Increasing of Carotene Production from Local Isolate when Using Additional Supernatant from Bacterial Culture in their Growth Medium

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Abstract
Screening of quorum sensing (QS) quencher takes many attentions because of resistances problems in the using of antibiotics to kill pathogenic bacteria. There are several reports that QS mechanism controls more than one metabolism that not very closes relatively each others. Because of these reasons research in finding of quencher molecule should take into account about side effect of this molecule. *Chromobacterium violacein* Tn5 and lasR-lasI-GFP plasmid recombinant that uses in the QS research seems to be difficult to detect side effect on using QS quencher molecule, because of engineering on their DNA make the reporter gene express higher than the normal gene, this make difficulties to observe other gene which is effect by quencher molecule. In this research we used wild type local isolate of bacteria that produce carotene. We also reported that carotene production in our isolate was driven by quorum sensing. Adding of supernatant from bacterial culture in bacterial growth medium until 25; 50 and 75% gave increasing of carotene production until 64; 50 and 50%. Extracting supernatant from bacterial culture using ethyl acetate and adding to the growth medium until 40; 50 and 60%, gave 31; 40 and 22% increasing of carotene production. In this research we reported that adding of supernatant or ethyl acetate extract supernatant from bacterial culture also decreasing cell mass production. Based on this data we concluded that increasing of carotene production causing by increasing of carotene production per cell not by increasing of number of cells.
Keywords: *Quorum sensing, carotene, local isolate.*

INTRODUCTION

Quorum sensing is communication mechanism among bacteria to regulate their metabolite through the environmental condition. Quorum sensing also used as defend mechanism against other bacteria or host immunity in the pathogen bacteria.

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Recent researches in pathogen quorum sensing tend to explore novel substances that inhibit this quorum sensing mechanism. Auto inducer molecules in quorum sensing become a target to find quorum quenching substances.

There are two well known reporter that used for quorum sensing research, *GFP/lux* gene that conjugated with lasR and las I and *Chromobacterium violacein* that mutated using transposon. These two reporters are recombinants that allow high expression of protein/substances reporter, while another protein in the host cell expressed in the normal condition.

Violacein molecule as reporter in *Chromobacterium violacein* will be not produce when this bacteria grown in the complex media. This fact shown that this pigment is not important for the bacteria growth or defense. Violacein is important in respiration process, in the regulation of tryptophan synthesis and take a role in the defend mechanism against UV radiation.

These function above will be disturbed when auto inducer (AI) molecule inhibited by quorum quenching molecule that researcher try to find. Based on this fact, it is important to investigate another function in the cell that will be impact when using quorum quenching molecule. High expression of protein molecule in the reporter will inhibit us to observe another protein that may impact, because level of expression of reporter molecule very sensitive with quorum quenching concentration in the media, while level of expression normal molecule will react in the normal condition. Because of this reason we have difficulties to observe of another effect of quenching molecule when using recombinant reporter.

This research had a goal to find natural reporter in the quorum sensing research. Hopefully this natural reporter will be detect the entire gene that impacted by quorum quenching molecule. In the previous research we isolated local bacteria producing carotene. (Emantoko *et al.*, 2005). This local isolate can produce astaxanthin until 8.98 ng/ml in the growth culture. Our studies showed this isolate was

Pseudomonas sp. As one of the secondary metabolite, carotene production can be determined by quorum sensing mechanism (Demain *et al.*, 1998). Research by Whiteley *et al.* (1999) showed that zeazanthin production, one of the carotene group, determine by AI concentration in the growth media.

MATERIALS AND METHODS

Microorganisms: Local isolate bacteria (*Pseudomonas* sp.) used in this research was obtained from previous research (Emantoko *et al.*, 2005). *Escherichia coli* XL1 pSB1075 as a biosensor was from Institute of Infection, Immunity and Inflammation, School of Molecular Medical Sciences, Centre for Biomolecular Sciences, Nottingham University, United Kingdom.

Growth of bacteria in the supernatant medium and ethyl acetate extract supernatnat medium: Supernatant medium was fresh medium containing supernatant obtained from *Pseudomonas* sp. culture. This supernatant was 7 days supernatant culture of *Pseudomonas* sp. sterilized by membrane filtered 0.2 μm. Supernatant medium was prepared by adding fresh medium to old supernatant until concentration of 25, 50 and 75%. Ethyl acetate extract supernatant medium was prepared in the same method with extraction of old supernatant using ethyl acetate. One percent of starter culture was then added to these medium.

Carotene isolation: Three mililitres of bacterial culture was centrifuged at 10,000 rpm. Pellet cell was then harvested by adding methanol:chloroform (2:1) and shaked at 3000 rpm for 30 min. After centrifuge, supernatant was then prepared for spectrophotometer at 480 nm.

RESULTS AND DISCUSSION

When *Pseudomonas* sp. growth media was mixed with it's supernatan of culture, carotene production increased to certain level, specially in the early culture grown. Table 1 shows that carotene production increased to 64.28% for *P. aeruginosa* grown in the media containing 25% supernatant, while growth media containing 50 and 70% supernatant increased carotene production 50%. Our research also showed that carotene in the media with supernatant was produced faster than media without supernatant. Despite play a role to increase amount of carotene produce by local isolate, adding supernatant in the growth media also changed time to produce maximal carotene as shown in Table 1.

Table 1. Time to produce maximal carotene.

Curve supernatant concentration	Pigment	
	Time (h)	A_{480nm}
Supernatant 0%	173	0.42
Supernatant 25%	213	0.69
Supernatant 50%	173	0.63
Supernatant 75%	221	0.63

Table 1 shows that carotene production was smaller in the media containing supernatant in the high concentration than carotene production in the media containing low concentration of supernatant. *P. aeruginosa* culture in the 25% supernatant containing growth had highest carotene production, while culture in the 75% supernatant had smallest carotene production. This result shows that supernatant contained some substances that inhibited carotene production to certain level. Another researcher also showed that supernatant contains some inhibitors, that operate in the end of lag phase or early stationer phase. The inhibitor substances will inhibit the function of AI (Withers & Nordstrom, 1998; Dong *et al.*, 2005).

To eliminate inhibitor in the media, we extracted the supernatant using ethyl acetate. Amount of supernatant: amount of ethyl acetate used was 2:3 and 1:1. The influence of supernatant extract in the amount of carotene production and time to produce is shown in Table 2.

Table 2. Maximal carotene produce and time to produce it.

Amount of supernatant : amount of ethyl acetate	Time (h)	A_{480}
Normal	165	0.4585
1:1	125	0.6415
3:2	149	0.561
2:3	125	0.6015

Table 2 shows that extract supernatant increased carotene production. The carotene production increased to 40%, in the media containing supernatant extract came from 1:1 (supernatant : ethyl acetate) extraction. The second larger increasing in carotene production was 31% resulted in growth media containing supernatant extract 2:3 and the third increasing to 22% came from supernatant extract 3:2. The phenomena shown that auto inducer molecule in the supernatant culture was already extracted by ethyl acetate (Pearson *et al.*, 1994).

In the extraction using 2:3 (supernatant : ethyl acetate), not all auto inducer in the supernatant could be extracted, because of this reason carotene

production in the media containing supernatant extract in the 1:1 extraction, had more carotene than culture in the media containing 1:1 extract of supernatant. More increase amount of supernatant in the 3:2 extraction could not increase carotene production, this because of ethyl acetate that remained in the culture media inhibited bacterial growth. This reason told us that decline in the carotene production from 3:2 extract to 1:1 extract because of amount the bacteria in the 3:2 extract was less than 1:1 extract.

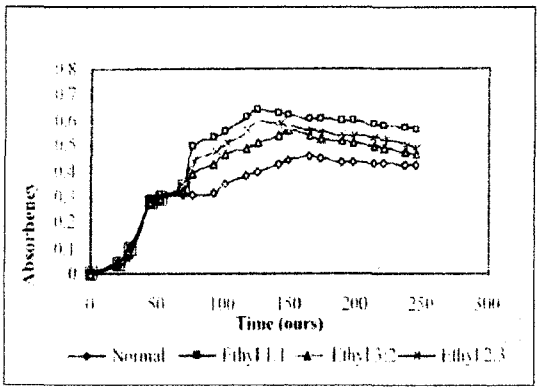

Fig. 1. Carotene production in the media containing ethyl acetate extract.

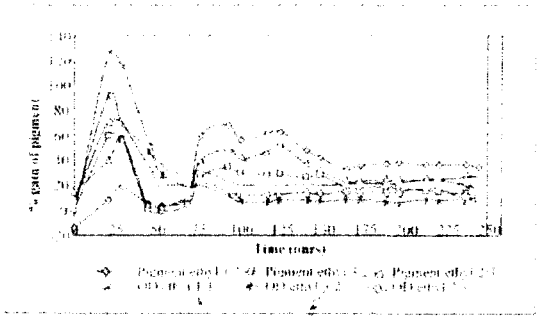

Fig. 2. Increasing amount of cell and amount of carotene when adding ethyl acetate supernatant in their growth media.

To answer whether the increase in the carotene amount because of the increase of cell member or carotene production, we do another research. We observed the amount of carotene and member of cells during bacterial growth. As shown in the Figs. 1 and 2, from starting growth until 60 h, increasing amount of cells was followed by increasing amount of carotene. There were no carotene production increase until 60 h after bacterial growth. From 60 to 125 h of growth, there was no amount of cell increase, but the carotene amount increased in these experiment. This means that carotene production is increase. Auto inducer in the growth media resulted from ethyl acetate supernatant extract already given their influence to increase the carotene production in the bacteria. Research done by Demain (1998) also gave the same result. A 125 h after bacterial growth, there was decline in the carotene production, because all nutrient in the media was already consume by bacteria and auto inducer would be also consumed in the end. Because of decreasing auto inducer in the growth media, carotene production decreased too (Roche *et al.*, 2004). In conclusion that carotene production in the local isolate was induced by ethyl acetate extract supernatant.

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JOURNAL of APPLIED AND INDUSTRIAL BIOTECHNOLOGY in TROPICAL REGION

ISSN: 1979-9748
http://www.biotechindonesia.org

ISSN 1979-9748
9 771979 974814

JOURNAL of APPLIED AND INDUSTRIAL BIOTECHNOLOGY in TROPICAL REGION Vol.1, 2008 (special edition)

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