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Identification of Lactic Acid Bacteria Isolates from Intestine of Milkfish (Chanos-Chanos) Potential Activity against Pathogen Bacteria Used PCR 18s Rrna Methode

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Abstract

The research aimed to identify the lacted acid bacteria from intestinal of milkfish (Chanos chanos) potential acvity pathogen anbacteria used PCR 18s Rrna Methode, The lactic acid bacteria were isolated using selective media MRS aggar for lactic acid bacteria. Identification isolates PCR 18S rRNA and sequencing method that are DNA isolation, DNA amplification use PCR, DNA visualizasion from amplification with electroforesis, Basic Local Alignment Search Tool (BLAST) and Tree view program. In view of PCR analysis and tree view program, LAB is Lactobacillus acidophilus strain ATTC 4796.

Keywords: *Lactic Acid Bacteria, Milkfish, PCR*

1. Introduction

Lactic acid bacteria (LAB) constitute a group of bacteria that have morphological, metabolical and physiological similarities, and they are also relatively and closely related phylogenetically. They are gram-positive, non-sporulating, non-respiring cocci or rods, which ferments carbohydrate to produce lactic acid as their major end product (Dike,Sanni, [1]). Lactic acid bacteria are widely distributed in the nature. Representatives of the genus Lactobacillus, Lactococcus, Pediococcus and Leuconostoc are be largs this group. According to many reports, lactic acid bacteria are normal flora in gastrointestinal (GI) tract of healthy animals like mammals and aquaculture animals (Nikoskelainen, et al, [2]) with no harmful effects(Ringo, et al, [3]).

The milkfish that breed in brackish waters has slim bodied characteristics, fin forked, fleshy scales like glass and white. It has a uniqueness, that his mouth is toothless and seaweed base food consumtion. In addition, the intestinal length of the milkfish 9 times longer than the body length (Murtidjo [4]). In the long intestine, there are many different types of bacteria including lactic acid bacteria (LAB), which helps the food digestion process. LAB also function as antagonistic bacteria against pathogenic bacteria. Lactic acid bacteria can be isolated and tested its antagonistic activity against pathogenic bacteria and can be developed as a new antibiotic.

Lactic acid bacteria (LAB) are known as microorganisms that have probiotic properties. They can produce inhibitory compound such as lactic acid, hydrogen peroxide, diacetyl, acetal dehyde and bacteriocin. These compounds are able to inhibit the growth of harmful microorganisms (Ringo, Gatesoupe [5]), (Gatesoupe [6]). Lactic acid bacteria are widely distributed in the nature. Representatives of the genus Lactobacillus, Lactococcus, Pediococcus and Leuconostoc are be largs this group.

The research was an experimentally conducted that treatment with filtrate lactic acid of Lactobacillus concentration 50 to 100% effective can inhibition the growth of bacterial pathogen were Bacillus cereus, Staphilococcus aureus, Salmonella paratyphi and E.coli.

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The acid lactic from culture Lactobacillus can be used as bio-preservative isolate from intestinal of milkfish (Sulistijowati, Mile, [7]).

The determination of lactic acid bacteria genus as potential biopreservative was conducted performed according to their morphological, cultural, physiological and biochemical characteristics as described in Bergey's Manual. The rusults after characterization, based on Bergey's manual, seven of the isolates (RS1,RS2,RS3,RS4,RS5,RS8 and RS10) were determined as representatives of refferred to genus *Leoconostoc* were cocci, Gram +, gas production, hetero/homo fermented and others (RS6, RS7 and RS9) were representatives of reffered to genus *Lactobacillus* were rod, Gram +, gas production, hetero fermented (Sulistijowati, [8]).

The 16S rRNA gene sequence is a molecular technique to identify microorganisms up to species level. This method has been considered as one of the advanced tool at molecular level for identifying isolated bacteria. 16S rRNA analysis has facilitated the study of microbial populations without cultivation which has made quantitative assessment of microbial diversity now conceivable (Subramani, Aalbersberg [9]).

The aim of this study was to develop PCR 18s Rrna in order to simultaneously detect Lactobacillus species in single reaction.

The polymerase chain reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. Developed in 1983 by Kary Mullis,[1][2] PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications.[3][4] These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and DNA paternity testing); and the detection and diagnosis of infectious diseases. In 1993, Mullis was awarded the Nobel Prize in Chemistry along with Michael Smith for his work on PCR.[5]

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase, which the method is named after, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated was itself used as a template for replication, setting in motion a chain reaction in which the DNA template exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations.

2. Material and Methodes

2.1. Materials and Equipment

This research used lactic acid bacteria isolate potential biopreservative from Pohuwato District were cultivated in deMan Rogosa Sharp (MRS) Agar at 35°C for 24 hours, reagen PCR: *deoxyribonucleotide triphosphate* (dNTP) mix, larutan *buffer*, LA Taq, *primer* 1141R dan 765R , dH_2O, *Big Dye Teminator*, gel agarosa, *buffer* TAE (1x) Merck 106023), DF *buffer* (Merck.71340), *wash buffer*, *lotion buffer*, SyBr *safe*, EDTA (Merck.324503), natrium asetat (Merck. 106268), etanol (Merck.104025), alkohol 70% (Merck.117946). The equipment machine PCR *Thermal cycler* Takara, machin elektroforesis, UV *Transluminator* Gel Doc Sys-Vilber Loumart, sequensing ABI 3130 XL *Genetic Analyzer*

2.2. Reasearch Methodes

The determination of Lactobacillus species was permormed DNA genom total isolation, DNA amplification, electrophoresis, gel electrophoresis purification, PCR cycle

sequencing, cycle sequencing purification, sequencing and Basic Local Aligment Search Tool (BLAST) and Tree view program (Maniatis, et al,[10]).

DNA Genom Total Isolation use PrepMan Ultra Reagent

Samples were inserted into the tube was added 20 µL PrepMan Ultra then homogenized using vortex for 10-30 seconds. Furthermore incubated at a temperature of 100°C for 10 minutes on the digital heatblock, then centrifuged at 16,000 x g for 3 min. A total of 12 µL of the supernatant was transferred into 1.5 mL tube and stored at a temperature of 40°C.

DNA Amplification

Total genomic DNA that has been obtained from the isolation was amplified using the Polymerase Chain Reaction (PCR) Thermal cycler in the area Internal Transcribe Spacer (ITS). The total gene DNA used as a template and added with another reagent-reagent with the following composition: 25 mM $MgCl_2$ 5 mL, 2.5 µM dNTPs mix 4 µL, 10 X buffer free Mg 2+ 5 µL, LA Tag 0.25 µL , template 1 µL , 10 µM primer 1141R (base sequence 5'-TCC GTA GGT GAA CCT GCG G-3 ') 2 µL , 10 µM R 765 primer (base sequence 5' TCC GCT TAT TGA TAT GC-3') 2 µL and 30.75 µL dH_2O. The total volume of the reaction mixture of 50 µL, then put in a PCR machine with the program: Heating beginning of 94^{0}C for 3 minutes, denaturation at temperature 940C for 30 seconds, annealing at a temperature 55^{0}C for 20 seconds, elongation of DNA at atemperature 720C for 1 min. After PCR 30 cycles were left at a temperature of 72^{0}C for 7 minutes later the temperature was lowered to 4^{0}C.

Electrophoresis

Done the 1% agarose gel, 0.25 g dissolved in 25 agarose TAE buffer (1x) and then heated to dissolve agarose. Cooled to at temperature 450C was added 0.25 µL of SyBr safe then poured into molds and allowed to stand until the gel hardens.

Electrophoresis; PCR products were subsequently examined by electrophoresis. Electrophoresis was carried out in TAE buffer (1x). A total of 50 µL PCR product and 6x loading dye 10 µL homogenised by means of repeated pipette 2-3 times and then put in a pipette and gel well. Loading dye serves to increase the density of the sample so it is not out of the well and the samples be colored to facilitate observation of the current migration process. Also incorporated into the gel other wells as a comparison (marker) 4µL 1 Kb DNA Ladder Mix fermentas GeneRuler DNA ladder to facilitate the determination of the size of the DNA. Electrophoresis is run at a voltage of 100 volts for 25 minutes, then the gel electrophoresis results checked over transluminator UV and photographed for documentation. Then the DNA bands appear at 600 base pairs was cut to enter the stage of purification.

Purification

Purifying Gel Electrophoresis with GeneAid Gel / PCR DNA Fragments Extraction Kit DF buffer gel added Pieces of 300 µL, incubated at 60^{0}C for 15 minutes on a heat block until later dispindown soluble gel and then inserted into a filter column and centrifuged at 13000 x g for 1 minute. Then the supernatant was discarded, and then added 600 µL wash buffer into the filter column is then centrifuged at 13000 xg for 1 minute, remove the supernatant and centrifugation in an empty column to remove residual wash buffer at 13000 xg for 3 minutes and then move the filter column into eppendorf new and added a solution of 40 µL lotion buffer and incubated for 5 min at room temperature. Then was centrifuged at 13000 x g for 2 minutes. Remove the filter column. Store the supernatant at temperature 40C.

PCR Cycle sequencing

Cycle Sequencing PCR was performed use a primer, ie 1141R and 765R. PCR tube inserted in the Big Dye Terminator 2 mL , 4 mL 5x sequencing buffer , 1.6 pmol / µL primer 1141R much as 4 µL, 6 µL DNA template, and 4 µL dH_2O. Mixture reaction volume 20 uL, then put in a PCR machine with the program: Preheating 96^0C for 10 seconds , annealing (annealing) at temperature 50^0C for 5 seconds, and DNA elongation at temperature 60^0C for 4 minutes. After 25 cycles of PCR then the temperature lowered to 4^0C. Furthermore, do the same thing on the primer 765R.

Purification Cycle Sequencing

A total of 20 mL samples of cycle sequencing equivalent dH2O was added (20 µL), then transferred to 1.5 mL tube. EDTA then added 4 µL, 4 µL of sodium acetate, and 100 µL of absolute ethanol, incubated at room temperature for 15 minutes in a state covered with aluminum foil. Then centrifuged at 5000 x g for 30 minutes, then the supernatant removed and added to 140 mL of 70% alcohol, followed by centrifugation at 3000 x g for 15 minutes. Subsequently the supernatant was discarded, spindown and dried with a tissue, and then use a vacuum desiccator for 10 minutes. Then added 16 µL of dH2O and spindown. The last heated use a heat block at temperature 52^0C for 10 minutes. Samples ready to prepare sequenced.

Sequencing and Basic Local Aligment Search Tool (BLAST) Analize

Cycle Sequencing DNA purified and then sequenced used a sequencing ABI 3130 Genetic XL analyzer to determine the nucleotide sequence of the DNA fragment. In this sequencing a lengthening or extension cords of DNA which begins at specific sites on the DNA template using short oligonucleotides, called primers. Primer used 1141R as 765 as forward and reverse. Sequencing was run for 1 hour. Furthermore, the results of sequencing the DNA sequences do assembly, namely the incorporation of bases of reading both directions (forward and reverse). Furthermore, DNA sequences that have been in the assembly copied to the notepad in the form of FASTA for purposes of analysis Basic Local Alignment Search Tool (BLAST). The analysis performed using the program's website: http: /www.ncbi.nih.nlm.gov/BLAST/ by copying the DNA sequence that will be in BLAST, and then pasted into the field that the DNA sequences in the BLAST window then the window appears on the monitor that contains the results of BLAST similarity diagram sequences that we enter with sequences in the gene bank. To determine the kinship of bacteria identified, selected sequences which have a percentage of similarity (Query) high, then the sequence data stored on a notepad in the form FASTA. Analysis of the sequence data using Clustal-X program and GeneDoc to edit sequences. The results of the analysis will be obtained in the form of family tree (phylogenetic tree) that can be opened with the program Tree View. From the family tree can be known species and strains of bacteria were identified.

3. Results and Discussion

DNA Isolation

Total genomic DNA has been isolated from BAL isolates that possess antibacterial activity on pathogenic bacteria. Total genomic DNA isolation used Ultra PrepMan Reagents.

DNA Amplification

Total genomic DNA isolates BAL amplification technique Polymerase Chain Reaction (PCR) used PCR Thermal Cycler. DNA amplification process consists of three stages, namely DNA denaturation, primer extension or DNA template and primer elongation by

the enzyme DNA polymerase. LAB isolates DNA amplification using primers 1141R and 765R produce DNA fragments were very clear with a size of about 600 base pairs.

Electroforesis

The results of DNA amplification used primer 1141R and 765R produce DNA fragments very clearly with a size of about 597 base pairs checked by electrophoresis which one of the main techniques in molecular biology .

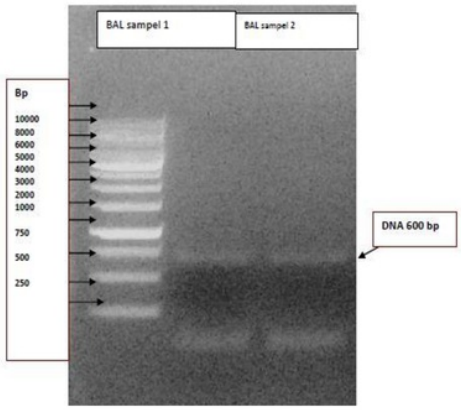

Figure 1. Electroforesis

Sequensing and Basic Local Aligment Search Tool (BLAST) Analize

Cycle Sequencing DNA tool was run on the ABI 3130 Genetic XL analyzer to determine the nucleotide sequence of the DNA fragment. In this sequencing a lengthening or extension cords of DNA which begins at specific sites on the DNA template using the primer 765R 1141R as a forward and as reverse. Sequencing running for one hour. Here was the results of assembly, was read from two directions (forward and reverse) the DNA sequences of the isolates BAL (597 bp) which has antibacterial activity.

Sequence isolate LAB

Lactobacillus acidophilus 30 SC

CCTGCCCCATAGTCTGGGATACCACTTGGAA
ACAGGTGCTAATACCGGATAAGAAAGCAGAT
CGCATGATCAGCTTATAAAAGGCGGCGTAAG
CTGTCGCTATGGNNTGGCCCCGCGATTGGTG
CATTAGCTAGTTGGTAAAGTCGAGCGAGCTG
AACCAACAGATTCACTTCGGTGATGACGTTG
GGAACGCGAGCGGCGGATGGGTGAGTAACA
CGTGGGGAACCTGCCCCATAGTCTGGGATAC
CACTTGGAAACAGGTGCTAATACCGGATAAG
AAAGCAGATCGCATGATCAGCTTATAAAAGG
CGGCGTAAGCTGTCGCTATGGGATGGCCCCG
CGGTGCATTAGCTAGTTGGTAGGGTAACGGC
CTACCAAGGCAATGATGCATAGCCGAGTTGA
GAGACTGATCGGCCACATTGGGACTGAGACA
CGGCCCAAACTCCTACGGGAGGCAGCAGTAG
GGAATCTTCCACAATGGACGAAAGTCTGATG
GAGCAACGCCGCGTGAGTGAAGAAGGTTTTC

Lactobacillus acidophilus ATCC 4796

AAAAACGAGAGTTTGATCCTGGCTCAGGACG
AACGCTGGCGGCGTGCCTAATACATGCAAGT
CGAGCGAGCTGAACCAACAGATTCACTTCGG
TGATGACGTTGGGAACGCGAGCGGCGGATGG
GTGAGTAACACGTGGGGAACCTGCCCCATAG
TCTGGGATACCACTTGGAAACAGGTGCTAAT
ACCGGATAAGAAAGCAGATCGCATGATCAGC
TTATAAAAGGCGGCGTAAGCTGTCGCTATGG
GATGGCCCCGCGGTGCATTAGCTAGTTGGTA
GGGTAACGGCCTACCAAGGCAATGATGCATA
GCCGAGTTGAGAGACTGATCGGCCACATTGG
GACTGAGACACGGCCCAAACTCCTACGGGAG
GCAGCAGTAGGGAATCTTCCACAATGGACGA
AAGTCTGATGGAGCAACGCCGCGTGAGTGAA
GAAGGTTTTCGGATCGTAAAGCTCTGTTGTTG
GTGAAGAAGGATAGAGGTAGTAACTGGCCTT
TATTTGACGGTAATCAACCAGAAAGTCACGG

DNA sequences that have been copied to the notepad in the form FASTA used for the analysis (BLAST) using the program on the website http: /www.ncbi.nih.nlm.gov/BLAST/, phylogeny of fungi identified, selected from a sequence that has a percentage high similarity> 90% and in the program tree view derived phylogenies (filogenetic tree), it was found that the species and strains of bacteria. The philogenetic tree diagram of the LAB isolate is Lactobacillus acidophilus ATTC 4796 on figure 2.

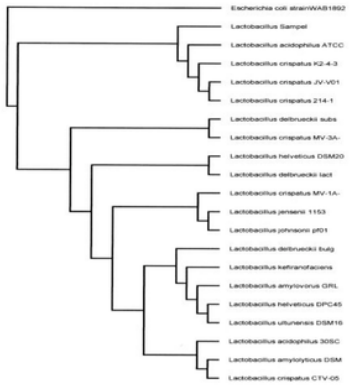

Figure 2. Philogenetic Similarity Lacidophilus

Based on philogenetic tree diagram known that LAB isolate resemblance> 98% with bacterial species Lactobacillus acidophilus ATTC4796.

Tacsonomy of Lactobacillus acidophilus :

Kingdom	:	Bacteria
Divisio	:	Firmicutes
Classis	:	Bacilli
Ordo	:	Lactobacillales
Familia	:	Lactobacillaceae
Genus	:	*Lactobacillus*
Species	:	*Lactobacillus acidophilus* (Moro 1900)
Strain	:	ATTC 4796

4. Conclusion

As a conclusion this study had successfully identified based on 16S rRNA gene sequence analysis of the Lactic Acid Bacteria isolate from intestinal milkfish as Lactobacillus acidophilus ATTC4796 (98% similarity).

Acknowledgment

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