Screening of Indonesian marine bacteria and their potential for D-tagatose production

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Abstract. A rare natural ketohexose, D-tagatose, has attracted an increasing interest as sucrose substitute due to its low caloric value and similar properties to sucrose. D-Tagatose is enzymatically produced by using L-aldose isomerase (LAI) as catalyst and D-galactose as substrate. Therefore, a continuous effort to obtain a new isolate with high LAI activity is needed. Therefore, our collection of marine bacteria was assayed by employing designed minimum culture media with 0.5% (w/v) D-galactose as the sole carbon source. D-Tagatose production ability of bacterial strains were investigated and quantified by high performance liquid chromatography (HPLC). Our investigation showed that from 23 Indonesian marine bacteria, a total 18 bacteria possessed the ability to grow in the designed media. This result was subsequently confirmed using HPLC and showed that a total of four isolates, namely isolates LBF 110, 138, 108, and 131, exhibited a D-tagatose yield. Isolates LBF 110 and 138 were identified in this study according to molecular identification 16S rRNA method as Bacillus xiamensis, and Brevibacterium sediminis, respectively. The other two isolates, LBF 108 and 131, were already identified in previous study as Shewanella algae and Pseudomonas stutzeri, respectively. This result suggests the potential of these isolates as D-tagatose producing bacteria.

Keywords: marine bacteria, ketohexose, D-tagatose, D-galactose, L-aldose isomerase.

1. Introduction

Rare sugars can be defined as monosaccharides and their derivatives that occur in small amounts in nature, these sugars can be found in small amounts in various food sources such as honey, certain fruits, vegetables, and whole grains. More than 40 types of rare sugars have been identified and have little difference in their chemical structure compared to natural sugars such as D-glucose [1]. D-tagatose is a ketohexose monosaccharide sugar which is categorized as a rare sugar and has a sweetness level of 92% of sucrose, a caloric value of 1.5 kcal/g or 38% of sucrose, has a low glycemic index and high solubility at 20°C [2]. D-Tagatose provides various health benefits including prebiotic, anti-diabetic, and good for teeth [2].

D-Tagatose can be applied in foods and beverages as a flavor enhancer and can be a healthy sweetener even when used in large quantities [2]. D-Tagatose have the potential to be used as alternative sugars because they have almost the same characteristics as sucrose. D-Tagatose have been declared by the Food and Drug Administration (FDA) as safe food ingredients with Generally Recognized As Safe (GRAS) certification. D-tagatose can be produced in two ways, namely enzymatically and chemically. However, chemical production has the disadvantage that it requires a complicated purification process and produces chemical waste and by-products [3]. Therefore, the most widely used production method
is enzymatic production. D-Tagatose can be produced from the conversion of D-galactose using an enzyme called L-arabinose-isomerase.

The availability of microorganism resources in nature can be used as an exploration material to determine the potential of a bacterium that has the ability to produce a certain compound. Some bacteria have enzymes that can convert D-galactose to D-tagatose using L-arabinose-isomerase, such as E. coli [4], Lactobacillus fermentum [5], Bacillus coagulans [6], Geobacillus thermoelectrificans [7], Geobacillus statherothermphilus [8], Thermota neapolitana [9], Thermodota maritime [10], and Anoxybacillus flavithermus [11].

In this study, a collection of marine bacteria available in the form of pure isolates was used to search for bacteria that have the ability to produce the rare sugars D-tagatose. Screening and identification are steps to determine bacterial isolates that have the potential to produce D-tagatose. Screening was performed by culturing bacteria on selective growth media, namely by using D-galactose as substrates. The resulting D-tagatose can be identified using High Performance Liquid Chromatography (HPLC) methods. Bacteria that have the ability to produce these compounds can be identified using the 16S ribosomal Ribonucleic acid (16S rRNA) method. HPLC method can be used for the analysis of organic compounds and 16S rRNA methods can be used to determine taxonomy, phylogeny (evolutionary relationships) and estimate the rates of species divergence between bacteria.

2. Material and Methods

2.1 Sample and Reagents

The materials used in this study consisted of test materials and chemicals. Samples used were 23 isolates of marine bacteria that were obtained from several marine areas in Indonesia, i.e., Pari Island, Jakarta, Kamal Port, East Java and Cilacap Bay, Central Java [12]. Chemicals used in this study were listed as follows: standard D-allulosa (Sigma), standard D-tagatose (TCI), zobell marine agar (Himedia), artificial sea water (Marine Art SF-1), yeast extract (Himedia), D-galactose (Nacalai Tesque Inc.), magnesium chloride (100 mM), agarose (Genetika Science), tris borate EDTA (TBE) (Genetika Science), 1 kbp DNA marker (Thermo Scientific™), dreamtaq green PCR master mix (Thermo Scientific™), and Quick-DNA™ fungal/bacterial miniprep kit (Zymo Research).

2.2 Screening for Bacteria with the Ability to Produce D-tagatose

The screening method was in accordance with [13] with several modifications. A total of 5 mL of sterile preculture medium containing 3.6% (w/v) artificial sea water, 0.05% (w/v) yeast extract, and 0.5% D-galactose was transferred into 23 test tubes, then a single colony transferred into each preculture medium and incubated in an incubator shaker for 16 hours at 30°C with shaking at 200 rpm. The culture medium made were adjusted to the number of bacteria that could grow in the preculture media. A total of 200 μL of preculture (1%) was inoculated into the prepared culture media containing 3.6% (w/v) artificial sea water, 0.1% (w/v) yeast extract, and 0.5% D-galactose. Cultures were incubated in an incubator shaker for 24 hours at 30°C with shaking at 200 rpm. Bacterial cells grown in culture media were harvested by centrifugation at 4°C, 8000 rpm for 20 minutes. A total of 1 mL 25 mM tris-HCl buffer (pH 8) was added into a tube containing harvested bacterial cells and then the cells were disrupted by sonication for 6 minutes. Subsequently, the cells were frozen for 5 minutes in a refrigerator at -80°C and immediately thawed for 5 minutes at 60°C. To obtain cell-free extract, samples were centrifuged at 4°C, 8000 rpm for 20 minutes. To confirm the strain with the ability to produce D-tagatose, high performance liquid chromatography (HPLC) equipped with a Bio-Rad Aminex HPX-87P, 9 μm, 300 x 7.8 mm column, and RID-20A detector (Shimadzu) was performed. The mobile phase employed was water at a flow rate of 0.6 mL/min, and a sample volume of 20 μL.

2.3 Molecular identification of strain using 16S rRNA method

Genomic DNA of the bacterial isolates were extracted using Quick-DNA Miniprep Kit (ZYMO RESEARCH) according to the manufacturer’s instruction. The amplification of 16S rRNA gene was carried out using two universal primers: (Integrated DNA Technologies, IDT) 9F (5’-
GAGTTTGATCTGCTGCTAC3') and 1541R (5'-AAGGAGGTGATCCAGCC3') [14]. The PCR reaction was conducted in a total volume of 25 µl with DreamTaq Green PCR Master Mix (Thermo Fisher Scientific) consisting of 12.5 µl of PCR Master Mix (2X), 1 µl of Primer 9F (10 µM), 1 µl of Primer 1541R (10 µM), 1 µl of genomic DNA template (270 ng/µl), and 9.5 µl of Nuclease-Free Water. The thermocycler program for each PCR reaction was started with an initial denaturation step set at 95 °C for 3 min, followed by 30 cycles of denaturation steps at 95 °C for 30 sec, annealing step at 65 °C for 30 sec, extension step at 72 °C for 60 sec, and then for final extension step at 72 °C for 5 min. All PCR products were run on 1% agarose gel in Tris-Boric EDTA (TBE) buffer using an electrophoresis system. DNA bands were visualized with SYBR green using an UV Transilluminator to check the success of PCR amplification. For DNA sequencing, PCR products and primers (forward and reverse) were sent to First-Base company service. The obtained sequences of 16S rRNA gene were assessed the quality, trimmed, and assembled using Geneious Prime version 2021 (http://www.geneious.com) software [15]. The generated consensus sequences were compared with the most closely related species available in the GenBank database using the BLAST online server from The National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.4 Construction of Phylogenetic Tree
The phylogenetic tree of bacterial isolates was constructed based on the 16S rDNA gene sequences using Geneious Prime version 2021 software. Selected sequences with the high similarity (97-100 of percent identity) were multiple aligned and then constructed using the Neighbor-Joining (NJ) algorithm method [16, 17, 18]. As an outgroup, Arthrobacter enclensis strain NIO-1008 was used in this study. The resampling tree was performed to assess the stability of the relationship using Bootstrap method for 1000 replicates and 50% of threshold.

3. Results and Discussion
3.1 Screening of Bacterial Strains with D-tagatose Production Ability
Carbon sources are utilized by cells as substrates in metabolic pathways, carbon is degraded to provide amino acids and other components used for cell growth [19]. Zobell marine broth is a growth medium used for marine bacteria. This medium contains peptone which serves as a source of carbon and nitrogen. Previous results have reported that D-galactose was converted to D-tagatose using L-aldose isomerase, particularly L-arabinose isomerase [20]. Thus, to obtain bacteria capable of producing D-tagatose, peptone in the growth medium was replaced by D-galactose as the only carbon source and yeast extract used as a nitrogen source. Bacterial isolates grown on selective media were bacteria that had been rejuvenated. The results of bacterial rejuvenation can be seen in Figure 1. A total of 23 isolates of marine bacteria were grown on selective media. The results of screening for marine bacteria on selective media can be seen in Table 1. A total of 18 bacteria was able to grow on the selective media. Marine bacteria that grow on selective media are characterized by changes in the media from clear to become cloudy, indicating that these bacteria are able to metabolize available carbon sources.

3.2 Qualitative and quantitative analysis of D-tagatose
To confirm whether D-galactose were metabolized into D-tagatose or other compounds, HPLC analysis was performed. HPLC analysis can be used for qualitative and quantitative sample identification. Qualitative analysis was carried out by comparing the retention time of the sample with the retention time of the standard, while quantitatively by substituting the sample area into the regression equation. According to qualitative analysis of HPLC, D-tagatose was detected in cell-free extract of bacterial samples which can be seen from the peak that appears at similar retention time as the D-tagatose standard. According to [21], the relative retention time of the analyte should correspond to a standard solution with a tolerance of ± 0.5% for Gas Chromatography and ± 2.5% for Liquid Chromatography. Therefore, if the analyte retention time is still in the tolerance range of ± 2.5%, then the analyte can be concluded to be the same as the standard. The average retention time of the D-tagatose standard series is 41,238 minutes, so the accepted retention time range is 40,207- 42,269 minutes, which was shown in
cell-free extract with codes LBF-108, LBF-110, LBF-131, and LBF-138 (Table 2). The levels of D-tagatose produced from samples were shown in Table 2, having produced D-tagatose in the range of 18.5-32.2 g/L. This result indicates that the bacterial isolates possess the ability to produce D-tagatose. Similar study performed with lactic acid bacteria showed a much lower D-tagatose yield with 3.134 g/L for *Lactobacillus salivarius* [22].

![Cell-free extract with codes LBF-108, LBF-110, LBF-131, and LBF-138](image)

**Figure 1.** Rejuvenation of marine bacteria.

### 3.3 Molecular identification of marine bacteria with the ability to produce D-tagatose

The 16S rRNA gene can be used to identify bacteria because 16S rRNA is a specific coding gene and is not easily mutated, so it can be used to determine taxonomy, phylogeny (evolutionary relationships) and estimate the distance of diversity between bacterial species [23]. Bacteria that showed positive results on HPLC analysis were then identified by the 16S rRNA method. The marine bacteria that were identified in this study were isolates with codes LBF-110 and LBF-138, whereas isolates with codes LBF-108 and LBF-131 were identified as *Shewanella algae* strain KJ-W37 and *Pseudomonas stutzeri* strain ATCC 17588, respectively, in a previous study conducted by [12] which can be seen in Table 3. Amplification result was checked using electrophoresis as quality control. The results of the electrophoresis of the 16S rRNA gene amplification product can be seen in Figure 2. The quality control function at the electrophoresis stage is proven by the results of the analysis which can be seen in Figure 2, which shows that the amplified gene produces a single band in the range of 1500 bp. According to [24], the single band seen in the visualization results of PCR products using agarose gel indicates that the primer used work well and was specific for the target gene, namely the 16S rRNA gene. Therefore, the amplification product was used for the sequencing process.
Table 1. Screening of marine bacteria on selective media.

| Isolate Code | Growth Indication |
|--------------|-------------------|
| LBF 9        | +                 |
| LBF 18       | +                 |
| LBF 76       | -                 |
| LBF 82       | +                 |
| LBF 101      | +                 |
| LBF 102      | +                 |
| LBF 105      | +                 |
| LBF 106      | -                 |
| LBF 108      | +                 |
| LBF 109      | +                 |
| LBF 111      | +                 |
| LBF 113      | +                 |
| LBF 114      | +                 |
| LBF 115      | +                 |
| LBF 118      | -                 |
| LBF 128      | -                 |
| LBF 129      | +                 |
| LBF 131      | +                 |
| LBF 132      | +                 |
| LBF 133      | +                 |
| LBF 138      | +                 |
| LBF 141      | -                 |
| LBF 142      | +                 |

*Note: (-) indicating no growth (clear culture); (+) indicating growth (cloudy culture)*

Table 2. Cell-free extract analysis using HPLC.

| Isolate Code | Retention Time of D-Tagatose (min) | D-Tagatose (mg/L) |
|--------------|-----------------------------------|-------------------|
| LBF-108      | 40.354                            | 18471.73          |
| LBF-110      | 40.210                            | 28957.25          |
| LBF-131      | 41.272                            | 26176.07          |
| LBF-138      | 40.237                            | 32209.45          |

Table 3. Identification of marine bacteria with D-tagatose production ability.

| Isolate | Closest taxonomic species            | Accession number | % Identity | Reference |
|---------|--------------------------------------|------------------|------------|-----------|
| LBF-108 | *Shewanella* algae strain KJ-W37    | JQ799131         | 95.00      | [12]      |
| LBF-110 | *Bacillus xiamenensis* strain        | NR_148244.1      | 99.78      | This study|
|         |                                      | MCCC 1A00008     |            |           |
| LBF-131 | *Pseudomonas stutzeri* strain        | NR_041715        | 98.00      | [12]      |
|         |                                      | ATCC 17588       |            |           |
| LBF-138 | *Brevibacterium sediminis* strain    | NR_153678.1      | 99.88      | This study|
DNA sequencing is the determination of the exact sequence of nucleotides in a DNA molecule [25]. The resulting sequences were then aligned with the database in NCBI, so that the closest strains between the identified bacterial isolates and the bacterial strains in the database could be identified. The results of the identification of marine bacteria can be seen in Table 3. Analysis of identification showed that there was isolate with a percentage identity value lower than 96%, namely LBF-108 with the closest strain being *Shewanella algae* strain KJ-W37, which was identified by [12]. The percentage identity value lower than 96% can be assumed as a candidate for new species [26], so further research is needed on morphology and biochemical tests for isolate with code LBF-108 [12]. Bacterial isolates with codes LBF-110, LBF-131, LBF-138 had an identity percentage value greater than 96%, which indicated that the bacterial isolates were the same species as the closest strain. On the basis of results of the analysis, the bacterial isolates with codes LBF-110, LBF-131, and LBF-138 were *Bacillus xiamenensis* bacteria,
**Pseudomonas stutzeri**, and **Brevibacterium sediminis**, respectively. The relationship between LBF-110 and LBF-138 bacterial isolates with other types of bacteria was shown in Figure 3. The phylogenetic tree showed that LBF-110 had the closest relationship with **Bacillus xiamenensis** strain MCCC 1A00008 and **Bacillus aerius** strain 24K, while LBF-138 has the closest relationship with **Brevibacterium sediminis** strain CGMCC.

There are several bacteria of the same genus and have been reported to possess the ability to produce D-tagatose from D-galactose, namely from the genera **Shewanella**, **Bacillus**, and **Pseudomonas**. However, there are no bacteria from the genus **Brevibacterium** that have been reported to have the ability to produce D-tagatose from D-galactose. Strains of the three genera reported to have the ability to produce D-tagatose from D-galactose was shown in Table 4. Previous study showed that L-arabinose isomerase is the most commonly reported enzyme for the production of D-tagatose from D-galactose, except for **Pseudomonas aeruginosa** PA-01 which uses phosphoglucose isomerase to produce D-tagatose from D-galactose. This study reports for the first time the use of **Bacillus xiamenensis**, **Pseudomonas stutzeri**, and **Brevibacterium sediminis** for the production of D-tagatose, so further research is needed on the enzymes involved in the production of D-tagatose from these species.

**Table 4.** Microorganisms from the genus **Shewanella**, **Bacillus**, and **Pseudomonas** that produce D-tagatose from D-galactose.

| Strain                        | Enzyme that converts D-galactose to D-tagatose | Reference |
|-------------------------------|---------------------------------------------|-----------|
| **Shewanella sp. ANA-3**      | L-arabinose isomerase                        | [27]      |
| **Bacillus coagulans 2-6**    | L-arabinose isomerase                        | [28]      |
| **Bacillus thermoglucosidasius** KCTC 1828 | L-arabinose isomerase                        | [29]      |
| **Bacillus stearothermophilus US100** | L-arabinose isomerase                        | [30]      |
| **Bacillus licheniformis ATCC 14580** | L-arabinose isomerase                        | [31]      |
| **Bacillus subtilis** strain 168 | L-arabinose isomerase                        | [32]      |
| **Bacillus halodurans** DSM 497 | L-arabinose isomerase                        | [33]      |
| **Bacillus stearothermophilus IAM 11001** | L-arabinose isomerase                        | [34]      |
| **Pseudomonas aeruginosa** PAO1 | phosphoglucose isomerase                     | [35]      |

4. **Conclusion**

The recent study succeeded to screen 4 out of 23 marine bacteria of Indonesia origin showing the potency to produce D-tagatose. The three isolates consist of **Bacillus xiamenensis**, **Pseudomonas stutzeri**, and **Brevibacterium sediminis**, whereas one isolate with code LBF-108 identified as **Shewanella algae** needs further research on morphology and biochemical tests to confirm the isolate species. The D-tagatose producing bacteria showed the ability to assimilate D-galactose as a sole carbon source. This is the first report showing the potency of the four isolates in producing the rare sugar D-tagatose, and further research is required to study the enzymes involved in the production of the rare sugar D-tagatose from these species.

**Author Contributions**

F.A.L., E.A. and I.N. were equally contributed to this work. F.A.L. designed the study. E.A., I.N., E.Y., S.M and H.W. performed data collection, analysis, and interpretation. F.A.L. prepared the manuscript and supervised thoroughly this work.
References

[1] Amna A, Tauseef A K, D Dan R, Cyrill W C K and John L S 2021 Nutrition Reviews. 1-16
[2] Mitchel, H 2006 Sweeteners and Sugar Alternatives in Food Technology (United Kingdom: Blackwell Publishing Ltd)
[3] Wenli Z, Shuhuai Y, Tao Z, Bo J and Wanneng M 2016 Trends in Food Science & Technology. 54 127-37
[4] Sang-Hyun Y, Pil K and Deok-Kun O 2003 World Journal of Microbiology & Biotechnology. 19 47-51
[5] Zheng X, Yujia Q, Sha L, Xiaohai F, Hong X and Pingkai O 2011 Journal of Molecular Catalysis B: Enzymatic. 70 1–7
[6] Wending M, Lu W, Ying Zaohuan Z and Jia O 2016 BMC Biotechnology. 16 1-11
[7] Hye-Jung K and Deok-Kun O 2005 Journal of Biotechnology. 120 162-73
[8] Dong-Woo L, Eun-Ah C, Seong-Bo K, Soo-Hyun E, Young-Ho H, Sang-Jae L, Han-Seung L, Dong-Yun L and Yu-Ryang P 2005 Archives of Biochemistry and Biophysics. 434 333-43
[9] Byoung-Chan K, Yoon-Hee L, Han-Seung L, Dong-Woo L, Eun-Ah C, Yu-Ryang P 2002 FEMS Microbiology Letters. 212 121-6
[10] Dong-Woo L, Hyeung-Jin J, Eung-Ah C, Byoung-Chan K, Sang-Jae L, Seong-Bo K, Young-Ho H and Yu-Ryang P 2004 Applied And Environmental Microbiology. 70 1397-404
[11] Yanjun L, Yueming Z, Anjun L and Yuanxia S 2011 Extremophiles. 15 441-50
[12] Elvi Y, Ahmad T and Yopi 2016 Biodiversitas. 17 857-64
[13] Akihide Y, Taro K, Tomoya S, Ryo M, Kouhei O, Tetsuo I, Kazuya A, Ken I and Pushpa K G 2017 Journal of Bioscience and Bioengineering. 123 170-6
[14] Bruce J P, Susan K B, Jamie L G, Rebeccia E E, Carol N L, Valerie A L, Ashish S and Floyd E D 2001 Journal of Bacteriology. 183 3770-83
[15] Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, Thierer T, Ashton B, Meintjes P, and Drummond A 2012 Bioinformatics. 28(12) 1647-49
[16] Naruya S and Masatoshi N 1987 Mol. Biol. Evol. 4 406-25
[17] Koichiro T and Masatoshi N 1993 Mol Biol Evol. 10 512–26
[18] Koichiro T, Masatoshi N and Sudhir K 2004 Proc Natl Acad Sci U S A. 101 11030–35
[19] Xin W, Kang X, Xiaojing Y and Chao T 2019 Nature Communications. 10 1-7
[20] Laksma F A, Arai S, Tsurumaru H, Nakamura Y, Saksono B, Tokunaga M, Ishibashi M 2018 Biochim Biophys Acta Proteins Proteom. 1866(11) 1084-91
[21] European Communities 2002 Official Journal of the European Communities. 1:8-36
[22] Li W, Zhou Z, Zhang G, Awad F N, Chandankere R, Guo Q and Qi X 2019 BJJ. 23(2) 1-6
[23] Trisia R 2011 Jurnal Kedokteran Syiah Kuala. 11 172-7
[24] Cahyadi M, Taufik I M, Pramono A and Abdurrahman Z H 2019 Journal of the Indonesian Tropical Animal Agriculture. 44 10-18
[25] Indu R, Mamta B and Jyoti S 2014 Advances in Biotechnology (New Delhi: Springer)
[26] George E F, Jeffrey D W and PETER J, JR 1992 International Journal of Systematic Bacteriolo. 42 166-70
[27] Moez R, Goran B, Rimeh I, Samira B, Emmanuelle M, Richard H and Nushin A 2011 Microbial Cell Factories. 10 1-11
[28] Xingding Z and Jin C W 2012 World Journal of Microbiology and Biotechnology. 28 2205-12
[29] Myung-Ji S 2013 Bioscience Biotechnology Biochemistry. 77 385-8
[30] Moez R and Samir B 2006 Biochim Biophys Acta. 1760 191-9
[31] Ponnandy P, Manish K T, Mariomuthu J, Paramasamy G, In-Won K and Jung-Kul L 2008 Applied Microbiology and Biotechnology. 81 283-90
[32] Jin-Ha K, Ponnandy P, Mariomuthu J, Manish K T, Hye-Jung M, Raushan K S and Jung-Kul L 2010 Applied Microbiology and Biotechnology. 85 1839-47
[33] Dong-Woo L, Eun-Ah C, Seong-Bo K, Soo-Hyun E, Young-Ho H, Sang-Jae L, Han-Seung L, Dong-Yun L and Yu-Ryan P Archives of Biochemistry and Biophysics. 434 333-43
[34] Lifang C, Wanmeng M and Bo J 2010 Journal of Science and Food Agriculture. 90 1327-33
[35] Manisha J P, Arti T P, Rekha A, Samir D and Darshan H P 2016 Appl Biochem Biotechnol. 179(5) 715-27.