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Molecular Identification of A Local Lactic Acid Bacteria (LAB) Isolate (B21) And Primer Confirmation For D-Lactate Dehydrogenase (D-LDH) Gene Isolation

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ABSTRACT

This study aims to identify a lactic acid bacteria (BAL) isolated from native chicken (Gallus gallus domesticus) intestinum (B21) based on 16S rRNA gene sequence and also to confirm primers for PCR-based D-Lactate dehydrogenase (D-LDH) gene isolation. The genomic DNA of B21 was isolated and then amplified using 16S rRNA. The PCR product was sequenced and aligned. The sequencing was done in 1st Base Pte. Malaysia. The sequence was aligned using the Basic Local Alignment Search Tool (BLAST) in NCBI to know the similar genome species. Isolation D-LDH gene was done through PCR using 5 primers (idb 0101, idb 1147, idb 0813, idb 1010, and idb 2021) of B21 and also J15 isolate genome. The PCR products were then sequenced and aligned with the D-LDH gene sequences in NCBI. The results of molecular identification based on 16S rRNA markers showed that B21 has 99.45% similarity to Bacillus proteolyticus. Amplification of the LDH gene with idb 1010 primer on J15 isolate yielded 4 fragments (i.e. 650, 1400, 1500, and 1700bp). The results of phylogenetic tests showed that the 1700bp fragment has a high similarity to the D-LDH gene in Myobacterium tuberculosis species with a bootstrap value of 84%. In comparison, other size fragments cannot be trusted to have similarities to the LDH sequence because they have not reached the minimum bootstrap value that meets the qualifications.

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1. INTRODUCTION

Lactic acid bacteria (LAB) is a type of microorganism that is widely used in various industrial fields because of its ability to decompose carbohydrates (glucose) into lactic acid in large quantities (Widyastuti and Sofarianawati, 1999). In the food industry, lactic acid bacteria play an important role in almost all food and beverage fermentation processes. Its main function is to acidify raw materials by producing lactic acid, a small part of acetic acid, ethanol and carbon dioxide (Nur, 2005). Until now, this study continues to be conducted to identify and characterize new BALs with their potential utilization in various industrial fields (Perin et al., 2014; Fguiri et al., 2015).

The important potential of LAB to produce lactic acid is formed in the process of sugar metabolism or glycolysis. It is characterized by the degradation of pyruvate molecules by the enzyme Lactate dehydrogenase (LDH) caused due to unavailability of oxygen. During this series of

processes, the degraded pyruvic acid is then reduced by NADH to produce lactic acid. The LDH gene plays an important role because it is a key gene encoding the LDH enzyme and can be used as a catalyst for the conversion of pyruvate to lactic acid. The LDH gene in lactic acid bacteria is very important and has the potential to be used in biotechnology/DNA-based industries. Many studies have shown LAB can produce both D-lactic and L-lactic types from the LDH gene, and the ratio of both isomers varies according to the species and its strain in the growth cycle (Garmyn et al., 1995). Today we have entered the molecular era (genomics, transcriptomics, proteomics, and genetic engineering). Various biological problems that have social impacts can be solved through molecular approaches. Genetic engineering technology has been widely used to produce goods and services that benefit humans and the environment. The utilization of this technology continues, both to produce innovative products/services and to study regulation and genetic function. The study and research of LAB genetics and its applications ranging from gene cloning is expected to meet the needs of the industry with a wide range of benefits.

Several important protein/enzyme coding genes of LAB (e.g. lactate dehydrogenase which is a key enzyme in lactic acid biosynthesis, bacteriocins (antibiotics), amylase, polylactic acid (PLA) depolymerase which is an enzyme in plastic biodegradation, and genes involved in pigment biosynthesis) have been successfully cloned by previous researchers, but gene exploration and genetic modification of new LAB strains is ongoing. Identification based on 16S rRNA markers on LAB isolates genome is the initial stage carried out in this study then continued with the isolation of LDH genes based on the PCR method. In the future, the identification of the D-LDH gene could be used for genetic engineering to increase lactic acid production from BAL and other organisms.

2. RESEARCH METHOD

The research was conducted from September 2020 - April 2021, at the Microbiology Laboratory, Biotechnology Laboratory, and Basic Biology Laboratory of Mathematics and Natural Science Faculty, UNY. The sequencing was conducted at 1st BASE Laboratories, Aprical Scientific, Malaysia through DNA isolation and amplification method. Total genomic DNA was isolated from B21 and J15 cells that have been subcultured in MRSB media. DNA isolation was done using a TIANamp Genomic DNA kit.

Molecular identification was only done for the B21 isolate (the J15 isolate has been identified before) based on 16S rRNA gene sequences. The genes were isolated by amplification using specific primers through PCR. The PCR was performed using PCR Kit KOD-Plus Neo with conditions: 3 minutes at 94ºC for pre-denaturation, 30 seconds at 94ºC for denaturation, 30 seconds at 55oC for annealing, 2 minutes at 72ºC for extension, and 5 minutes at 72ºC for final extension. The PCR cycle (denaturation-annealing-extension) was repeated 25 times. The PCR products were then sequenced in 1st BASE Laboratories, Aprical Scientific, Malaysia.

The PCR products were then in 1st BASE Laboratories, Aprical Scientific, Malaysia. For sequencing preparation, there need steps, include: (1) DNA preparation, (2) amplification process through PCR using 16S rRNA primers and D-LDH gene-specific primers, (3) DNA purification, (4) electrophoresis, (5) electrophoregraph readings of sequencing results. The sequence was analyzed using bioedit application and BLAST NCBI. LDH gene data was analyzed using the MEGA X application by alignment using ClustalW to perform phylogenetic testing.

3. RESULTS AND ANALYSIS

3.1 Molecular identification of B21 isolate based on 16S rRNA gene sequence

Molecular identification was only carried out on B21 isolates because J15 isolates had been sequenced with 16S rRNA genes in the previous study. Based on the results of electrophoresis visualization of BAL B21 isolates showed that the quality of amplified DNA is very good indicated by the appearance of fragment amplicon with a size of 1500 bp which is the expected size (Figure 1). DNA multiplication occurs in certain areas beyond by 16s rRNA primers, these primers will

recognize conserve areas and amplify hypervariable regions, thus specific sequences of the organism will be obtained (Rinanda &; Tristia, 2011).

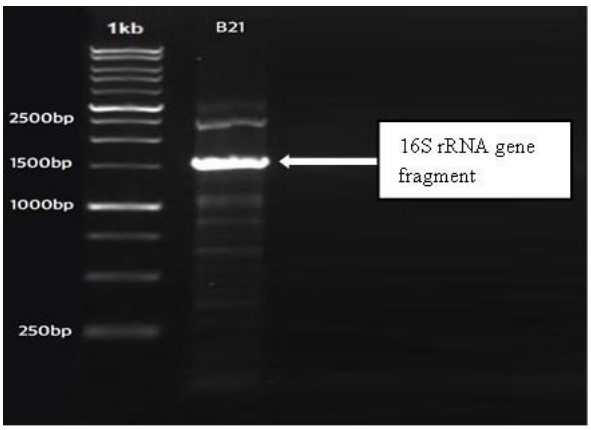

Figure 1. Amplification result of 16S rRNA gene electrophoregraph

The data obtained from the sequencing results are in the form of electrophoregrams in the form of ABI files, where each nucleotide is shown with a different color. Adenine (A) are shown in green, Guanine (G) is shown in black, Thymine (T) is shown in red, and Cytosine (S) is shown in blue (Figure 2).

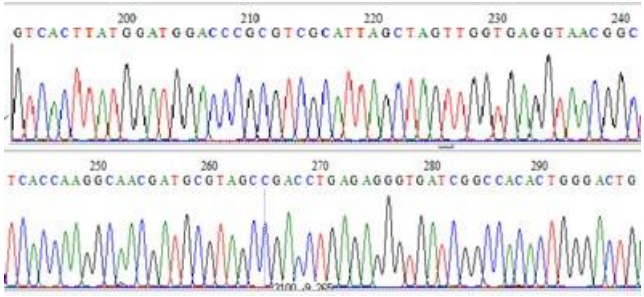

Figure 2. Sequencing result of 16S rRNA gene

The alignment result of the sequence to Genebank data using the BLAST show that B21 similar to *Bacillus proteolyticus* with access codes MZ_317473.1 (Table 1). Stackebrandt & Gobel (1995) state that a species of bacteria can be said to be the same if it has a homology greater than or equal to 97%. B21 isolate has an identity value above 97%, which is 100.00%, so this data can be trusted. This is in accordance with NCBI (2015), that BLAST will find areas of similarity from sequences. The query coverage is an important parameter because it assesses the percent of database sequences that cover the query, this is to ensure that the sample is covered all by the database sequence. Acceptable percentages are at least 95%, except for sequences where lower readings are applied at least 75%. (Narita, et al., 2012).

Table 1. BLAST result of 16S rRNA gene

Scientific Name	Max Score	Total Score	Query Cover	E Value	Per. Ident	Acc.Len	Accession
Bacillus cereus	2689	2959	100%	0.0	98.81%	1577	KR185831.1
Bacillus pumilus	2085	2085	98%	0.0	91.99%	1507	EU586782.1
B.proteolyticus	1720	2774	100%	0.0	100%	1490	MZ317473.1

Confirmation of Primers for D-LDH genes isolation

In preliminary experiment; the PCR process succeeded in amplifying D-LDH gene from *Bacillus cereus* and *Bacillus proteolyticus* genome, but still produced very thin bands and some still yielded multiband and excess primers. It showed that the amplification process was not optimal. Therefore, it is necessary to optimize the PCR process in order to improve the quality of amplification products so that thick bands are obtained through optimal PCR formulations and conditions.

Optimization of annealing temperature are presented through visual analysis of DNA bands that appear on the electrophoresis gel (Figure 3). Amplification in almost all annealing temperatures used yielded PCR product (amplicon), except at 56 ºC and 57 ºC by using Idb 2021 primer (Figure 3.e). There was an excess primer or primer dimer in amplification using Idb 1010 (Figure 3.c). This primer dimers is characterized by the presence of shadow bands with a size of less than 100 bp. Primary dimers are unamplified primary remains, formed due to the absence of mixed DNA or due to the high concentration of primers used (Gonzalez, *et al.*, 2015). Primer dimer can be avoid by arrange the GC content (at 40 – 50 %), primer length (18 – 30 bp), no hairpin and no repeat more than 4 bases (Diffenbach &; Dveksler, 1993).

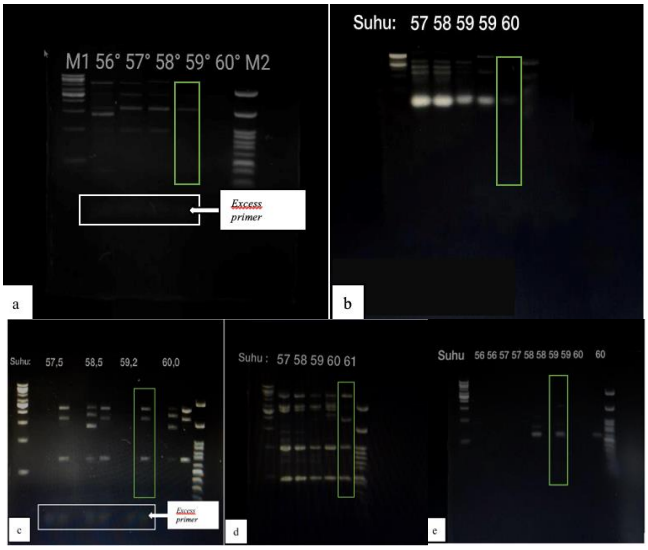

Figure 3. Annealing temperature optimization electrophoregraph. (a) Idb 0101 primer, (b) Idb 0813 primer, (c) Idb 1010 primer, (d) Idb 1147 primer, (e) Idb 2021 primer

The result shows that each primer has the own optimal annealing temperature, between 57 – 58 ºC. The optimum annealing temperatures are determined by their melting temperature (Tm) which related to the primer base composition and length. This optimum temperature can avoid interactions between primers such as self-homology *and* cross-homology *in* the *annealing* process when performing DNA amplification. These results relate to Handoyo and Ari (2000) that Tm generally ranges from 50 - 65ºC

Amplification of *LDH* genes from *Bacillus cereus* and *Bacillus proteolyticus* was performed to identify the D-LDH gene from these LAB isolates. Primer screening (*Idb 0101, Idb 0813, Idb 1010, Idb 1147, Idb 2021)* to isolate D-LDH from *B. cereus* and *Bacillus proteolyticus* show that fragments amplified more than 1 fragment (*multiband*). According to Yuenleni (2019), *the optimal band is a thick, live/*single-band *band and according to the target size. Multi-band* in the results of this study

occurred because of several possibilities, the first of which was possible because in this study the test samples used were *Bacillus cereus* and *Bacillus proteolyticus*, while it is known that the reference primers used are specific primers that code for the D-LDH gene in *Lactobacillus delbrueckii* subsp *bulgaricus* so that the success rate of PCR amplification can be different, this is supported by a statement from Putri Gea (2020) the chemical content of test samples and primers that have certain specifications sometimes have incompatibility to amplify various types of samples. The use of primers in non-specific species can also cause homodimers, the statement is supported by Handoyo & Rudiretna (2001) that low primary specificity can cause the formation of loop structures (*hairpins*) and bonds between primers (*dimers).*

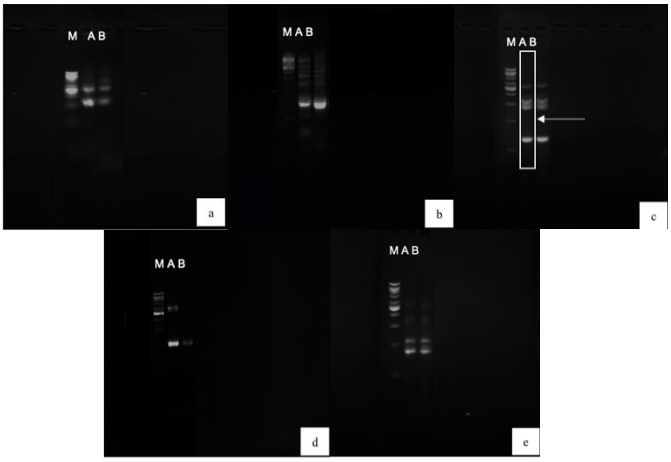

Figure 4. Electrophoregram of gene amplification results D-LDH. (a) idb 0101, (b) idb 0813, (c) idb 1010, (d) idb 1147, (e) idb 2021

Primer annealing to unspecific region of DNA template could be caused by restriction site sequence in each primer used. That is not to the expected gene site (mispriming) so that various fragments appear (multi-band). This is in accordance with the theory according to Chuechill (1989) which says that the distribution of restriction sites *in E. coli* bacteria is dominated by eight restriction enzyme sites scattered in the genome BamHI restriction site are most widely spread across the genome. The heterogeneity in the distribution of PstI sites is seen that the site is a small clearing (<50kb) site. The HinII site has a larger distribution area (>100kb) in the genome.

Another possibility that could cause multiband is because the gene target (LDH) is one of the superfamily genes, e.g. LDH-A has 5 isoform forms. Lactate dehydrogenase A (LDHA) is one of five isoforms of the lactate dehydrogenase family that catalyzes the conversion of pyruvate to lactate under anaerobic conditions and is key in glycolytic metabolism while D-LDH is a gene included in the specific 2-hydroxyacid dehydrogenase family - isomer D (Tocris, 2008). The fact that LDH is a superfamily gene causes the presence of proteins with many types that have similar sequences in certain regions so that in one bacterial genome can be amplified at several points with different fragment lengths. Family genes have many homologous genes (having similar sequences) when expressed can produce protein that is similar in structure and function (Mitha, 2021). To confirm which fragments that contain D-LDH gene, we sequence several thickest bands amplified by *idb* 1010 primer (Figure 5).

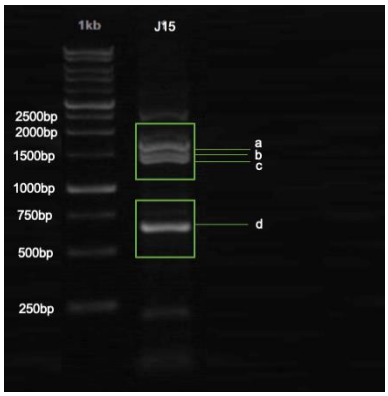

Figure 5. Multiband yielded in amplification using Idb 1010 of *Bacillus cereus* genome

There are 4 fragment yielded ini amplification of B. cereus genome using Idb 1010 primer (i.e. 650, 1400, 1500, and 1700 bp). Furthermore, sequence editing is carried out with a bioedit application to get the sequence with the best reading. At the time of editing, data processing and unification (contig analysis) of the D-LDH sequence were also carried out to obtain a consensus sequence from the original sequence. This analysis aims to find overlapping regions of both sequences so that they can produce one sequence unit (Nurkholidah, 2019).

Each sample sequence that has been edited using Bioedit is then used in searching for the nearest relative sequence with *Molecular Evolutionary Genetics Analysis* (MEGA X) software version 6.06 by aligning using *ClustalW Multiple Transfer* between BAL Bacillus *cereus sample sequences* and some LDH sequences obtained from the NCBI database. *ClustalW* is a widely used program in aligning nucleotide sequences with progressive methods, homologous sequences with the best values are aligned first, followed by sequences that are further similar until global alignment is obtained (Tamura *et al.,* 2011). The most common method of performing The approach used in constructing the phylogenetic tree in this study is the *maximum likehood approach with* a bootstrap *value analysis* of 1000x. Hall (2001) states that *bootstrap* values of 100 to 1000 repetitions are used to estimate the confidence level of a phylogenetic tree. In addition, Ubaidillah & Sutrisno (2009) stated that the greater the *bootstrap* value used, the higher the trust in the reconstructed tree topology based on the distribution of characters in the data. While the *maximum likehood* method uses calculations to find trees that have the best count of variation in multiple *sequence alignment* is to first line up closely related sequences and then sequentially add related but more distinct sequences. The alignment sequence will show homologous characters, namely characters who have the same ancestor (Kemena and Notredame, 2009).

The alignment results showed that all amplicon sequenced has not identical to several LDH gene sequences from the NCBI database. This could be possible because the LDH is a superfamily gene so that each sequence has various types of protein motifs (conserv motifs*)* that cause the sequence of the genes could varies in the alignment results. According to Acquaah (2007), bacteria are unicellular prokaryotic organisms with a short life cycle and very high regeneration. It is likely that there has been a mutation that causes the replacement of several amino acids and protein structures due to mutations (Yatnita &; Hilda, 2017).

There are many gaps in the results of the alignment. The gaps indicate that there are no similarity in these region. It could be there are insertion or deletion on one or more region of DNA. In addition, gaps in sequence alignment represent mutational changes in sequences, including rearrangements of genetic material. In biological systems, the process of evolution involves genetic mutations and recombinant processes within a species to form a new species.

The evolutionary history of an organism can be identified by changes in its character. The same character is the basis for analyzing the relationship of one species to another (Schmidt, 2003). The phylogenetic tree is a logical approach to show evolutionary relationships between organisms, can

also be interpreted as a model to represent the relationship around the organism's ancestors, molecular sequences or both (Brinkman &; Leipe, 2001). One of the purposes of compiling a phylogenetic tree is to construct precisely the relationships between organisms and estimate the differences that occur from one ancestor to offspring (Li *et al.*, 1999).

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While the *maximum likehood* method uses calculations to find trees that have the best count of variation in the sequence set by considering for each tree, the number of sequence changes or mutations that occur that cause sequence variation. This method can be used to explore relationships between more diverse sequences (Schadt *et al.*, 1998). The selection of reference species was carried out by looking for LDH sequences in various bacterial species that are still in the same genus as *Bacillus cereus* and its closest species in the NCBI GenBank database.

4. CONCLUSION

Molecular identification based on 16S rRNA gene sequences found that B21 isolates is similar (has 100 % similarity) to *Bacillus proteoticu*s. The optimum annealing temperature for each primers are 50 ºC for 16s rRNA, 56.15 ºC for idb 0101, 57.5 ºC for idb 0813, 56.4 ºC for idb 1010, 58.3 ºC for idb 1147, and 56.5 ºC for idb 2021. Idb 1010 primer yield thickest fragment (PCR product). There are 4 fragments yielded in Bacillus cereus DNA amplification using Idb 1010 primer

(650, 1400, 1500, and 1700 bp). The 1700 bp have a highest percentage of kinship with the D-LDH gene in *Myobacterium tuberculosis* with a bootstrap value of 84 %. While other size fragments cannot be trusted to have close kinship with the comparison LDH sequence obtained from the NCBI database.

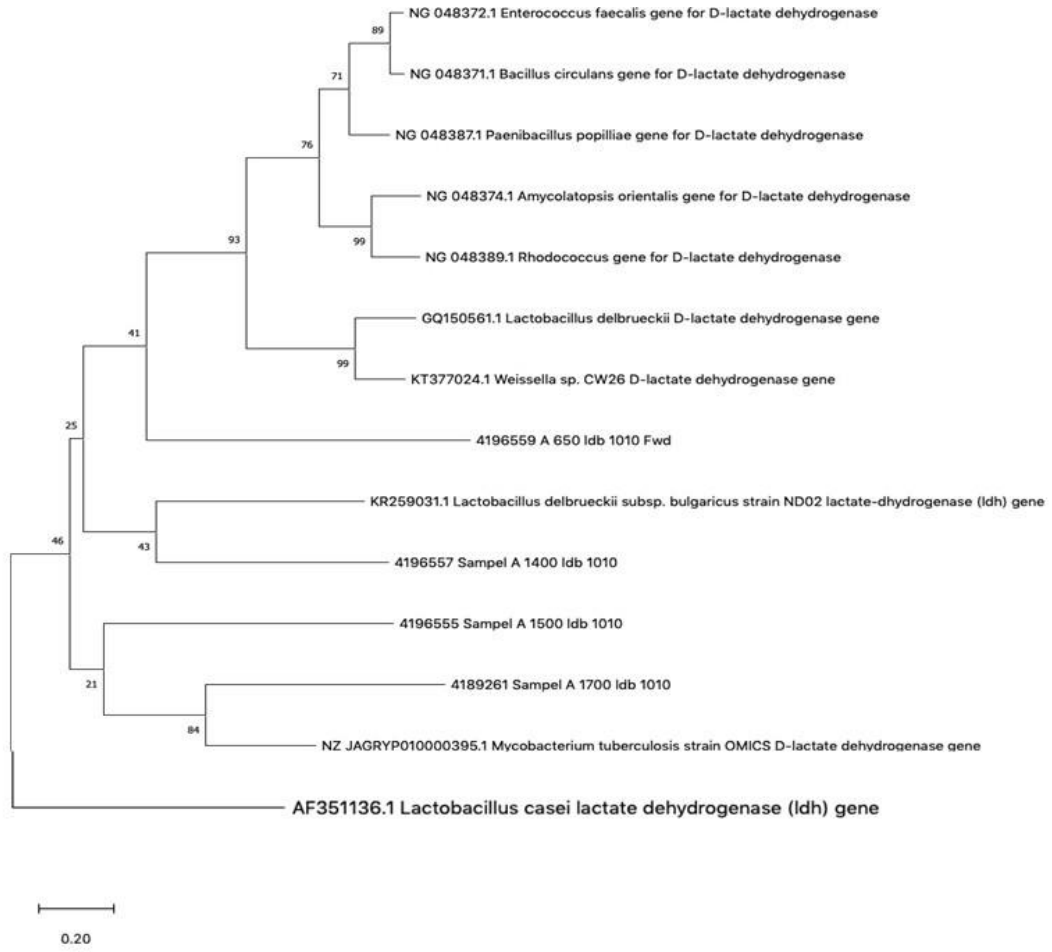

Figure 7. Results of analysis with phylogeny tree on LDH idb 1010 on *Bacillus cereus isolate* and several reference sequences from GenBank database

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