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CARBON SOURCE OPTIMIZATION FOR ANTIBIOTIC PRODUCTION FROM AAPTOS-ASSOCIATED BACTERIA *Rhodobacteracea bacterium* SP.2.11

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ABSTRACT

Marine sponge *Aaptos-aaptos* is thought to produce antimicrobial aaptamine and its derivatives. To investigate whether its associated bacteria are in fact the producer of such bioactive compounds, a study of antibacterial compounds derived from *Aaptos*-associated bacteria was conducted. In this research, approximately 10 bacterial colonies were isolated from the sponge *Aaptos aaptos*. Among the bacteria isolated, the one that showed the most potential for producing antibacterial compounds was Rhodobacteracea bacterium. Extra and intra cellular bacterial extract from this strain strongly inhibited the growth of pathogenic bacteria *Staphylococcus aureus* and *Vibrio eltor,* while were moderately effective against *Bacillus subtilis*. Optimization of antibacterial activity was conducted by culturing *Rhodobacteraceae bacterium* in various carbon sources such as glucose, lactose, amylum, molasses and glycerol. The highest production of biomass was obtained by culturing this bacteria in SYP (Seawater Yeast Peptone) medium, enriched with 1% glycerol as the carbon source and with a harvesting time of around 56-104 hours. The highest activity (8 U/ml) was reached when culturing this strain in SYP medium without any adding of carbon sources. Data analysis using a statistically tool indicated that carbon sources added to medium do not have a significant effect to antibacterial activity. Characterizing the compound responsible for the antibacterial properties will be the topic of further work.

Keywords : carbon source, *Rhodobacteracea bacterium*, *Aaptos aaptos*, associated bacteria

INTRODUCTION

Since discovering many problems in producing drugs from sponge metabolites on a large scale, researches on their associated microorganisms have flourished. Antibiotics are secondary metabolite products that inhibit pathogenic microorganisms. In particular, *Rhodobacteraceae bacterium* is a sponge-associated microorganism that has the potential to produce antibiotics (Murniasih *et al.*, 2013). However, the production of antibiotics using ordinary marine media have been very low. As extracelluler metabolites, the production of antibiotics is influenced by nutrient contained in the media. Carbon catabolisms have been shown to interfere with the production of antibiotics that are produced by the genus *Streptomyces* (Sanches *et al.*, 2010). Therefore, the optimization of carbon source may be very important to enhance the antibiotic production of *Rhodobacteraceae bacterium*.

Several carbon sources can be used to optimize antibiotic production. Glucose has been shown to be the best carbon source to

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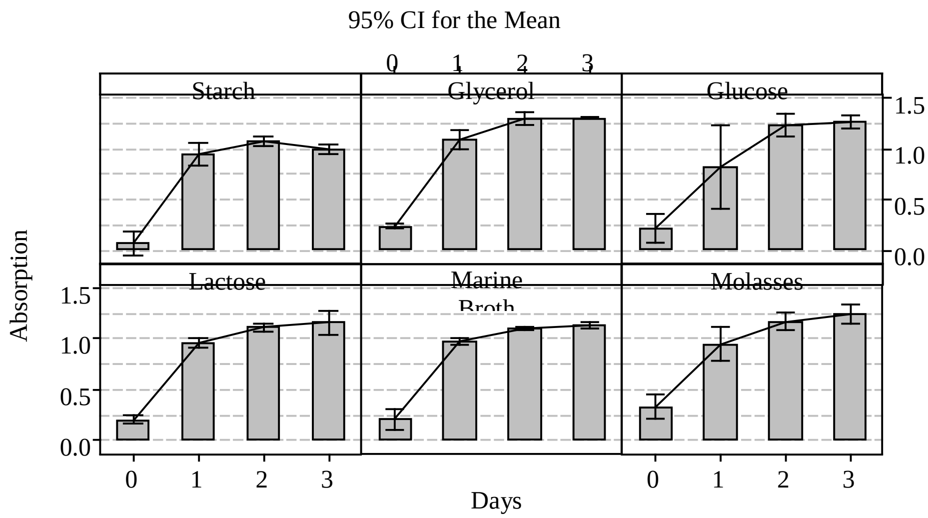


Figure 1. The growth curve of R. bacterium in various carbon sources with Internal Plot of absorbance at 600 nm

produce the poliketide antibiotic from *Streptomyces psammoticus* (Sujatha *et al.*, *2004)*. Glucose is also the central carbon for producing antibiotic actinorhodin and undecyl prodigiosin in *S. lividans* (Butler *et al*., 2002). Glucose is converted to Glucose-6-phosphat and induces the glycolisis process. Acetil CoA and amino acids produced in glycolisis are the precursor for secondary metabolite compounds. The Acetil CoA is converted to malonyl CoA. By using the enzymatic process in microorganism/ organisms, some isoprenoid compounds are produced. Through intermediate biosyn-thesis, amino acid as shikimic acid and alkaloid precursor are produced (Butler *et al.*, 2002). The production of antibiotics by *Cephalosporium acremonium* increases when glucose as a carbon source is exhausted (Kennel and Demain, 1987). Not only small saccharide but also polysaccharide could induce the antibiotic production. An early study reported that the production of penicillin using oligo and polysaccharide as carbon sources is better than glucose (soltero and Johnson, 1953 in Demain and Solomon, 1983). Very significant effects on antibiotic production by regulating the carbon source were reported in detail by Konig *et al*. (2005).

Optimizing the carbon source for the production of *Rhodobacteraceae bacterium* has not been reported before. Therefore, the aim of this study is to find the optimal nutrient, especially the carbon source, for antibiotic production.

MATERIALS & METHODS

The medium used for culturing *Rhodo-bacteraceae bacterium* in this study was sea water peptone bacterium broth (SYP) that consisted of 5g peptone (Himedia), 1 g yeast extract (Himedia), 1 g $Na_2HPO_4.12H_2O$ (Merck), 15 g agar (Frisconina) in 1 L fresh seawater. The carbon sources used for nutrient enrichment were glucose (Himedia), lactose (conda pronadisa), glycerol (Applichem), starch (Merck) and natural molasses. The nitrogen sources used were tryptone, soybean, urea, and protease. The organic solvents used in this research were ethyl acetate p.a. (Merck), acetone p.a (Merck), methanol p.a (Merck). The equipment used included visible spectrophotometry (Shimadzu), laminar air flow, incubator, centrifuge (Biofuge) and shaker.

Screening Variable for Carbon Source

The carbon sources used for variable screening were glucose, maltose, lactose, molasses, glycerol and amylum. Exactly 1 g of each carbon source was added into a 90 mL medium SYP that was placed on a 250 mL erlenmeyer and followed by sterilization using an autoclave. Each solution was then mixed and inoculated with 10 mL of R. bacterium. The cultures were incubated at room temperature for 3 days in a shaker incubator. The experiment was carried on triplicate. The response of each variable was observed for growth and bioactivity data. Figure 1 describes the experiment on carbon source variation.

Bacterial Extraction

Rhodobacteraceae bacterium used in this research was isolated from *Aaptos* sp. collected from Barang Lompo Island, South Sulawesi in May 2013. Approximately 2 loops of *R. bacterium* colony were added to 30 mL of sterile MB medium and incubated at room temperature for 2 days, while agitated at 110 rpm. The initial concentration of bacteria was equal to 10^4 cell/mL. The growth was determined by measuring the turbidity of the solution using a spectrophotometer at λ=600 nM. About 5 mL of the culture solution was taken every day during a 4-days incubation period to measure their turbidity and bioactivity. The negative control used was Marine Broth medium without any inoculating *R. bacterium*.

The evaluation of antibacterial activity was applied to the pellet and supernatant extracts of *R. bacterium*. Approximately 5 mL of bacterial broth was centrifuged (4^{o}C, 6000 rpm, 10 min) to separate the biomass (pellet) from the supernatant. The pellet was extracted using 3x 5 mL acetone, while the supernatant was extracted using 3x 5 mL ethyl acetate. The organic solvent was removed from extracts using a rotavapor. The extracts were measured and dissolved in 100 μL methanol.

Antibacterial assay

The bacterial activity test was carried out using the agar diffusion method (Bauer, 1996). Approximately 20 µL of extract was dripped onto a paper disk laid on the nutrient agar medium containing 10^5 cells of pathogenic bacteria (turbidity equal to macfarland solution). The pathogenic bacteria used in this study were wild strain of *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Vibrio eltor* collected by Microbial Lab of the University of Indonesia. As a positive control, the antimicrobial suceptibility test-disc contained 10 µg of ampicillin was used. The cultures were incubated overnight at 30° C. The diameter of inhibition was measured using calipers. The value of activity was adjusted to the Maxwell methode.
Bioactivity (Unit/mL) = diameter of inhibition - diameter of paper disk in 1 mL extract (Maxwell *et al.*, 1994).

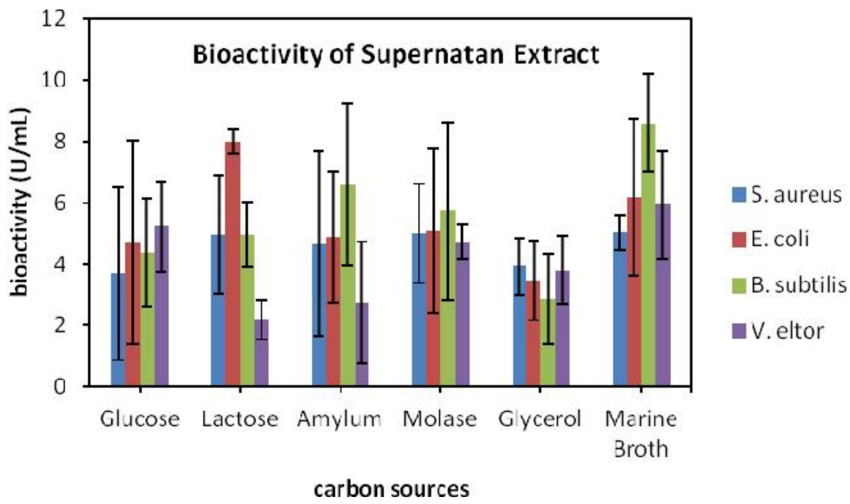
Data analysis

Experimental research design and statistical analysis were performed using Minitab® 16 software.

RESULTS

The Growth of Bacterial Cells

The curve growth of *R.Bacterium* is shown in Figure 1. The carbon source addition to the SYP medium has a significant effect (p-value < α = 5%) on the biomass of *R.bacterium.* The growth of *R. bacterium* in SYP broth without carbon source reached a maximum after 3 days fermentation. The presence of starch showed to decrease cell production. Based on the value of confidence interval (CI), glucose, lactose and molasses did not deliver a result that was significantly different from the original SYP medium, nor did these three saccharides significantly increase *R. bacterium* growth. Increased biomass was shown when the SYP broth was enriched with glycerol. Glycerol gave a significant effect for increasing bacterial cell after 2 days fermentation.



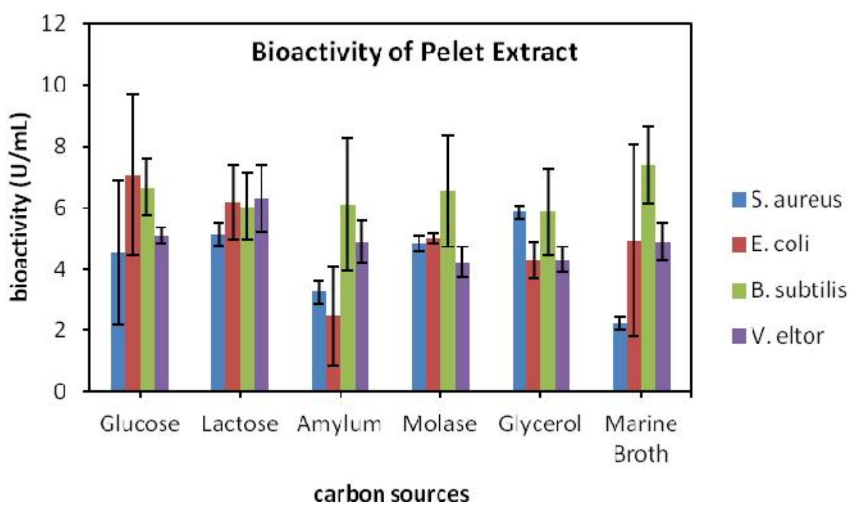


Figure 2. The profile of antibacterial activity by culturing *R. bacterium* in various carbon sources (a. supernatant extract; b. pellet extract)

Data shown in Fig. 1 was measured at the average value of three replicates with the confidence interval at 95% and confidence level (α = 5%). The ANOVA results also illustrate that the effect of fermentation duration was significant (p-value < α = 5%) on the growth of *R. bacterium*. In this case, the growth of the *R. bacteria* took place at log phase until the first day of fermentation, and continued to a stationary phase starting on the second day of fermentation. The best carbon source for increasing the biomass of *R. bacterium* was glycerol (Fig 1.), followed by glucose and molasses.

The Evaluation of Antibacterial Activity

The evaluation of antibacterial activity of pellet and supernatant extracts of *R. bacterium* indicated antibiotic activity against all pathogenic bacteria tested. Figure 2 shows the profile of antibiotic activity of *R. bacterium* cultured in various carbon sources. The supernatant extract (Fig.2a) showed high activity when *R bacterium* was grown in SYP (Marine broth) medium without any carbon source. The highest activity was against *Bacillus subtilis* with the number of activity >8 U/mL. Activity against *S. aureus*, *E. coli* and *V. eltor* were also high in *R. bacterium* culture without being enriched with other carbon source. The second model was shown when *R. bacterium* culture in SYP was enriched with lactose. The anti-*B. subtilis* was highest in this medium with the number of activity 8 U/mL. Generally, if we consider all of phatogenic bacteria, the better model for bioactivity substance production of extracellular was in SYP medium without enrichment with carbon source.

The bioactivity profile of pellet extract is shown in Figure 2b. The highest anti-*Bacillus subtilis* (8 U/mL) showed when culturing *R. bacterium* in SYP (marine broth) without adding carbon source. Adding glucose and lactose also gave high activity for almost all pathogenic bacteria.

Based on the ANOVA results (see supporting data), it can be seen that the CI value of glycerol is significantly different with others carbon sources. Moreover, it shows the highest result than others either in 600 nm or 680 nm turbidity measurements. Hence, glycerol can be a good nutrient for *R. bacteraceae*. In these context, the ANOVA assumptions were acceptable since the residuals are normally and independently distributed with a mean zero and variance σ^2, NID(0, σ^2).

DISCUSSION

The productivity of *R. bacterium* when amylum was used as carbon source was less than in the original marine broth medium, probably because amylum has large molecules and low solubility in a seawater medium. The bacteria need more energy when using amylum as a nutrient. Considering the value of confidence interval, a long chain carbon source like amylum gives a lower growth than short chain sources. *R. bacterium* finds amylum very difficult to digest and it provides less energy. In general, mono- or disaccharide carbohydrate is an effective source of energy to support the metabolism of microorganisms (Vogel and Todaro, 1997). Therefore, the short chain carbon sources are an ideal nutrient for the growth of *R. bacterium*.

The glycerol produced the highest relative growth with CI values significantly different from the other carbon sources. Although not common, some microorganisms may use glycerol as an energy source for growth (Wendisch, Lindner and Meiswinkel, 2011). Therefore, glycerol is a good carbon source of nutrients to increase the growth of *R. bacterium*.

The highest antibiotic production was achieved by culturing *R. bacterium* without adding the carbon source. Several antibiotics were produced when the bacterium was in a stressed condition as a result limited nutrients, high salinity or high pressure (Demain *et al*., 1983; Doul & Vining ,1990; Sanches & Demain, 2002 dalam Gesheva *et al*., 2005)

The presence of glycerol in the medium culture could increase growth rate. Otherwise, the production of antibiotic compounds was not significantly affected, whether a carbon source was added or not. These additions had no significant effect (p-value> α = 5%) on the bioactivity of the extracts. This data indicate that glycerol induced the growth of *R. bacterium* in the log phase, but after the stationery phase glycerol or other carbon source did not affect the production of antibiotic compounds.

It could be concluded that the selected carbon source for increasing biomass production was glycerol. Although the bioactivity test showed no significant effect on antibiotic production, it could increase antibiotic productivity by increasing biomass concentration.

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