Isolation and Characterization of Hexokinase Gene from *Pseudonocardia carboxydivorans* 18A13O1

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Abstract. Marine Actinobacteria, including *Pseudonocardia carboxydivorans*, is known for producing secondary metabolites with antibacterial activity. Hexokinase, a key enzyme in the glycolytic pathway, catalyzes the conversion of glucose to glucose-6-phosphate and is crucial in cellular energy metabolism. Conversely, in *Penicillium chrysogenum* strain dogR5, the activity of this enzyme is known to increase during penicillin production, a bioactive compound. Studying the gene encoding hexokinase in marine actinobacteria can provide insights into the regulation of metabolism. This study aimed to isolate and characterize the gene encoding hexokinase from *P. carboxydivorans* 18A13O1. The sequences were obtained through several main stages, including DNA extraction, DNA amplification, sequencing, and characterization analysis using in silico methods. In the amplification process, PCR optimisation was carried out using HRM to obtain the ideal annealing temperature based on melting temperature and the ideal temperature was 52°C Sequencing used the Sanger method at PT Genetika Science Indonesia. In silico analysis was performed with BioEdit, Open Reading Frame Finder (NCBI), SwissDock, and ChimeraX. The results showed that the isolated gene encodes Hexokinase-1 (HXK1), with active sites identified at ASP 116, LYS 117, GLU 248, ASP 152, GLU 214, and GLY 216. These findings contribute to biotechnology by enhancing our understanding of hexokinase in marine Actinobacteria, with potential applications in enzyme production and drug development.

Keywords: gene isolation, 3D-visualization, Hexokinase, HXK1, *P. carboxydivorans.*

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1 Introduction

Antibiotics are the "magic bullet" against bacteria and are considered the most incredible medical discovery of the 20th century. The introduction of antibiotics has changed the paradigm of medicine and continues to save millions of lives from bacterial infections [31]. With increasing use and misuse, microorganisms have developed antimicrobial resistance. Infections caused by antimicrobial-resistant organisms are not only difficult to treat, but there is also always an increased chance of serious disease and even death from these infections [28]

Antimicrobial resistance has been recognized as one of the three greatest human health challenges by the World Health Organization (WHO) [4]. According to a substantial study published in January 2022. The number of deaths due to resistance is expected to rise to 10,000,000 per year by 2050, far exceeding cancer deaths [28]. This is no more a reality than science fiction, there is an urgent need for new antibiotic active ingredients to ensure effective treatment of infections that are resistant to old antibiotics.

Hexokinase (HK) is an enzyme that is involved in glucose metabolism, it functions to catalyze glucose and convert it into glucose-6-phosphate (G6P) with the mechanism = glucose + MgATP → G6P + MgADP. Hexokinase is the gatekeeper of glucose entry into metabolism, where G6P is used for glycolysis of all tissues except for glycogen synthesis in the liver and skeletal muscles in the absorption phase and gluconeogenesis in the liver and kidneys in the fasting phase, which can be interpreted that the existence of the hexokinase gene is a crucial part that determines whether the cellular metabolic process of each body tissue is good or bad, especially the determination of blood sugar levels [24]. Hughes et al. [18] in 2017 showed that the existence of the hexokinase gene in immune cells affects the level of immune metabolism. Bantug et al. [6] research in 2018 showed that abnormalities in the hexokinase gene have a significant effect on the activation of inflammatory cells. Exploration of new microorganisms as an alternative to producing the hexokinase gene is considered a wise step as one of the therapeutic options for metabolic disorders caused by the hexokinase gene.

One of the potential microorganism candidates is actinobacteria, a group of Gram-positive bacteria with high G+C content that has an important role in various ecosystems, including the marine environment. Marine actinobacteria are identified as producers of bioactive compounds with antimicrobial, anticancer, and anti-inflammatory properties [12], [9], [7]. Actinobacterial symbiotic with other marine organisms have a role to protect the host from harmful microorganisms [5]. In addition, actinobacteria also play a role in the bioremediation process of marine pollutants (oil, heavy metals, pesticides and plastics) through degradation, biostimulation, and bioaugmentation strategies [10].

One of the actinobacterial genus is *Pseudonoardia*. *Pseudonocardia* species are known to produce various secondary metabolite compounds that exhibit biological activity as antibacterials [25]. This species has been known to have the ability to metabolise complex organic compounds and symbiotic relationships with other marine organisms. Previous research found that Pseudonocardia carboxydivorans symbiotic with marine sponges has antibacterial potential [21]. This ability suggests the existence of

specialised adaptations and unique metabolic mechanisms, which are interesting for further investigation.

DNA isolation is the first step that needs to be done, followed by gene characterization based on genomic technology as the most effective method available to increase yield, efficacy, and success rate in the development of new antibiotic agents. Moreover, understanding genomics is not limited to identifying innovative targets for new antibiotics. It also provides an opportunity to understand the fundamental interactions involved in the therapeutic use of antibacterial agents. Moreover, it has led to the acquisition of data on genes responsible for several important processes, such as bacterial adaptation, host vitality, pharmacokinetics, and multi-drug resistance, as well as metabolic processes that help in understanding secondary metabolites, nitrotoluene degradation, and lipopolysaccharide biosynthesis [2].

2 Experimental Section

2.1 Materials

The materials used in this research were ISP-2 liquid actinobacterial specific media consisting Malt Extract (Merck KgaA, Darmstadt, Germany), Granulated Yeast Extract (Merck KgaA, Darmstadt, Germany), Dextrose, and artificial seawater provided by the Microbiology Laboratory, Department of Biology, University of Lampung. Pseudonocardia carboxydivorans 18A13O1 was collected from a marine sponge and identified at UPT LTSIT, University of Lampung. Genome data has been verified in Gene Bank with access code LC647653. DNA extraction kit used in this study are Wizard® Genomic DNA KIT protocol (cat. no. A1120, Promega, Madison, WI, USA), Lysozyme (Geneaid), EDTA (Merck KGaA, Germany), Choloform, 70% Alcohol, Isoprophyl Alcohol (MP Biomedicals LLC, New Zealand). The solvent used was Water, Ultra Pure (iNtRON, USA). For PCR reagents, PCR Master Mix Solution I-TAQ (iNtRON, USA) was used for PCR and RealMod Green W2 2X QPCR Mix (iNtRON, USA) for HRM PCR. For electrophoresis, SeaKem LE Agarose (Lonza, ME USA) and RedSafe as a staining agent were used. Instruments used included Qiagen Tissue Lyser LT to maximise the lysis process. Analysis of the quality and quantity of DNA extraction results used Nanophotmeter P360 (Implen, Germany). The PCR process used LineGene MiniS (Bioer, Hangzhou). The electrophoresis was performed using Mupid-exU electrophoresis instrument (Mupid, Japan).

2.2 Sample Preparation

Isolates from stock were rejuvenated to ensure the quality and purity of the isolates. *Pseudonocardia carboxydivorans* isolate 18A13O1 was inoculated aseptically to the surface of colloidal chitin agar media by single streak method and then incubated at room temperature for ± 14 days. The growing colonies were observed microscopically by Gram staining to confirm the purity of the isolates. Pure isolates were sub-cultured onto liquid ISP-2 media. ISP-2 was prepared with 0.08 g yeast extract, 0.2 g malt extract, and 0.08 g dextrose dissolved in 100 ml sterile Artificial Sea Water (ASW) with

the composition of ASW (g/L); 27 g NaCl, 5. 6 g MgCl2.6H2O, 1.5 g CaCl2.2H2O, 1 g KNO3, 0.07 g K2HPO4, 6.6 g MgSO4.7H2O, and 0.04 g NaHCO3 with a pH between 7.4-8.2 [3]. The medium was sterilised by autoclave for 15 minutes at 121℃ pressure 1 atm. Afterwards, *P. carboxydivorans* isolate 18A13O1 was inoculated and incubated in a shaker incubator (50rpm) at 28℃ for 7-14 days.

2.3 DNA Extraction

Colonies of cultured isolates were taken with a micropipette and put in Eppendorf tube (2 ml) until the volume was 1 ml, then centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded. Added 500 µl of 0.05 M EDTA. To maximise the lysis process, 2 bullets were added, then crushed with a Tissue Lyser for 3 minutes. The bullets were then removed. Then added 20µl Lysozyme which is useful for degrading proteins and maximising cell lysis. then incubated at 37°C for 30 minutes. Next, the solution was centrifuged at 10,000 rpm for 5 minutes. The supernatant was discarded and 400 µl Nucleic Lysis Solution, and 100 µl Chloroform were added. Incubate at 80°C for 5 minutes. After incubation, the solution was equilibrated to room temperatur. After that, 250 µl Protein Precipitation Solution was added to the mixture and then vortexed for 20 seconds. incubated on ice for 5 minutes, then centrifuged at 10,000 rpm for 5 minutes.

The supernatant was collected and put into a new Eppendorf tube containing 600 µl isopropanol. The DNA pellet was separated by centrifugation at 10,000 rpm for 5 minutes. Discard the supernatant carefully. Afterward, the DNA pellet washed with 600 µl 70% Ethanol. Centrifuged at 10,000 rpm for 5 minutes. Discard the supernatant and then added 25 µl DNA Rehydration Solution to the pellet. Incubated for 1 hour at 65°C (the tube was gently tapped every 15 minutes to homogenise the solution). The extraction results were analysed with a P360 Nanophotometer at λ 260 nm and 280 nm. The results of DNA extraction were stored in a freezer at -20℃.

2.4 Amplification and Visualisation

The DNA extraction products that have been obtained are amplified with HRM PCR. Primer P1F 5'-ATGCCCGACGAACAGAAGC-3' and P1R 5'-GAGGTTGGACTTCACGGAG-3' were added 0.25 µl into a 0.2 mL PCR tube. 5 µl DNA template, 4.5 µl ddH2O, and 10 µl RealMod Green W2 2X QPCR Mix (iNtRON, USA) were added. Then the thermocycler was set with a pre-denaturation stage at 95°C for 1 minute, denaturation at 95°C for 1 minute, primer attachment or annealing stage at 52°C for 1 minute, elongation stage at 72°C for 1 minute, post elongation stage at 72°C for 5 minutes, and cooling stage of 20°C for 10 minutes. The cycle was performed 35 times. Immediately after PCR amplification, HRM occurred; amplicons were gradually melted at 0.2 °C increments from 51°C to 57°C, with fluorescence acquisition every 1 second. The results of the HRM PCR were analysed for specific melting temperature graphs and used as an approach for subsequent PCR annealing temperature optimisation. PCR was performed with a similar amount of composition as HRM PCR,

but using I-TAQ Master Mix Solution (iNtRON, USA) as the reactant. The PCR samples were then stored in a refrigerator at 4°C to avoid the DNA degradation. The PCR products were run on a 1.2% agarose gel and visualised under UV light on Geldoc.

2.5 Sequencing

PCR products that obtained amplicons were sent to PT Genetika Science Jakarta for sequencing using the Sanger method. Alat: ABI sekuensing.

2.6 In Silico Analysis

The sequencing results were analysed using BioEdit and Open Reading Frame Finder (NCBI), followed by protein modelling and docking using SwissModel and SwissDock.

3 Result and Discussion

3.1 Sample Preparation

Psedonocardia carboxydivorans 18A13O1 was successfully rejuvenated on colloidal chitin agar media with a total incubation time of 11 days. The morphology of the growing colonies shown in **Figure 1** is similar to that reported in previous studies [20], [29]. The staining results showed that the cells and hyphae were purple, indicating that the cell wall of *Psedonocardia carboxydivorans* 18A13O1 had a thick peptidoglycan layer and was able to bind crystal violet dye and was classified as Gram-positive bacteria. Microscopic observation also showsed uniformity of cell shape. Therefore, the isolate is confirmed as pure and can proceed to the next stage. The microscopic results are shown in **Figure 2**.

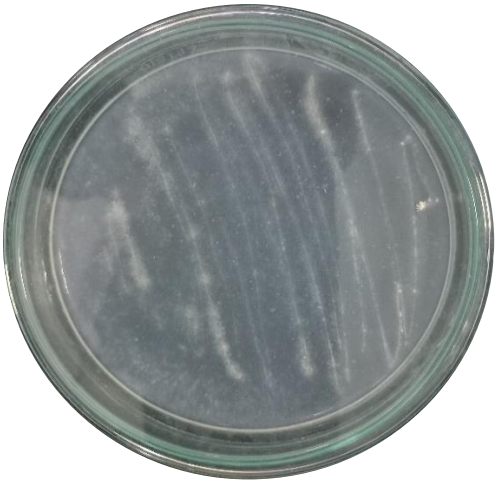

Fig. 1. *Pseudonocardia carboxydivorans* 18A13O1 in colloidal chitin agar medium.

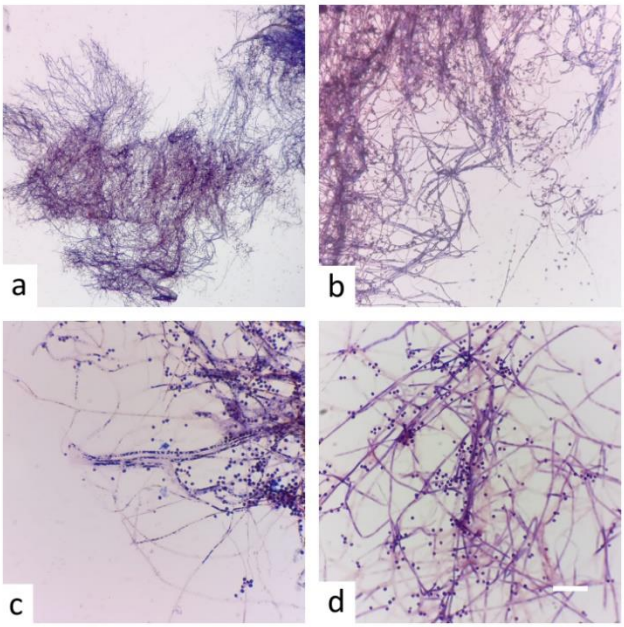

Fig. 2. Microscopic observation of *P. carboxydivorans* 18A13O1 after Gram staining. (a) 10× magnification; (b) 40× magnification; (c,d) 200× magnification, 40 micron bar.

Pure isolates were subcultured on liquid ISP-2 media. After 14 days of incubation, growth of white granular colonies was observed. Consistent colonies indicate that the isolate growth is uncontaminated and can be processed for DNA isolation (fig. 3).

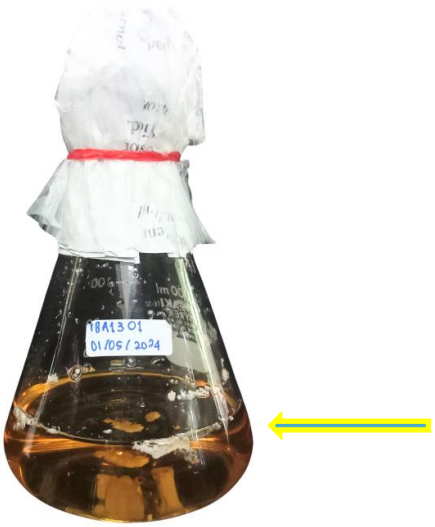

Fig. 3. *P. carboxydivorans* 18A13O1 in ISP-2 medium after 14 days of incubation (Blue arrow showed white granular colonies).

3.2 DNA Extraction

DNA was isolated from these samples using the Promega Wizard® Genomic DNA KIT protocol. The purity and concentration of the isolated genomic DNA, analysed using a P360 Nanophotometer (IMPLEN, Germany). The purity of DNA obtained from the isolation of *P. carboxydivorans* showed A260/A280 ratio values between 1.98-2.06 with an average purity value of 2.02. The results of quantitative analysis showed that the concentration of DNA obtained ranged from 223-284 ng/µl with an average concentration value of 245.3 ng/µl. Complete data as presented in **Table 1**.

Table 1. *P. carboxydivorans* 18A13O1 Genomic DNA Purity and Concentration .

Isolate	Nucleic Acid Concentration (ng/µl)	A260	A280	Absorbance Ratio
P1	284	0.577 A	0.283 A	2.065
P2	223	0.455 A	0.234 A	1.987
P3	229	0.461 A	0.230 A	2.022
Average	245.3			2.02

DNA is considered to be pure when the absorbance value of A260/A280 is in the range of 1.8-2.0 [16]. The results of the DNA concentration obtained are greater than 20 ng/µl, allowing the DNA extract to be used for amplification [19], [26].

3.3 PCR and Visualisation PCR Products

The results of the first amplification step, HRM PCR, showed that the melting temperature of the template DNA was ±52°C **(Fig.4**). The melting temperature in HRM PCR indicates the specific temperature at which the DNA melts into a single strand. This can be an approach to determining the ideal annealing temperature, as the separation of double-stranded DNA is related to primer annealing. The HRM graph is shown in Figure 4.Then, amplification was performed by conventional PCR at 52°C annealing temperature. The PCR products were visualised by electrophoresis (**Fig.5**).

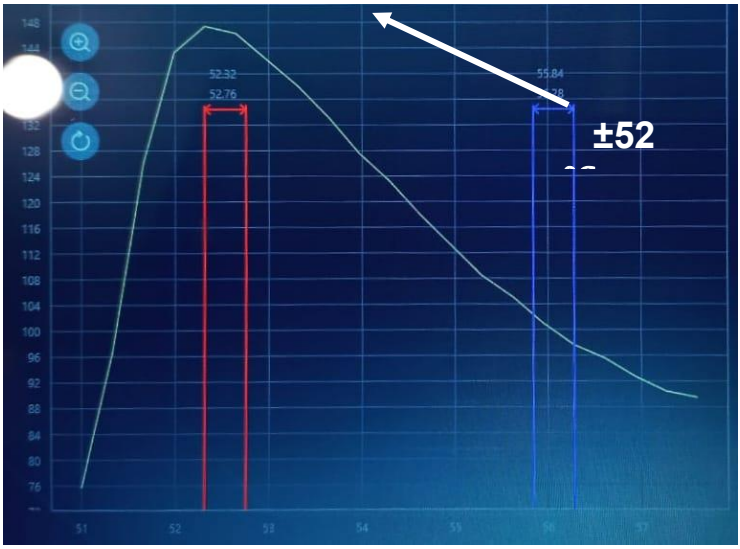

Fig. 4. HRM PCR Graphic.

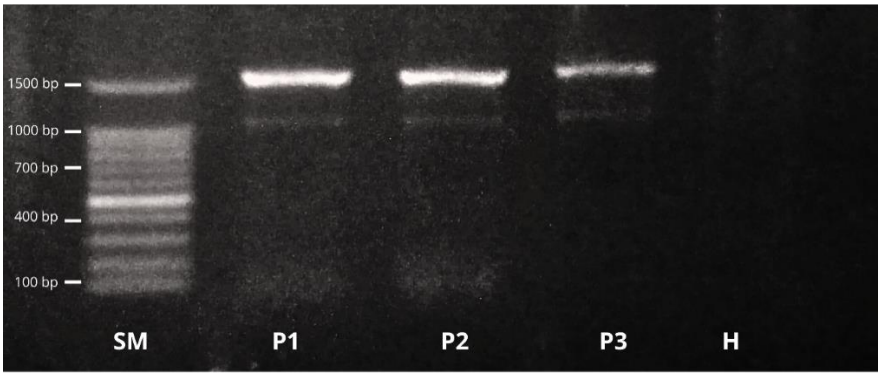

Fig. 5. Result of DNA band visualisation; [P1, P2, P3] PCR sample codes; H, H_2O.

The results of the visualisation showed the formation of double bands. The amplicons were ±1500 bp and ±1200 bp in length. The formation of these double bands can be caused by non-specific primer annealing [8]. The ±1500 bp band is clear and thick, whereas the ±1200 bp band is faint and thin.

Accordingly, the two bands formed are separated based on their size using the elution technique. The elution technique is a method used to extract and purify DNA fragments after separation by agarose gel electrophoresis [1]. The standard method for isolating DNA from agarose gels was presented by Grey & Brendel [13]. This resulted in two DNA samples with different band sizes, 1200 pb and 1500 pb. The elution and sequencing process was performed at PT. Genetika Science Indonesia.

3.4 Characterization of Hexokinasse

The nucleotide sequences were edited using BioEdit [15], to remove the parts of the sequence curve that were not read properly during the sequencing process, as indicated by the flat and overlapping peaks at the beginning and end of the sequence. The two gene sequences obtained were analysed using Open Reading Frame Finder (ORF Finder) to predict the DNA region that potentially encodes the hexokinase protein. The results showed that only sequences with a length of ±1500 bp (**Figure 6.**) had a potential hexokinase region. This was the protein query (ORF1), with a length of 1104 nt. The results showed that ORF1 has a similarity to hexokinase 1 (Hxk1) from *Saccharomyces cerevisiae* S288C (accession number NP_116711.3) (**Figure 7.**) with a percentage identity of 37.43%, indicating that both protein bases have a similar structure and function. Protein sequences are shown in **Figure 8**.

Fig. 6. Complete sequencing results with a length of 1150 bp after editing with BioEdit.

Fig. 7. ORF-Finder results showed similarity of the protein sequence with hexokinase 1 protein from *S. cerevisiae* S288C.

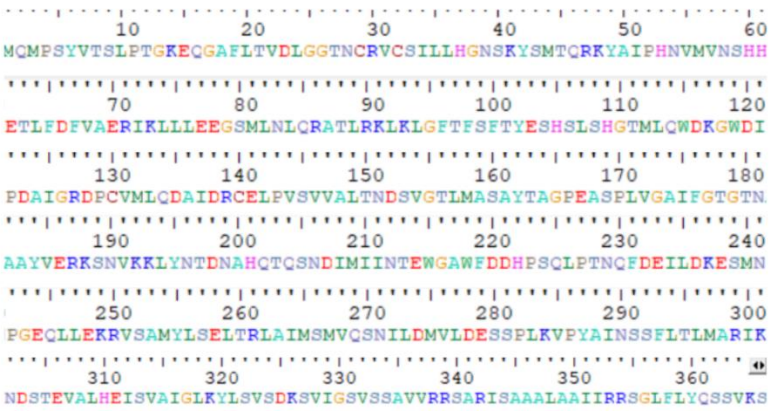

Fig. 8. Amino acid sequence from ORF1.

ORF1 protein sequences were used for protein modelling using SwissModel (http://swissmodel.expasy.org). SwissModel is an online server for automated comparative modeling of three-dimensional (3D) protein structures (**Figure 10.**) [27]. The results of modelling with SwissDock were downloaded in PDB format. In order to conduct analysis of 3D structure and molecular interactions a comparative docking approach was utilized, employing SwissDock 2024 (https://www.swissdock.ch/) [14], followed by visualisation of the protein-ligand docking result using the ChimeraX application, standard procedure according to Meng et al. [22].

The ligand utilised was alpha-D-hexose-6-phosphate ($C_6H_{13}O_9P$), the file was downloaded from PubChem in SDF format and converted to SMILES using Format Convert from Cheminfo (https://www.cheminfo.org). The structure of alpha-D-hexose-6-phosphate is shown in **Figure 9**.

The free energy result (ΔG) of the ligand docking molecule with the most negative value is -6.7429, located in cluster number 1, cluster member 1. This region visualised with Chimera X. The 3D model with an active site is shown in **Figure 11.** The ligand alpha-D-hexose-6-phosphate showed possible hydrogen bonding with ASP 116, LYS 117, ASP 152, GLU 214, GLY 216 and GLU 248 residues, as displayed in **Figure 12**.

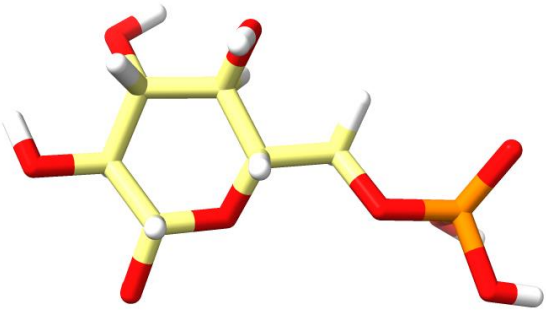

Fig. 9. 3D Structure of alpha-D-hexose-6-phosphate ($C_6H_{13}O_9P$), atoms represented by colour; yellow= C; red= O; white= H; orange= P.

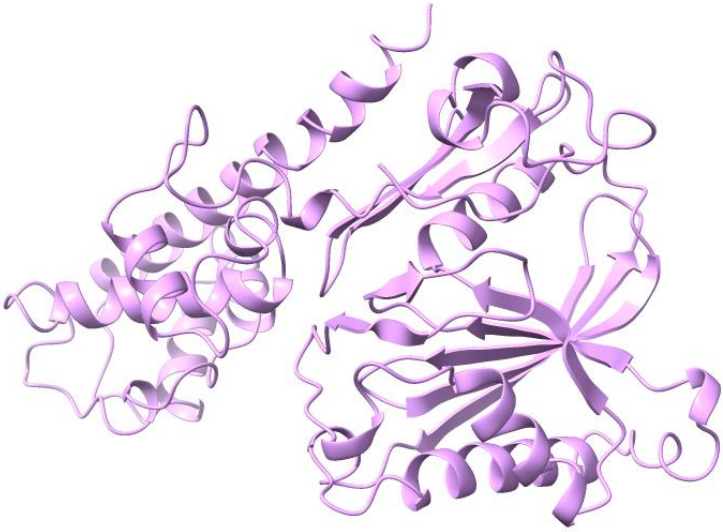

Fig. 10. 3D model of hexokinase-1 (HKX1) from P. carboxydivorans 18A13O1.

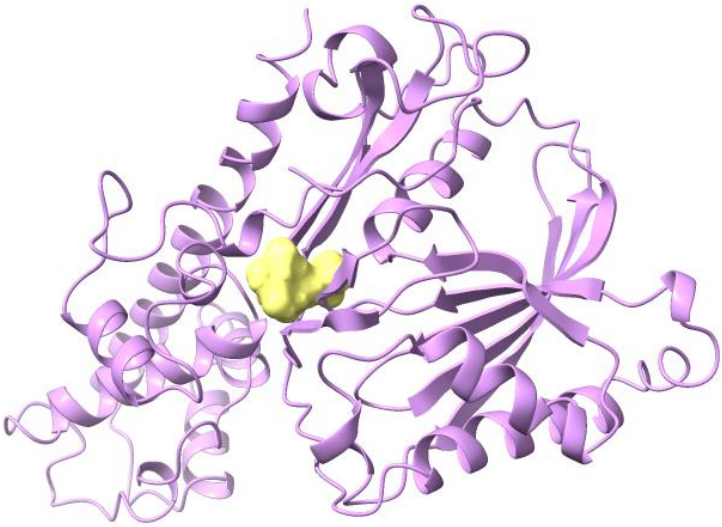

Fig. 11. Cartoon representation of the HKX1 model (purple colour) together with the predicted binding site in sphere format (yellow colour).

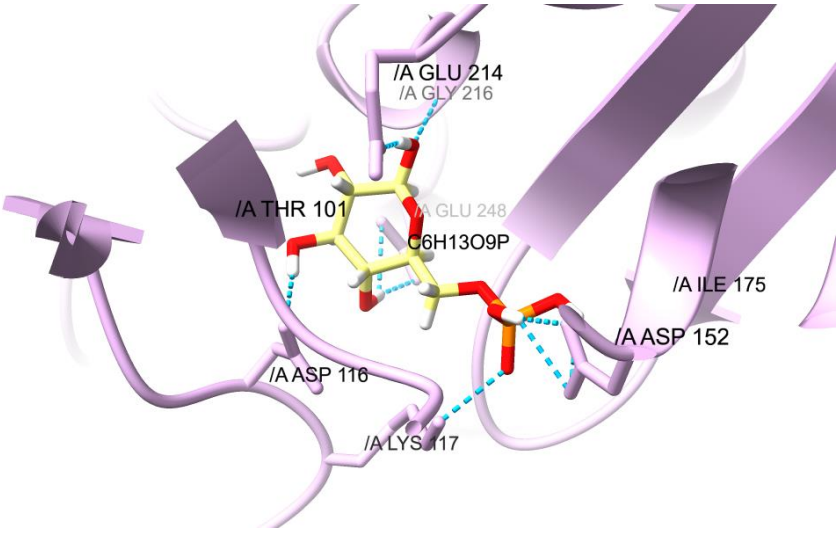

Figure 12. Interactions of (C6H13O9P) with HKX1 predicted by molecular docking. Interacting amino acid residues are shown in stick format in purple colour. The ligand is shown in yellow colour. Hydrogen bonds between protein and ligand are shown as blue dashed line.

Pseudonocardia carboxydivorans is known to be an actinobacteria, in which actinobacteria are distinguished from other prokaryotes by their G+C content, which can be as high as 70% [30]. The G+C content is a major factor that determines the amino acid composition and influences their metabolism and survival, potentially contributing to the diverse secondary metabolites they produce [11]. Metabolically, hexokinase is a key enzyme in glycolysis, converting glucose to glucose-6-phosphate for various pathways including anaerobic fermentation and oxidative phosphorylation [17].

Results of in silico tests showed that the isolated hexokinase sequence is similar to hexokinase from *Saccharomyces cerevisiae* S288C. Hexokinase from *S. cerevisiae* is known to be involved in penicillin biosynthesis. Previous research by Pérez et al. [23], *Penicillium chrysogenum* strain dogR5 mutant derived from strain AS-P-78. Both strains did not respond to glucose regulation of penicillin biosynthesis and β-galactosidase. The dogR5 strain was transformed with the hexokinase gene (HXK2) from *S. cerevisiae*. The transformants successfully restored glucose regulation of β-galactosidase, penicillin biosynthesis and acquired hexokinase activity previously absent in the dogR5 and AS-P-78 strains. The *Pseudonocardia carboxydivorans* 18A13O1 hexokinase sequence therefore has the potential to be developed for antibiotic production. This is supported by previous research by Setiawan et al. [29] who found that *Pseudonocardia carboxydivorans* 18A13O1 has antibacterial activity, inhibiting the growth of *S. aureus* resistant to clindamycin (CDM), ciprofloxacin (CFX), erythromycin (ERH), lincomycin (LC) and amoxicillin (AMX). Knowing the antibacterial potential of *P. carboxydivorans* 18A13O1 and the hexokinase that plays a role in it, hexokinase can in future be used as a target for overexpression or induced more. so that it can massively support antibacterial production in *P. carboxydivorans* 18A13O1.

4 Conclusions

In this study, the isolation of the hexokinase gene from *Pseudonocardia carboxydivorans* 18A13O1 was successfully carried out. The obtained gene sequence has a length of 1104nt. Based on the results of in silico analysis, the obtained gene sequence was confirmed to be similar to hexokinase from *S. cerevisiae* S288C, with predicted active sites located at amino acid residues ASP 116, LYS 117, ASP 152, GLU 214, GLY 216 and GLU 248. This new information is very important for further research into the application of hexokinase in the production of antibacterial compounds. Further research is needed to determine the relationship between hexokinase and the amount of bioactive compounds produced by *Psedonocardia carboxydivorans* 18A13O1.

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