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Optimization of Carbon and Nitrogen Source to Enhance Antibacterial Activity from a Sponge-derived *Bacillus tequilensis*

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Abstract

In our endeavor to find antibacterial from marine bacteria, we screened the microbiota from a Lithistid sponge, collected from Seribu Island, Jakarta. One of the promising strains was K 2.4.2. Identification of the strain using 16S rRNA Sanger sequencing resulted a sequence similarity of 100% to *Bacillus tequilensis*. Response surface methodology was applied to optimize the culture conditions for enhancing antibacterial activity of the strain. The optimization was carried out using Box-Behnken design experiment to determine the optimum carbon and nitrogen sources in order to produce maximum antibacterial activity. The optimum media with high antibacterial activity was marine broth with the addition of carbon and nitrogen concentration source of sucrose 2.15 % and peptone 1.08%, respectively. In addition, the optimum temperature was 31.18°C. The antibacterial activity response was predicted using a statistical model and compared to the activity in the experiment. The difference between the model and the laboratory experiment was less than 5% which showed a good accuracy. The results of this study once again showed that optimization method effectively reduced time, chemicals, and energy

Introduction

Marine bacteria from genus *Bacillus* are a promising source of novel active pharmacological substances with activity against MDR bacteria. Secondary metabolites produced by that genus was active in inhibiting Methicillin-Resistant *Staphylococcus aureus* (Mondol et al., 2013). These microorganisms have been reported to produce more than 200 active secondary metabolites (Mohanrasu et al.,2020; Kiran et al., 2018). For example, a marine bacteria *Bacillus tequilensis* MS145 isolated from marine sponge *Callyspongia diffusa* produced pyrrolo[1,2-*a*]pyrazine-1,4-dione, hexahydro that was active against MDR *S. aureus* with a MIC of 15±0.2 mg L^{-1} and MBC of 20±0.1 mg L^{-1} (Kiran et al., 2018). Unfortunately, most of the secondary metabolites produced by microorganisms are usually obtained in a low quantity.

To gain a better understanding of the bioactivities, absolute structure, and mechanism of action of marine bacteria's secondary metabolites, it is necessary to increase their yield. The production of bioactive compounds by marine bacteria is influenced by their nutrition and environment, with carbon and nitrogen being essential for bacterial growth and affecting the biosynthesis of primary and secondary metabolites (Ruiz

et al., 2010). The selection of carbon and nitrogen sources determines the formation of metabolites and affects the activity of certain compounds. For example, when *Bacillus licheniformis* was fed with glucose and soybean meal, it produced more bacitracin (Suzuki et al.,1988). Similarly, using glucose as a carbon source and ammonium sulfate in the batch culture of marine *Bacillus megatorium* increased the production of polyhydroxybutyrate (Mohanrasu et al.,2020).

Marine sponge was known as the treasure house of active pharmacological substances, such as antibacterial, antiviral, ancancer, antimalarial etc (Anjum et al., 2016). About 800 antibacterial secondary metabolites were isolated from the marine sponges (Tores et al., 2002). The antibacterial secondary metabolite produced by sponge some cases were claimed to be produced by associated microorganisms. The secondary metabolite biosynthesis interaction between host-symbiont also become the focus of investigation nowadays. Marine microorganisms harbored in the sponge body and lived in it, triggering issue about the origin of the active secondary metabolite.

Enhancing the associate microorganisms was more reliable than culturing marine sponge. Recenly the study was move from the sponge to the associated microorganisms. Such as our study about the evaluation of antibacterial activity showed that the bacterium *Bacillus tequilensis* isolated from *Lithistida* sp sponge inhibited the growth of *S. aureus*. *Bacillus tequilensis* was reported produced antibacterial lipopeptide (Goreishi et al., 2023). Therefore, the production of antibacterial substances was very low.this study aimed to determine the optimum antibacterial activity of the bacterium using Response Surface Methodology (RSM) based on the composition of carbon and nitrogen source.

Materials and Methods

Isolation and Culture of the Bacterium

The bacterium strain M1SP3.121015.101.a was isolated from a Lithistid sponge obtained from Untung Jawa Island, Kepulauan Seribu, Jakarta. The 1 x 1 cm of sponge voucher was washed with sterile water and dipped in sterile seawater, then stirred using sterile copper. Approximately 100 µL of sponge solution was dilluted until 10^{-4} concentration. About 50 µL of each concentration was spread on M1 media . After 3-6 days incubation at 28oC, Each colonies was transfer in each M1 media plate and purified until single colony. M1 medium was consisted of 16 g agar (Himedia), 10 g starch (Merck), 4 g yeast extract (Himedia), and 2 g Peptone (Himedia), 1 L tropical sea water, 50 mg nystatin (AppliChem). Ethyl acetate p.a (Merck) was used for bacterial extraction.

Identification of the Bacterium Using 16S rRNA

Isolation of DNA

Bacterial DNA isolation was carried out using ZR Fungal/Bacterial DNA Kit. The extraction process was carried out by inserting 50-100 mg (wet weight) of bacterial cells that had been suspended with 200 µL of PBS buffer. It was centrifuged for 1 minute at 10,000 rpm. Then 400 µL of the supernatant was loaded to the Zymo-SpinTM IV Spin column Filter. The solution was centrifuged at 7000 rpm for 1 minute. Approximately, 1200 µL of bacterial DNA binding buffer were added. The 800 µL of suspension was loaded into the Zymo-SpinTM IIC column, and the solution was centrifuged for 1 minute at 10,000 rpm. The liquid was discharged into the collection tube. The 200 µL of pre-washed DNA buffer was loaded into Zymo-SpinTM IIC column and the solution was centrifuged for 1 minute at 10,000 rpm. About 500 µL of DNA bacterial wash buffer was loaded into Zymo-SpinTM IIC column and the solution was centrifuged for 1 minute at 10,000 rpm. The suspension was transferred to 1.5 ml of a new microtube and 100 µL of DNA elution buffer was added and centrifuged for 30 seconds at 10,000 rpm. The DNA concentration and purity was measured using nanodrop

PCR for amplifying the16S rRNA gene of the bacterium was carried out using MyTaq Red Mix (Bioline) with primers of 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The PCR process was carried out using 25 µL solution that consist of 9.5 µL aquabidest, 1 µL 27F primer, 1 µL of 1492R primer, 1 µL of DNA template and 12.5 µL master mix liquid, all of reagent was added into a 0.2 ml microtube. The thermal cycler machine was run with the following temperature settings: initial denaturation at 95^{o}C for 1 minute, denaturation at 95^{o}C for 15 seconds, annealing at 52^{o}C for 15 seconds, and extension at 68^{o}C for 45 seconds. The PCR process was carried out for 35 cycles (Susana, 2017).

The purification process of PCR results using Zymoclean Gel DNA Recovery Kit (Zymo Research). DNA fragments were inserted into 1.5 ml microtubes, then 300 µL ADB was added and incubated for 10 minutes at 55^{o}C. Centrifugation process was carried out for 60 seconds at 10,000 rpm and the liquid was discarded. The 200 µL of DNA wash buffer was added to the column and centrifuged for 30 seconds at 10,000 rpm. Approximately 6 µL of DNA elution buffer was added into the column matrix and transferred the column to a 1.5 ml tub and centrifuged for 30 seconds at 10,000 rpm to elute the DNA.

The purified DNA was tested by electrophoresis on 1.2% agarose gel (to see DNA fragments). About 100 ml of tris acetate EDTA (TAE) solution was added into electrophoresis until the agarose gel is submerged. The 1 µL of DNA marker was prepared, 2 µL of PCR results were mixed with 1 µL of loading dye. The solution was

injected into the wells using a micropipette. The voltage set to 70 V for 25 minutes or until the bromophenol blue indicator moves part of the gel length. After the bromophenol blue color reached 75% of the gel length, the process was stopped and the agarose gel was immersed in ethidium bromide (EtBr) solution for 5 minutes. To stop the coloring process, the agarose gel was immersed in distilled water for 10 minutes. The agarose gel was placed on top of the UV transilluminator to observe the formed bands. Purified DNA was sent to Macrogen for Sanger sequencing.

The DNA sequences of bacterial isolates were compared with the sequences in the DNA Database Gen Bank through the BLAST system (Basic Local Alignment Search Tool) which was accessed at http://www.ncbi.nlm.nih.gov/blast. The phylogenetic three was build using clustal W in Mega-X version 10.2.6. software with neighbor joining method and 1000x bootstrap**.**

Bacterial Cultivation and Extraction

Bacterial strain M1SP3.121015.101 was maintained in M1 media. The seed culture for antibacterial production was prepared by inoculating a loopful of strain from M1 agar to 100 mL M1 broth incubated at 30^{o}C for 24 hours with shaker at 150 rpm. Approximately 10 mL of bacterial seed was transferred into 200 mL of M1 media and incubated at 30^{o}C for 72 hours with shaker at 150 rpm. The bacterial growth was determined based on OD_{600} every 24 h. The culture was harvested and extracted using ethyl acetate (Merck) with the ratio of 1:1 three times. The maceration with stirring during 24 x3 hours was applied for extraction. The ethyl acetate layer was collected and evaporated to remove the ethyl acetate solvent.

Antibacterial Assay

Antibacterial assay was performed using agar diffusion disk assay method. Gently, a loopful of *S. aureus* was put into 0,9% NaCl (Merck). The density was compared to 0.5 McFarland (1x 10^8 CFU/ml). The amount of 100 µl of the *S. aureus* was spread on the Muller Hinton Agar (Himedia). The amount of 100 µg in 20 µL of the bacterial extract was dropped on the paper disks. After dry, the paper disk were placed onto the MHA-agar medium that contained *S aureus* and incubated at 37^{o}C for 24 hours. Inhibition zone was measured as the clear zone outside the paper disk.

Optimization of Carbon and Nitrogen Source

Lactose (Pronadisa), sucrose (Himedia), maltose (Himedia), carrageenan (Oceapro), and glucose (Himedia) were used as carbon sources, while nitrogen sources were yeast extract (Himedia), peptone (Himedia), protease (Himedia), tryptone (Himedia), and urea (Merck). Response Surface Methodology (RSM) was performed to optimize the selected carbon and nitrogen nutrient value and the temperature that resulted in optimum antibacterial activity (Table 1). Experimental and statistical analysis was done using a Design Expert Software .

GasChromatography-MassSpectroscopy(GC-MS) Analysis

GC-MS analysis was carried out using Aglient Technologies 7890 Gas Chromatography with Auto Sampler and 5975 Mass Selective Detector and Chemstation data system. A 1 µL of the sample was injected into a GC-MS that was operated using a Hewlett Packard Ultra 2. Capilary Coloumn Length (m) 30×0,25 (mm) LD×0,25 (µm) film thickness. The operating condition was adjusted with an oven temperature between 70-270 °C, gradually increasing 10 degree / minute, and a pressurized Helium-carrying gas was 12 kPa, with a total speed of 30 mL/minute and a split ratio of 1:50.

Results

Bacterial Identification

The 16S rRNA gene sequence of strain M1SP3.121015.101.a showed high similarity to to the genus Bacillus. In order to find the closest relative of the bacterium, a phylogenetic tree was created in Mega X 10.2.6. version software with the Neighbor Joining method.

Phylogenetic tree was showing the relationship of strain M1.SP3.121015.101.a with several other bacterial species (Figure 1). Validation on the phylogeny tree was carried out using the Bootstrap of 1000. Based on the phylogenetic tree, strain M1.SP3.121015.101.a has a close relative with *B. tequilensis* strain K24.2.

Effect of Carbon & Nitrogen Sources on Cell Growth

The carbon source is vital in bacterial cell growth, especially for their biosynthesis of primary and

Table 1. The variable was used to optimize the fermentation condition.

Variable	Code Level		
	-1	0	1
Sucrose concentration (g/L)	10	20	30
Peptone concentration (g/L)	10	15	20
Temperature (^{o}C)	25	30	35

secondary metabolites. The former is a benefit to support their growth while the latter has a general role in their chemical defense. Therefore, carbon source in the media should be chosen concerned with the cell growth and potential antibacterial activity. Carbon sources such as lactose, maltose, glucose, carrageenan, and sucrose were applied in this study to cultivate *B. tequilensis* strain M1.SP3.121015.101.a.

Figure 2.a. shows that *B. tequilensis* grew was not significantly different, but when glucose was used as a carbon source in the culture media showed the slightly higher than others. A similar observation was also reported for the cultivation of *Bacillus* sp (Maheswaran et al., 2014).

Another factor that have significant effect to growth was nitrogen source ,as shown in Figure 2.b., various nitrogen sources were used to determine the best nitrogen source for growth cells. Similar to the previous research by Shayesteh (2014), Figure 4 describes that yeast extract increased the *B. tequilensis* growth.

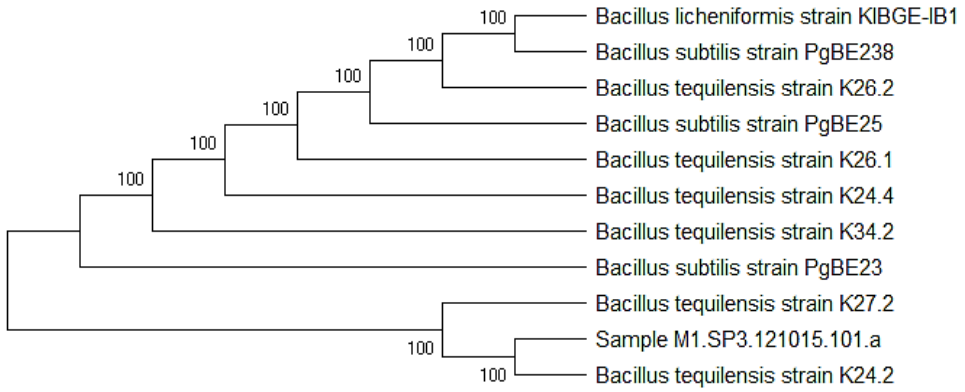

Figure 1. The phylogeny tree profile of strain M1.SP3.121015.101.a

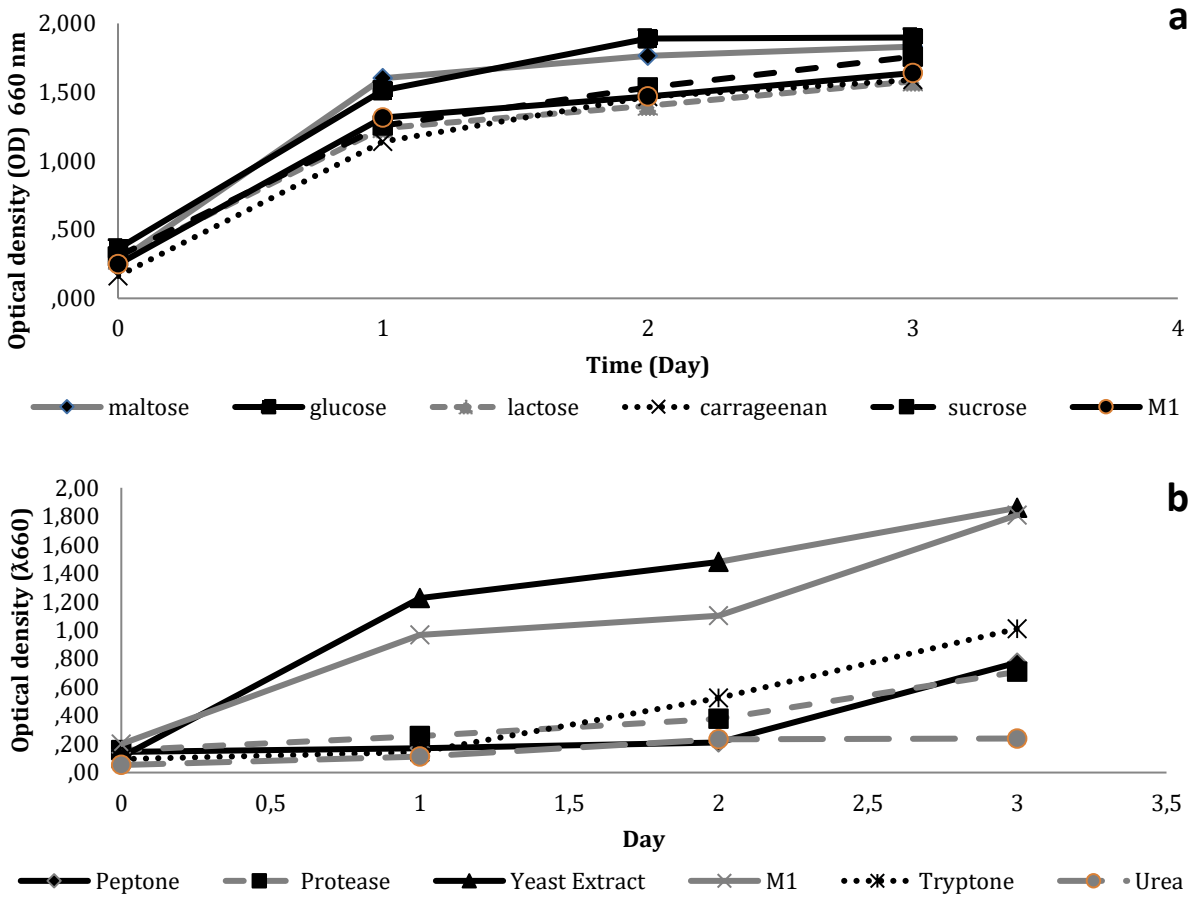

Figure 2. The cell growth of *B tequilensis* in various carbon source a)and nitrogen source (b)

Effect of Carbon & Nitrogen Source on Antibacterial Activity

Although it has essential for bacterial growth, carbohydrates often repress enzymes to produce secondary metabolites. As shown in Figure 3.a., adding a carbon source mainly resulted in the extract with slightly lower antibacterial activity than the original culture medium (M1). Even though it does not significantly increase the antibacterial activity, adding the sucrose and maltose showed a little bit higher than without those sugars (M1 medium).

Nitrogen, as a nutrient for the microbial, functions as a precursor to synthesizing amino acid, purine, pyrimidine, protein, DNA, and RNA (Vogel et al., 1997). Furthermore, it is vital in biomass growth cells and secondary metabolites such as peptides that are potent for antibacterial activity (Umeza, 1976). It was found that *B. tequilensis* grew well in yeast extract and M1 media. Nevertheless, the former media lead the growth faster than the latter. A previous study also reported that *Bacillus* sp. Sh10 could grow well in a medium containing yeast extract (Shayesteh et al., 2014). The rich nutrition of yeast extract, which contains vitamins, minerals, and amino acids, has the benefit of supporting cell growth (Li et al., 2011).

Figure 3.b. shows the effect of nitrogen sources such as yeast extract, peptone, protease, and tryptone on the antibacterial activity of the extract obtained from *B tequilensis* against *S. aureus*. It could be seen that all the used media did not have a significant effect on antibacterial activity even though peptone showed the highest activity than other nitrogen sources. The lowest activity was occurred when *B tequilensis* cultured in M1 media with added the urea as nitrogen source.

Optimization of Concentration of Carbon, Nitrogen Source and Temperature

In general, nutrition in culture media affects the cell growth of bacteria. It is required for the cell's metabolism to produce metabolites to support their lives. As such, we are optimizing the nutrient composition necessary to get the best composition for obtaining optimum cell growth with the potential antibacterial activity of the extract. In addition, some environmental factors, especially temperature, affect the growth of cells. Therefore, optimization of the

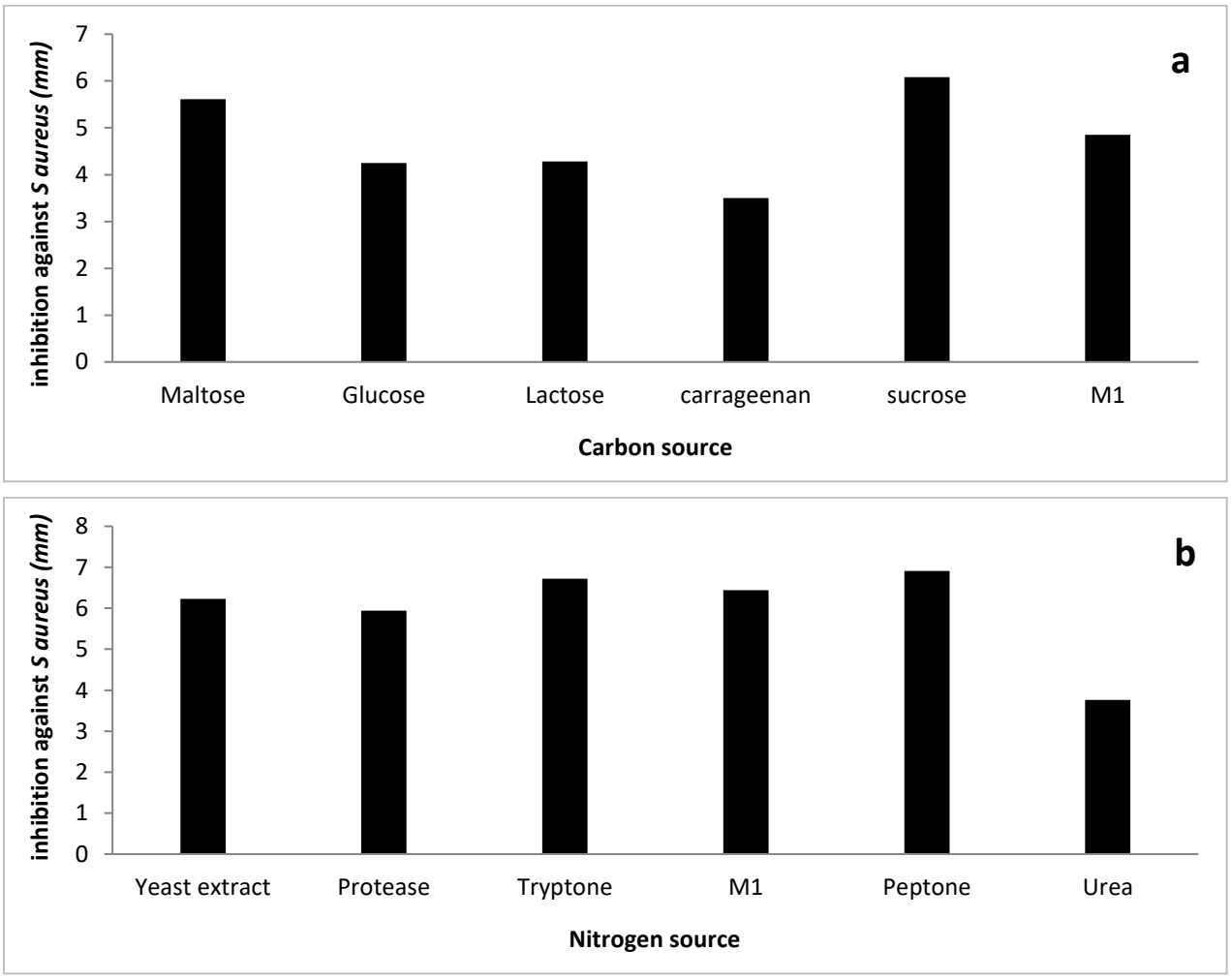

Figure 3. The anti-*S. aureus* activity of *B tequilensis* grew in various carbon sources (a) and nitrogen sources (b) after three days of cultivation

concentration of sucrose and peptone as carbon and nitrogen sources in medium culture was performed in correspond to temperature (Table 2). The range of each variable value was determined based on our previous results, and the optimization was carried out using Box-Behnken experiment design.

As shown in Figure 4, the obtained model fits the quadratic model with the high predicted-R square and the lowest PRESS number. The result indicates that the extract's optimum value of cell growth and antibacterial activity was achieved in the range of this experimental design (Figure 4).

The concentration of carbon and nitrogen sources in the media significantly affected the antibacterial activity of the obtained extract (Figure 4.A.), suggesting the possible variation of active compounds contained in the extract. However, the excess of these nutrients could also cause inhibition that resulted in the decreasing of antibacterial activity of the extract.

On the other hand, increasing temperature and sucrose concentration would enhance the antibacterial activity of the extract to a certain extent (Figure 4.B.).

Increasing peptone concentration and temperature would increase inhibition activity against *S. aureus.* (Figure 4.C.). However, there would be a decrease in inhibition activity at specific peptone concentrations and temperatures.

Validation of Predicted Model

Validation aims to prove that the optimum point solution given by Design Expert 7.1.5 software is similar to the experiment. The results obtained from the experiment were compared with the software's solution from antibacterial activity response to the predicted response with a verification value below 5%. A comparison of the results of verification with the results of calculations provided by the program can be seen in Table 3.

Tabel 2. Model Summary Statistic

Source	std. deviation	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS
Linear	0.80	0.2635	0.0936	-0.1394	13.00
2F1	0.91	0.2698	-0.1683	-1.0793	23.73
Quadratic	0.13	0.9897	0.9764	0.9468	0.61
Cubic	0.15	0.9922	0.9690		

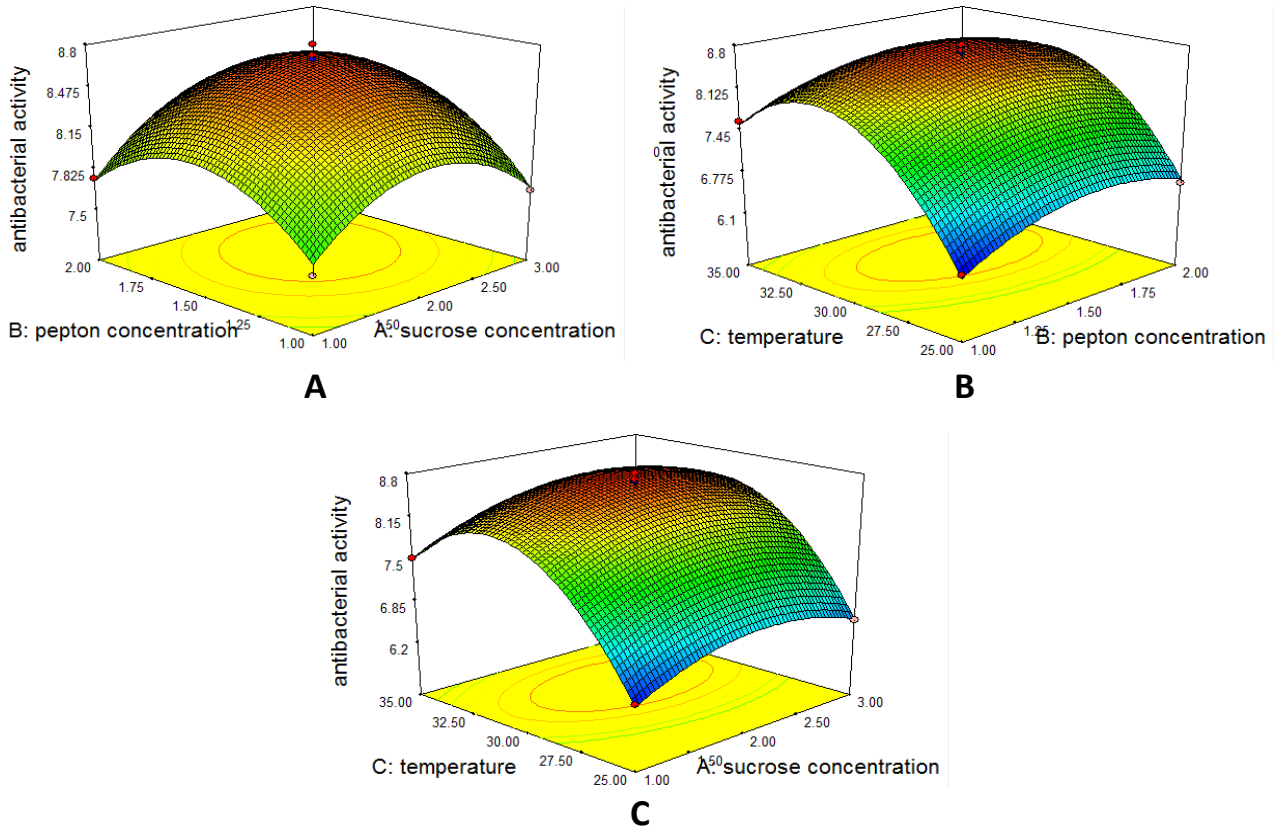

Figure 4. Curve plot between sucrose and peptone concentration (A), sucrose concentration and temperature (B), peptone concentration and temperature (C)

Table 4 showed that the predicted value from the optimum solution for the response of antibacterial activity was 8.72 mm, while the verification value was 8.85 mm. The difference in the response value of the antibacterial activity as a result of verification with the predicted results was 1.49%. The difference between the actual and predicted values was less than 5%, meaning verification and predicted values are not significantly different.

Gas Chromatography-Mass Spec (GC-MS) Analysis

Analysis of GC-MS was conducted to identify secondary metabolites produced by *B. tequilensis*. Table 4 presents that *B. tequilensis* produced phthalate and phenol. Phthalate can disturb DNA methyl transferase and methyl-CpG binding protein (Valinluck et al., 2004). Phenol compounds can damage the cytoplasmic membrane, increasing its permeability and releasing intracellular fluids such as proteins, nucleic acids, and inorganic ions (Amborabe et al.,2002). Phenol is lipophilic, making it easier to pass through microbial cell membranes and cause protein denaturation, which disrupts cell activity (Campos et al., 2016). In addition, phenol can damage the cell wall structure (Dodriquez et al.,2009).

Discussion

The molecular identifications using 16S rRNA sequencing indicated that the strain was belong to genus *Bacillus sp*. Phylogeny tree analysis showed that the strain was closed to *Bacillus tequilensis*. The profile of phylogenetic tree (Figure 1) showed that *Bacillus subtilis* and *Bacillus tequilensis* have a close relationship. Research conducted by Gatson et al.,(2006), identified the *B. tequilensis* as *B. subtilis* by using biochemical methods and sequencing analysis 16S rRNA, *B. subtilis* showed 99% similarity to *B. tequilensis*. The phylogeny tree also shows the relationship between *B. subtilis* and *B. licheniformis*. *B. licheniformis* has a close relationship with *B. subtilis* based on sequencing analysis of 16S rRNA and 16S-23S internal transcribed spacer (Xu & Cote, 2003). It is estimated that 80% of *B. licheniformis* genome sequences contain orthologous genes from *B. subtilis* (Rey et al.,2004).

Bacillus is one of the most common groups of bacteria found in marine sediments and invertebrates

Table 3. Comparison between Predicted and Actual Value

	Sucrose Concentration (%)	Peptone Concentration (%)	Temperature (°C)	Response
				Inhibition zone (mm)
Prediction*	2,15	1,08	31,18	8,72
Verification**	2,15	1,08	31,18	8,85±0,70
Residual				0,13
Error				1,49%

Table 4. The compounds detected using GC-MS containing in extracts of *B tequilensis*

carbon /nitrogen source	compound name
Sucrose	Pyridinium, 1-hexadecyl, chloride monohydrate
	1-hexadecanol
	methyl hexadecanon
	1-nonadecene
	ethyl hexadecanoate
	methyl octadecanoate
	1,2 benzene dicarboxylic acid, bis(2-ethyl hexyl) ester
	2H-pyrrole-2-on, 5-[[2-[4-aminophenyl methylene]-3,4-dimethyl-2H-pirol-5-il] methylene]-3-ethyl-1,5-dihydro-4-methyl.
	Cyclohexanol,
	2-[2-pyridyl]-5,7,9,11-tetramethyl-1,3- dihydro heptalene [1,2-C]furan-3-on
	4,4-ethylene di-m-toluidin
	2,6 dimethoxy-9,10-dimethyl-anthracene
	9-t-buthyl-2-nitro-9,10-dihydro-9, 10 anthranotracene
	hexadecyl hexadecanoate
	Piperazin,1-p-tolil-4(2,4,6,- trimethylbenzenesulphonil)
	Hexadecyl octadecanoate
	6H, 16H, 31H-5,9 15,19-dimethano-10, 14-methano-26, 30-nitrilo-5H, 25 H-dibenzo[B,S]1,21,4,8,14,18]ioctatetraazacyclooctacocine-34, 36-dion, 7,8,17,18-tetrahydro-35-methoxy-1,1,3,21,23-tetramethyl
Peptone	1,2-Benzedicarboxylic acid bis(2-ethylhexyl)ester
	Tris(2,4-ditert-butylphenyl) phosphate

(Pabel et al.,2003). *Bacillus* usually produces spores in extreme environmental conditions such as in aquatic ecosystems (Hentschel et al.,2002). In marine ecosystems, *Bacillus* is known to produce metabolites that can inhibit bacteria and fungi (Muscholl-Silberhorn et al., 2007)

The result showed that the best carbon source for growing *B. tequilensis* was glucose. The previous study reported that glucose has the benefit of accelerating the metabolism of bacteria and thus, inducing the cell's growth (Stanbury & Whitaker, 1984). This monosaccharide is also easier to digest rather than the long saccharides. In the logarithmic phase, glucose consumption will linearly increase the cell growth rate (Wang et al., 1980).

Even though glucose was the best carbon source for growth, it seems to decrease the production of antibacterial substances more than the original medium, M1.

However, using maltose and sucrose did not decrease the antibacterial activity against *S. aureus* of the extract. In this research, sucrose was the best carbon source for increasing the antibacterial activity of *B. tequilensis*. This result is similar to the previous study that presented the positive effect of sucrose on the antibiotic production of *Bacillus* sp. BH027 against *S. aureus* and *L. monocytogenes* (Zhao et al., 2014). The metabolism pathway of simple carbohydrates influences intermediates and primary and secondary metabolites (Jain & Gupta, 2012). Simple carbon sources such as fructose, sucrose, and glycerol have increased bacterial growth and secondary metabolites rather than starch, galactose, xylose, and mannitol (Calvo et al.,2002).

As described in Figure 3.b. by adding peptone in culture media showed more excellent antibacterial activity production than other nitrogen sources. In this study, peptone was chosen as the best nitrogen source that could inhibit *S. aureus* growth. Research by Supartono (2011) also used peptone as a nitrogen source in the composition of the medium for producing antibacterial compounds. This is related to the molecular chemical structure of the antibacterial compound, which is thought to contain nitrogen. Peptone contains many amino acids resulting from the hydrolysis of animal proteins. Therefore, peptone is widely used as a source of complex nitrogen in the fermentation process (Sunaryanto et al.,2011). Peptone is also rich in proline and tyrosine. It is used as a precursor for forming metabolite compounds i.e. biosynthesis of gramicidin antibiotics by *Bacillus* sp and *Pseudomonas*. Gramicidin production needs five amino acids as precursors, which are directly involved in biosynthesis (Sunaryanto et al.,2011). Therefore, the higher peptone concentration, the more likely the antibacterial metabolite is produced.

The differences between peptone as the best nitrogen source and the urea as the lowest antibacterial activity effect indicated that the variation of nitrogen would impact active secondary metabolites production by *B. tequilensis* (Gesheva et al., 2005). Some nitrogen sources can suppress gene expression related to secondary metabolite production and affect enzyme activity to produce metabolite (Jonsbu et al.,2002). These should affect the antibacterial activity of the obtained extract. The NCR (nitrogen catabolize repression) gene influenced the regulation of nitrogen sources for producing secondary antibacterial metabolites. If the nitrogen source is in excess, the transcription of the genes to transport and degrade the unfavorable nitrogen will be suppressed. When the preferred source is limited or non-existent, transcription of the genes sensitive to the NCR will be stopped, and cells will search for alternative nitrogen sources that may be available in their environment (Georis et al.,2005).

The optimization of sucrose concentration and temperature indicated that higher temperature and increasing sucrose until the concentration would increase the antibacterial activity. The temperature can induce genes to express or activate enzymes that play a role in synthesizing antibacterial metabolites (Wang et al.,2007). Raising the temperature will increase the action of enzymes in the metabolism of carbon sources. According to Stanbury and Whitaker (1984), the metabolic rate of carbon sources affects the formation of biomass and metabolites production (Standbury & Whitaker, 1984).

Optimization of the optimum concentration of sucrose as the best carbon source and peptone as the nitrogen source, as well as the temperature and pH, were described in table 3. The antibacterial zone inhibition of the *B. tequilensis* extract cultivated using the optimum condition showed no significant difference with the predicted value using the response surface statistical method. It indicated that the response surface method is beneficial for optimizing the *B. tequilensis* in producing secondary antibacterial metabolites.

The GC-MS analysis (table.4) figured out that the extract of *B. tequilensis* contained several secondary metabolites such as fatty acids, polycyclic aromatic hydrocarbon, and ketone. More than 50% of fatty acids are generally observed in the extract. These compounds have been reported to exhibit a bacteriostatic effect on disturbing the plasma membrane of *S. aureus* (Butcher et al.,1976). Fatty acids can cause membrane cells to lose potential activity because of the photophore effect, causing the lack of control of *S.aureus* in its internal pH. Furthermore, the presence of polycyclic aromatic hydrocarbon could inhibit bacteria and disturb their cell membrane permeability (Afshari, 2017). Interestingly, there is 1,2-Benzedicarboxylic acid bis(2-ethylhexyl)ester present in the bacterial extract. The previous research reported that this compound showed cytotoxic against several cell lines, PC3, MCF, HCT-116, A549, and MIAPACA (Save et al., 2015). In this study we reported that this compound also inhibit the *S aureus* growth.

Conclusion

The carbon sources suitable for cell growth are glucose, but for the antibacterial compounds production sucrose was better than other carbon source. The nitrogen source suitable for growth cells was yeast extract, while for the antibacterial compounds production peptone was the best. The optimum condition of fermentation media is produced at the point with the composition of the carbon source in the form of sucrose which is 2.15%, the source of nitrogen in the form of peptone is 1.08%, and the fermentation temperature is 31.18°C. The inhibition of *S. aureous* growth by *B.tequilensis* growth in optimum condition was 8.72 mm. Differences in predictive inhibition values with experimental data are less than 5%. The preliminary profiling of the group of active substances contained in the optimum carbon and nitrogen source were fatty acids, polycyclic aromatic hydrocarbon, and ketone. The 1,2-Benzedicarboxylic acid bis(2-ethylhexyl)ester also detected in the extract as the known active substance.

Ethical Statement

No ethical clearance was needed to running this reasearch

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Author Contribution

All authors have contributed equally as :Tutik Murniasih (TM), Nur Agustin Mardiana (NAM), Febriana Untari (FU), Joko Triwibowo (JTW) and Asep Bayu (AB). TM designed and performed experiments, analyzed GC-MS and bioassay data, coordinating the research, wrote the paper, editing and finalizing the manuscript. NAM collected data, analyzed statistical data and wrote the paper, FU was assisted the experiment, isolated bacteria, JTW identified bacterial & edited paper and AB analyzed statistical data & edited the paper.

Conflict of Interest

The authors declared that no conflict of interest regarding this publication

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