long consisting of several cloning regions with the specific sequence of the β-lactamase encoding gene located in the Multiple Cloning Site (MCS) region along the 861 pb from sequence number 18 to 879.

In silico exploration of several recombinant plasmid sources by the Haryono Research Team in 2021, informed that the recombinant plasmid pLA230 is one of the plasmids with an insert gene encoding the β-lactamase enzyme and confirmed that the protein from the gene has specific activity against penicillin antibiotics and their derivatives. In addition, the recombinant plasmid pLA230 can be utilized as a source of β-lactamase gene that is more specific than DNA isolation from wildtype organisms because the sequence of the target gene is known, so it can facilitate the DNA isolation process. However, the initial cultivation of pLA230 plasmid bacterial isolates showed

very low growth ability on selective media. This may be caused by several factors including improper storage of cell stock so that the cells are damaged, mutation of the gene encoding the β-lactamase enzyme so that it loses its antibiotic resistant properties, or the concentration of antibiotics used is not appropriate.

One of the properties of genes is that they can undergo mutations due to the influence of internal and external factors [6]. Checking the sequence of nucleotide sequences as a representation of gene conditions against mutation events to ensure gene conditions can be done through the alignment of the sequencing results of the β-lactamase gene source pLA230 with the origin sequence of the pLA230 plasmid map which will show the real and current conditions of the β-lactamase gene. Based on the preliminary information from the computational exploration that has been carried out in the previous research roadmap, namely as an initial step in screening β-lactamase gene source plasmid candidates, it can then be used as a basis for an approach to conduct direct gene exploration through isolation of β-lactamase gene from plasmid pLA230, and analysis of gene information through data processing of sequencing readings and translation into protein sequences to determine information related to the prediction of gene source organisms, tertiary protein modelling, protein similarity analysis with PDB data to determine the β-lactamase enzyme group, and prediction of Enzyme Commission number (EC). These data can then be compiled as gene data to be used to help select the most potential β-lactamase genes from several existing candidates to be used in further development stages.

In general, the storage of DNA isolates outsidethe cell for a long period of time can reduce the purity of the DNA [7]. In cloning technology, cloning vector is one of the gene storage media that can perform multiplicationthrough the process of host cell reproduction. The isolated β-lactamase gene that has been obtained can be stored and reproduced in the cloning vector and cloned in the appropriate host cell, so that it can be used as a medium for storing genes for a longer period of time.

## 2 Materials and Methods

### 2.1 Materials and Tools

The materials used in this study consisted of American Bacteriological Agar media, Luria Bertani media, agarose powder, TBE solution, sterile distilled water, Ethidium Bromide (EtBr), ampicillin antibiotics, DMSO, Biolab 1 Kb marker, DNA isolation kit (PrestoTM Mini Plasmid Geneaid), PCR kit (Thermo Scientific Dream Taq Green PCR Master Mix 2x), gel purification kit (ZymocleanTM Gel DNA Recovery), cloning kit (Toyobo Target Clone), PCR product, pTA2 vector, competent cells *E.coli* DH5α. The tools used in this study include; glassware namely test tubes, Scott bottles, 200 mL Erlenmeyer, 250 mL measuring cup; non-glassware equipment namely 1.5 and 2 mL centrifuge microtubes, PCR microtube, micropipette, microtip, ose needle, spatula, stir bar, petri dish; and instruments namely autoclave (Tomy SX-500), Laminar Air Flow (LAF), incubator (Soft Incubator SLI-600ND), vortex, analytical balance (Ohaus), hot plate (Oxone), centrifuge (Tomy MX-105), Nanodrop spectrophotometer (One Microvolume UV-Vis Spectrophotometer), PCR machine (Eppendorf Mastercycler Nexus

Gradient Thermal Cycler), electrophoresis machine (Mupid-exu AVR 500 P), and Geldoc (Documentation UV Tech Cambridge Fireder V10 Plus).

### 2.2 Isolation of pLA230 Recombinant Plasmid DNA

Cell harvesting from pLA30 bacterial cultures to obtain plasmid DNA. Bacterial cells that have been cultured in liquid media are then transferred as much as 500 µL into a 1.5 mL micro tube. Centrifugation was carried out at 16,000 x g for one minute at room temperature until a cell pellet was formed and the supernatant was discarded. The remaining bacterialcell stock was then re-added to the micro-tube containing the pellet and repeated the cell harvesting stage 6 times until the bacterial cells from the liquid culture were exhausted and resulted in a final total pellet [18]. DNA isolation was performed using the Geneaid Presto$^{TM}$ Mini Plasmid Kit.

### 2.3 Amplification of β-Lactamase Gene by PCR Method

The amplification process was performed using the Thermo Scientific DreamTaq Green PCR Master Mix Kit (2X). Amplification of the target β-Lactamase gene was performed using primers that have been designed based on the β-Lactamase gene sequence according to the pLA230 *insert* gene map [17]. The primers used were 20 bases forward primer and 20 bases reverse primer [17]. The forward primer was used to cut the target gene from segment 1, which is sequence number 19 to 879, and the reverse primer was used to cut segment 2 from sequence 879 to 19 according to the target gene sequence map.

### 2.4 Purification of Target DNA Fragment of PCR Result

Gel purification was carried out using the ZymocleanTM Gel DNA Recovery kit with the first step being cutting the target DNA fragment from the agarose gel using a scalpel / razor and transferring it to a 1.5 mL micro tube. The gel pieces were then added with ADB solution as much as 3 times the weight of the cut agar. Incubation was carried out at 50°C for 10 minutes until the gel slices were completely dissolved. The fully dissolved gel was then transferred to a Zymo-Spin™ column in a collection tube. Centrifugation was performed at 16,000 x g for 1 minute and the flow-through was discarded. A total of 200 µL of DNA wash buffer was added to the column and centrifuged at 8000 x g for 1 minute and discarded the flow-through formed (this washing process was repeated 3 times). 6 µL of DNA elution buffer was added to the column in a 1.5 mL micro-tube, then centrifuged at 16,000 x g for 1 minute to elute the DNA. The resulting supernatant is the final total yield of specific DNA fragments from gel purification with a concentration of 56.0 ng/µL.

### 2.5 Cloning β-Lactamase Gene into pTA2 Vector

TA Cloning begins with the ligation stage, namely preparing and mixing reagents containing 0.9 μL sterile distilled water, 2X ligation buffer 5 μL, pTA2 Vector (50ng/μL) 1 μL, PCR product 2.1 μL, and T4 DNA Ligase 1 μL until the final volume reaches 10 μL according to the calculation recipe in Appendix 5. The sample was incubated at room temperature for 5 minutes, thenincubated at 4°C overnight. The next step is to transform the ligation product that has been made by adding 10 μL of ligation product to 100 μL of E.coli DH5α competent cells, then inversion is slowly carried out. Samples containing ligation products and competent cells were then lysed using the heat shock method by placing them on ice for 30 minutes, then incubating for 30 seconds at 42°C and placing them back on ice for 2 minutes. The lysed samples were then added with 900 μL of SOC media and incubated at 37°C for 1 hour on an incubator shaker to help nourish the cells after treatment. After the incubation process, the samples were then centrifuged at 16,000 x g for 1 minute, then 700 μL ofsupernatant was discarded and 300 μL was left for use in blue and white screening.

### 2.6 Sequencing

Sequencing was carried out at Laboratory 1st Base Singapore with samples sent in the form of cloned DNA isolates as much as 60 μL with a DNA concentration of 154.1 ng/μL for 2 reactions (forward and reverse). The electropherogram of the sequencing reading results is the nucleotide of the β-lactamase gene isolated from pLA230, henceforth this information can be used as material for in silico data analysis.

### 2.7 In Silico Analysis

Data analysis was carried out by processing the sequencing data using bioinformatics software including Bioedit and Clustal Omega to perform sequence alignment, Expasy totranslate aligned clone sequences into protein sequences, Blastp to determine the homology of sample proteins with databases, and I-TASSERto predict protein structures and other information.

## 3 Results and Discussion

### 3.1 Isolation of pLA230 Recombinant Plasmid DNA

The β-lactamase gene isolation process begins with the rejuvenation of pLA230 bacterial isolates. This rejuvenation has the aim that the bacteria are in the optimalgrowth phase so that the optimal DNA concentration canbe obtained. Isolation of plasmid DNA using the alkaline DNA lysis method is based on the presence of NaOH (alkaline) which will cause all DNA molecules, both genomic and double-stranded plasmids, to denature into single strands [8]. Genomic DNA which is generally linear in shape can separate completely into single threads, while plasmid DNA which is circular in shape separates into single threads of circularly connected shape (like two linked rings). When

the pH of the solution is returned to normal, the H-bonds between the bases of the DNA single thread will re-form, allowing the DNA molecule to renature into a double thread. Large genomic DNA single threads cannot renature completely, so they form irregular tangled structures that are trapped and precipitated together with the complex KDS-lipid-protein complex, while the circular single thread of plasmid DNA which is relatively small in size and remains intertwined can renaturate completely to form a double thread that remains in solution. The structure and size characteristics of these two types of DNA are the basis for the separation of genomic and plasmid DNA. The presence of chaotropic salts causes plasmid DNA in cell lysates to bind to the glass fiber matrix of the Presto™ Mini Plasmid spin column. Contaminants that may still be carried are purified using Wash Buffer with ethanol content, then the collected plasmid DNA can be eluted using low-salt Elution Buffer, so that pure plasmid DNA isolates are obtained. Plasmid DNA isolation results were then quantified to determine the level of purity and concentration using a Nanodrop Spectrophotometer at a wavelength (λ) of 260 and 280 nm. The quantification results are shown in Table 1.

**Table 1.** Quantification Results of Plasmid DNA Isolate

| Concentration (ng/μL) | $A_{260}$ (AU) | $A_{280}$ (AU) | $A_{260}/A_{280}$ |
|---|---|---|---|
| 36,8 | 0,737 | 0,393 | 1,88 |

Table 1 shows that the isolated plasmid DNA shows a good level of purity because it is at a value of 1.88 where the value of DNA purity ranges from 1.8-2.0. Purity values below 1.8 indicate contamination from proteins, while values above 2.0 indicate RNA contamination [9].

### 3.2 Amplification of β-lactamase Gene with Polymerase Chain Reaction (PCR)

There are several important components involved in the amplification process using the PCR method, one of which is the primer used. Primers are a series of specific nucleotide bases as a barrier to the target DNA to be amplified. The primers used in this study are primers designed according to the nucleotide sequence of the target gene. The characteristics of the primers used are shown in Table 2.

**Table 2.** Characteristics of the Primers

| Primer | Base lentgh | % GC | Tm (°C) |
|---|---|---|---|
| F 5'-TTA CCA ATG CTT AAT CAG TG-3' | 20 | 35 | 47,9 |
| R 5'-TAT GAG TAT TCA ACA TTT CC-5' | 20 | 30 | 47,9 |

In this study, the primers used were 20 bases of forward primer and 20 bases of reverse primer. This is in accordance with the optimal primer length commonly used in PCR, which ranges from 17-28 bases. The shorter the primer used, the less specific the product, but also the use of primers that are too long can not always increase specificity

[10]. The successful amplification of a target DNA sequence is also strongly influenced by the selection of the melting temperature (Tm) of the primer which will affect the value of the annealing temperature (Ta). The annealing temperature used is generally 5°C below the melting temperature of the primer.

Amplification of the target gene from the recombinant plasmid source pLA230 was performed using the Dream Taq Green PCR Master Mix kit. Each PCR reaction performed with this kit contains several components including DNA polymerase enzyme, 2XDream Taq Green Buffer, dATP, dGTP, dCTP, dGTP,dTTP, and MgCl2 [8]. In each reaction using this kit, 10 pg - 1 µg of DNA mold can be used. Amplification was carried out through 35cycles with three stages, namely denaturation at 95°C, annealing at 42.9°C, and extension at 72°C for each cycle. The visualization results of the amplification are shown in Figure 1.

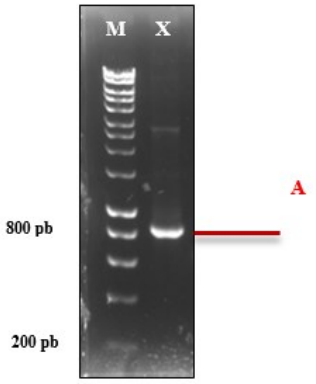


**Fig. 1.** Electrophoretic Visualization of Amplification Results; M: Marker Biolab 1 Kb; A: Target DNA.

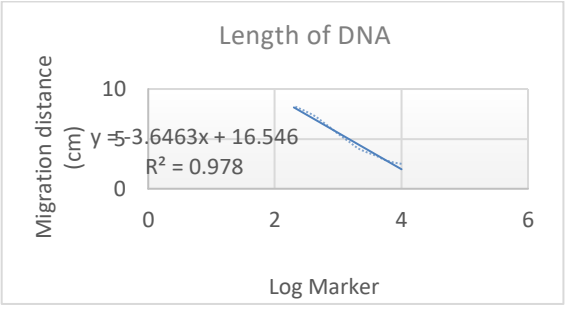


**Fig. 2.** Table 3. Migration Curve of Amplified DNA

Based on the results of electrophoresis visualization shown in Figure 1. shows that the DNA band obtained is quite thick and is at a size of 871 pb in accordance with the calculation of the DNA migration curve in Figure 2, but it is still in a double band state, which means that to get a specific DNA band, purification needs to be done to be used in the ligation process. Purification of target DNA from agarose gel was carried out using Zymoclean D4001T purification kit with the purification principle by cutting the

gel containing target DNA fragments and washing to remove impurities that may be carried in the purification process, where the components and process are quite short and do not require organic denaturing agents or chloroform. The success of target DNA purification can be known after electrophoresis visualization with purity parameters in the form of the presence of a single DNA band fragment without other non-target fragments. The results of visualization of DNA that has been purified can be seen in Figure 3.

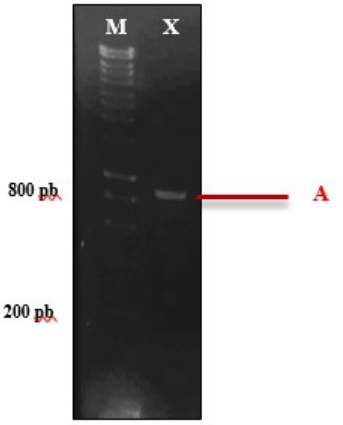


**Fig. 3.** Electrophoretic Visualization of Purification Results of Target DNA Bands where, M: Marker, A: Target Band.

From Figure 3 which is the visualization result after DNA purification from agarose gel has shown a better condition, namely a single band with a size of 871 bp based on the calculation of the electrophoresis migration curve. The size of the target DNA band as the expected insert gene is 861 bp which is the β-lactamase gene sequence. By looking at the target gene sequence in Appendix 1 and the results of the DNA band size that has been obtained, it can be predicted that the target DNA is likely to have been obtained even though the size of the DNA band has an excess of 10 bp, so that further quantification can be done to determine the purity and concentration of DNA before being used in the ligation stage. The results of the quantification can be seen in Table 3.

**Table 4.** Quantification Results of Purified DNA Isolate

| Concentration (ng/µL) | $A_{260}$ (AU) | $A_{280}$ (AU) | $A_{260}/A_{280}$ |
|---|---|---|---|
| 56,0 | 1,119 | 0,579 | 1,93 |

Based on the quantification results shown in Table 3, obtained information that the concentration of Nucleid Acid gel purification results amounted to 56.0 ng/µL. The DNA concentration has met the minimum target to be used in the cloning process of 50 ng/µL, but has not met the minimum concentration when used as a sequencing sample of 100 ng/µL, so that the multiplication through TA cloning method is expected to increase the quantity of genes to be higher.

### 3.3 Cloning β-Lactamase Gene

Cloning of the β-lactamase coding gene was performed using the TA cloning method. This method allows the ligation process to be simpler. The DNA mold is sufficiently derived from PCR products and does not require the addition of restriction enzymes for cutting the vector first. This cloning technique utilizes thepresence of two complementary bases namely adenine (A) and thymine (T) at the ends of different DNA fragments tobe able to hybridize together. PCR amplification products using Taq DNA polymerase will add one deoxyadenosine (A) to the 3' end of the PCR product, while the pTA2 vector used in this cloning TA has the following residues 3 deoxycytidine (T) which allows the *insert* DNA to be bound into the vector efficiently [11]. Ligation of the β-lactamase gene in the pTA2 vector was carried out withthe aim of forming a new recombinant plasmid with the β- lactamase coding gene from pLA230, so that it could be transformed into *E. coli* host cells to produce cell copies with the β-lactamase coding gene that could be propagated.

Recombinant transformation in *E. coli* DH5α hostcells was performed using the *heat shock* technique. The choice of this technique is based on the reason that t h e procedure is quite easy, which is done by using icebath and waterbath (42°C) as a temperature differencetreatment. Temperature changes that occur spontaneouslycan affect the state of the cell membrane. Cells become not stabilized,allowing foreign DNA to enter the cell [12]. To determine the success rate of the transformation process, it was checked using the *blue- white screening* method. *E. coli* transformants in SOC culture were transferred into LB agar media that had been added with ampicillin antibiotics to select bacteria carryingrecombinant plasmids (containing ampicillin-resistant genes) and the addition of IPTG and X-Gal compounds. The results of this transformation can be observed through the formation of cells with different colours of bacterial colonies, namely *blue* and white (*blue-white screening*) in Figure 4.

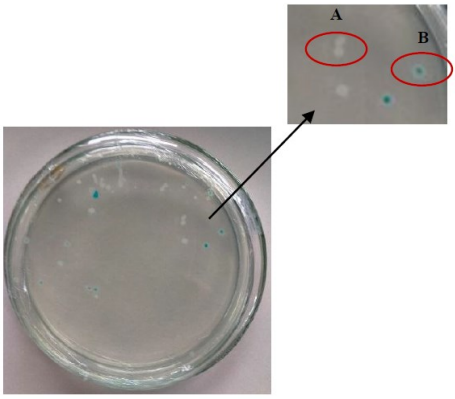


**Fig. 4.** Clone Isolates of Blue and White Screening Results Positive colonies (white in colour) that have been

Blue-white screening is a screening technique used in identifying the success of the cloning process. This technique is based on the activity of the enzyme β-galactosidase encoded by the lacZ α gene in E.coli host cells that can break down lactose into glucose

and galactose. This technique requires the addition of IPTG compound as an inducer and X-Gal as an analogue of lactose that can be hydrolysed into 5-bromo-4-chloro-indoxyl which can polymerize to produce a blue compound. The indication is when during the transformation process E. coli takes the plasmid in the absence of foreign DNA, the enzyme β-galactosidase will be formed and can hydrolyse X-Gal causing a blue colour on the colony. Vice versa, if E. coli takes the plasmid that has been ligated with DNA insert, the function of the lacZ gene will be disrupted, so that β-galactosidase is not formed and the colony remains white. The colonies obtained were then grown on selective LB media with the addition of ampicillin antibiotics to minimize contamination with other organisms. The positive colony culture results are shown in Figure 5.

**Fig. 5.** Culture of Positive Isolate on Selective Media

From the rejuvenation results shown in Figure 5, it shows that selective media with the addition of ampicillin antibiotics can grow colonies well with less possibility of contamination. This can be observed macroscopically as cells with a uniform colour, which is bone white, indicating that the cells that grow are the same cell type, where the addition of ampicillin antibiotics results in microorganisms without ampicillin-resistant genes most likely cannot grow. This is because the positive colony isolates already contain plasmids with selection marker sites that are specific to ampicillin antibiotics. These cells are then carried out plasmid DNA isolation for use in electrophoretic visualization. The results of electrophoretic visualization are shown in Figure 6

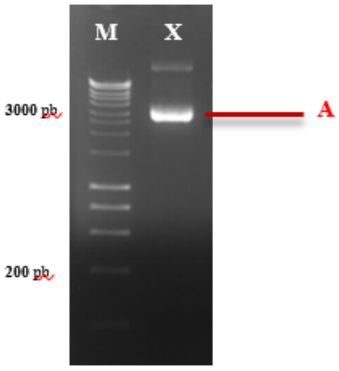


**Fig. 6.** Electrophoretic Visualization of Cloned Plasmid DNA; M: Marker Biolab 1 kb; A: Target DNA.

Based on Figure 6, it is known that the cloned plasmid DNA after electrophoresis visualization and calculation of the DNA migration curve shows an increase in size to 3,890 bp. The increase in the size of the DNA band is due to the merger between the insert DNA sequence along 871 bp with the 2981 bp pTA2 vector sequence used. In general, visualization of plasmid DNA bands will show 3 types of DNA bands that refer to the differences in the three conformational structures that can be owned by plasmids namely; (i) circular or open circular, (ii) linear and (iii) twisted or supercoiled. In the visualization results above, only two DNA bands were observed, it is possible that one of the other DNA bands was too thin, so it was not observed.

### 3.4 Cloning β-Lactamase Gene

Chromatograms obtained from sequencingreadings are still in the form of conservative sequences derived from reading forward and reverse primers separately, so consensus sequences must be obtained to facilitate molecular analysis. Consensus sequences are obtained from the unification of forward and reverse sequences in order to obtain one complete and complete sequence. The unification (Contig) of sequences was carried out using Clustal W software by starting with cutting the ends of each sequence that had poor qualitywith quality indicators in the form of overlapping curve positions and low peaks. Contig is done by modifying the sequence by performing reverse complement on thereverse result sequence until a consensus sequence is obtained for the next stage of molecular analysis. The contig result sequence was then aligned (*sequence alignment*) with the origin sequence of the target β- lactamase gene using Clustal W software in Figure 7.

```
ori     TTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCAT  60
klon    TTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCAT  60
        ************************************************************

ori     AGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCC  120
klon    AGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCC  120
        ************************************************************

ori     CAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA  180
klon    CAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAA  180
        ************************************************************

ori     CCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCA  240
klon    CCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCA  240
        ************************************************************

ori     GTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAA  300
klon    GTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAA  300
        ************************************************************

ori     CGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATT  360
klon    CGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATT  360
        ************************************************************

ori     CAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGC  420
klon    CAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGC  420
        ************************************************************

ori     GGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACT  480
klon    GGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACT  480
        ************************************************************

ori     CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC  540
klon    CATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC  540
        ************************************************************

ori     TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTG  600
klon    TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTG  600
        ************************************************************

ori     CTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCT  660
klon    CTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCT  660
        ************************************************************

ori     CATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATC  720
klon    CATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATC  720
        ************************************************************

ori     CAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAG  780
klon    CAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAG  780
        ************************************************************

ori     CGTTTATGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGAC  840
klon    CGTTTATGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGAC  840
        ************************************************************

ori     ACGGAAATGTTGAATACTCATA       862
klon    ACGGAAATGTTGAATACTCATA       862
        *********************
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**Fig. 7.** Alignment Results of the Origin Sequence withthe Cloned Sequence.

The alignment results show that the conserved region between the origin gene and the clone sequences with homology up to 861 bp is evidenced by the presence of the region. The sequence and composition of nucleotides with a similarity level of 100% are highly conserved. This means that the successfully isolated and cloned gene is a target β- lactamase gene with intact conditions in accordance with the sequence on the pLA230 sequence map, which means that there is no mutation in the gene. Analysis using Blastp software can reveal someinformation including gene description, organism of origin, as well as the percentage of confidence level and similarity. Based on the top three lines of data with the similarity which representative informs thatthe protein is a β-lactamase of the type TEM β-lactamaseclass A. TEM β-lactamase is type β-lactamase protein that is mostproduced by Gram-negative bacteria, in accordance with the prediction of the organism of origin, namely *Klebsiellapneumoniae, Escherichia coli, and Acinetobacter haemolyticus* with confidence percentages of 99.95%and 99.30%, where these organisms are all Gram-negative bacterial groups. If a new protein has a similarity percentage of about 50% with a protein in the database, it shows a high level ofsimilarity [13]. The data above also informs that the protein β-lactamase the protein is *Extended Spectrum Beta Lactamase* (ESBL) group of class A whichis a class of Serine Beta Lactamase (SBLs), where this β-lactamase group has a broader degradation activity with the active site of the enzyme being serine.

Protein secondary structure is a structure formed by a series of amino acids that will form a three- dimensional structure [14]. It is known that the protein is composed of *helix*, *strain*, and *coil-shaped* proteins with various confidence scores. It is from this structure that the next 3-dimensional model of the protein will be formed. The best results of protein modelling using I-TASSER are shown in Figure 8.

Model 1 (C-value: -0.07)

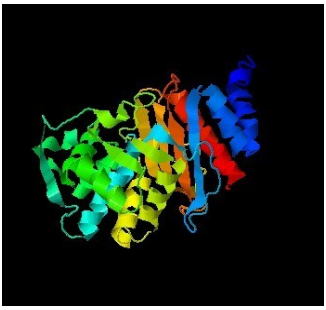

**Fig. 8.** Tertiary Model of Cloned Protein Tertiary model analysis using *software*

I- TASSER produces suggested protein models, with the parameter that if the analysis results show protein models >5 indicating a low level of model accuracy. The besttertiary model is based on the *Confidennce Score* (C) valuewhich indicates the confidence level of the resulting model. The C value has a range from 2 to -5, where the greater the C value, the higher the confidence level of the resulting model. Based on the C value. The protein shows that the best model is owned by the 1st model with a C value of -0.07 which is the highest value.

The data from the next I-TASSER analysis is the similarity structure of the protein analogue with the protein database based on the TM-score of the structural alignment

between the query structure and the protein structure from the PDB data source. TM-score or template modelling score is a parameter that measures the topological similarity between protein structures with a value range of 0-1, where the accepted value is >0.5. From the highestTM value that shows the greatest similarity with the SHV-1 β-lactamase type protein, where SHV (*Sulphydryl Variable beta* lactamase) is a type of β-lactamase enzyme that has specific activity on penicillin antibiotics [15]. The data analysed using the I-TASSER software then predicts the *Enzyme Commission* (EC) number and active site of the cloned protein shown in in Table 4.

**Table 5.** Predicted Enzyme Commission (EC) Numbers and Active Sites

| PDB-Hit | IDEN[a] | Cscore[EC] | EC Number |
|---|---|---|---|
| 3c4Pa | 0.677 | 0.642 | 3.5.2.6 |
| 1bsgA | 0.386 | 0.585 | 3.5.2.6 |
| 1iysA | 0.363 | 0.556 | 3.5.2.6 |
| 3lezA | 0.380 | 0.525 | 3.5.2.6 |
| 3bydA | 0.398 | 0.378 | 3.5.2.6 |

$^{EC}$Cscore (*Confident Score*) is the confidence score for predicting *Enzyme Commission* (EC) *Number*. EC number is an enzyme classification system based on the chemical reaction it catalyses. The C score value onEC *number* ranges from 0-1, where a score close to 1indicates a more accurate prediction of EC number. Based on the best Cscore data$^{EC}$ shown in the first row, namely with PDB-Hit 3c4pA which is the best data. From the comparison of several data with the highest C score value of 0.642 and the value below, all of them show the same EC number, namely 3.2.5.6 with various variations in the level of confidence. The EC number value consists of 4 letters where the first letter refers to the enzyme class, the second letter is an enzyme sub-class, the third letter is asub of the enzyme sub-class, and the fourth digit refers to the specific enzyme.

From the data above, it can be seen that the EC value of 3.2.5.6 means that the enzyme is a hydrolase enzyme class (3) with a sub-class that can work on carbon nitrogen bonds and peptide bonds (2) which has a sub-class of cyclic amides (5) and is a β-lactamase enzyme (6) [16].

## 4 Conclusion

Based on the research that has been done, it produces the following information that amplification β-lactamase coding gene from the source pLA230 using *Polymerase Charin Reaction* (PCR) method successfully obtained an intact gene of 861 pb according to the calculation using electrophoresis migration curve. Amplification of 861 pb β-lactamase coding gene usingTA cloning method in pTA2 vector produced positive transformants with target gene that can grow well on selective media. Sequence analysis of the sequenced β- lactamase gene using bioinformatics software *in silico* produced the following information;

- The alignment between the cloned sequence and the original sequence of pLA230 showed high homology with a percentage of up to 100%, which means that the β-lactamase gene has not changed its sequence due to mutation.
- Prediction of the source organism of the β- lactamase gene based on the results of analysis using Blastp *software* indicated that the gene most likely came from *Klebsiella pneumoniae, Escherichia coli, and Acinetobacter. The* analysis data also informs that the β-lactamase protein is a group of *Extended Spectrum Beta Lactamase* (ESBL) from class A which is aclass of Serine Beta Lactamase (SBLs).

Tertiary protein modelling showed the protein with the highest C score of -0.07 in the 1st model. Protein analogue similarity analysis with PDB data showed the greatest similarity with SHV-1 β-lactamase (*Sulphydryl Variable beta lactamase*) type proteins with specific activity on penicillin antibiotics. Predicted EC number3.2.5.6 with enzyme active side at sequence numbers 64, 102, 133, 158, 16, 160, 162, 167, 169,175, 234, 236, and 240.

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