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Chemotactic Motility and Growth of *Pseudomonas fluorescens* towards Glucose Concentration

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Pseudomonas fluorescens is plant growth promoting rhizobacteria (PGPR) often inoculated on plants as natural biocontrol agent capable of protecting the plants from soil-borne pathogens. Chemotactic motility allows populations of *P. fluorescens* to rapidly search for nutrients and is an important factor determining their competitive success to colonize plant root. Therefore, we investigated various glucose concentrations from 0% to 1% (w/v) to enhance chemotactic motility and growth of this rhizobia. Chemotactic motility was evaluated using swim plate assay and bacterial growth was measured using UV-Vis Spectrophotometer in LB and M9 medium. Glucose with low concentration (0.05%) showed to have optimum response in *P. fluorescens* chemotactic motility with colony diameter 38.3 mm in LB medium and 12.8 mm in M9 medium. Highest growth of *P. fluorescens* was seen in control condition of LB medium reaching a peak at 0.0246 OD_{600} (~±1.44x10^7 CFU mL^{-1}) while growth in M9 medium supplemented with 1% glucose was just slightly lower with 0.0227 OD_{600} (~±1.32x10^7 CFU mL^{-1}). Glucose in high concentration showed to repress chemotactic motility and first growth phase of *P. fluorescens* in LB medium due to catabolite repression.

Key words: catabolite repression, chemotactic motility, glucose, PGPR, *Pseudomonas fluorescens*

Pseudomonas fluorescens adalah bakteri PGPR yang sering diinokulasikan ke tanaman sebagai agen biokontrol alami yang mampu melindungi tanaman dari serangan patogen pada tanah. Pergerakan kemotaktik memungkinkan populasi *P. fluorescens* mencari sumber nutrisi dan merupakan salah satu faktor penting yang menentukan keberhasilan bakteri dalam mengkolonisasi akar tanaman. Glukosa diketahui sebagai kemoatraktan paling kuat bagi *P. fluorescens*. Oleh karena itu dilakukan pemeriksaan mengenai konsentrasi glukosa yang dapat meningkatkan pergerakan kemotaktik dan pertumbuhan bakteri rhizobia ini mulai dari konsentrasi 0% hingga 1% (w/v). Pergerakan kemotaktik dievaluasi melalui uji *swim plate* dan pertumbuhan bakteri diukur dengan Spektrofotometer UV-Vis pada media LB dan M9. Glukosa dengan konsentrasi rendah (0,05%) menunjukkan respons yang paling baik terhadap pergerakan *P. fluorescens* dengan diameter koloni sebesar 38,3 mm pada media LB dan 12,8 mm pada media M9. Pertumbuhan *P. fluorescens* paling baik terjadi pada perlakuan kontrol di media LB dengan absorbansi 0,0246 OD_{600} (~±1,44x10^7 CFU mL^{-1}) sementara pertumbuhan pada media M9 dengan glukosa 1% hanya sedikit lebih rendah yakni 0,0227 OD_{600} (~±1,32x10^7 CFU mL^{-1}). Glukosa dalam konsentrasi tinggi mampu menghambat pergerakan kemotaktik dan fase awal pertumbuhan *P. fluorescens* pada media LB karena adanya represi katabolit.

Kata kunci: represi katabolit, pergerakan kemotaktik, glukosa, PGPR, *Pseudomonas fluorescens*

The rhizosphere harbors a vast number of prokaryotic and eukaryotic organisms that interact and compete with each other and with the plant root, thus affects the plant's health. *Pseudomonas fluorescens* is one of the best colonizing group of PGPR (*plant growth promoting rhizobacteria*) often used as a research model for the study of root colonization and is inoculated on plant as biocontrol agent replacing the application of chemical fertilizer, in order to protect the plant from soil-borne pathogens (Capdevila *et al.* 2004; De Weert *et al.* 2002 and Vicario *et al.* 2015). However, successful inoculation of *P. fluorescens* in establishing a symbiotic relationship with the plant root is determined by the motility of the bacteria to colonize the plant root's surface.

Chemotaxis is the ability of motile bacteria to find source of nutrients that can be used to support growth. Which means that bacterial chemotaxis and growth play very crucial role in the process of root colonization. Glucose is known as the strongest chemoattractant that can induce *P. fluorescens* chemotactic motility among other forms of sugar (Arora and Gupta 1993). However, previous studies mentioned that glucose in high concentration could inhibit chemotaxis and growth of *E. coli* (Dobrogosz

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and Hamilton, 1971; Madigan *et al.* 2015) and motility of *A. hydrophila* (Jahid *et al.* 2013), due to the catabolite repression phenomenon. Meanwhile, the effect of glucose concentration on motility and growth of *P. fluorescens* needs further investigations. This research is aimed to study optimum concentration of glucose required for the induction of chemotactic motility as well as increasing the growth of *P. fluorescens*.

MATERIALS AND METHODS

Bacterial Strain. The strain used for the study was *Pseudomonas fluorescens* (FNCC 0070) obtained from Center of Food and Nutrition Studies, Gadjah Mada University (CFNS, Yogyakarta, Indonesia).

Culture Media and Growth Conditions. The media used for the chemotaxis and growth assay were LB (10 g L^{-1} casein enzyme hydrolysate, 5 g L^{-1} yeast extract, 10 g L^{-1} sodium chloride) and M9 medium (12.8 g L^{-1} $Na_2HPO_4.7H_2O$, 3 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NaCl, 1 g L^{-1} NH_4Cl) containing various glucose concentrations (0%, 0.05%, 0.2%, 0.5%, 1%) (w/v). Chemotactic motility assay was carried out in growth medium fortified with 3 g L^{-1} *Bacto agar*. *P. fluorescens* was then subcultured into 10 mL of LB for 18-20 hours of incubation in 30ºC. Bacteria were incubated in aerobic conditions with shaking overnight at 125 x g prior to the day of activity.

Motility Assay. Chemotactic motility assay was performed using swim plate method as described by DeLoney-Marino (2013) following the design by Kearns and Losick (2003) with slight modifications (Figure 1). For motility assay, 1.5 L aliquots of *P. fluorescens* from overnight cultures that had reached 1.7 OD_{600} (equivalent to 10^9 CFU mL^{-1}) were spotted at the center of plates containing ±25 mL LB or M9 with bacto agar and various glucose concentrations. Petri plates were incubated in 30°C for 6 hours. The assay was performed in triplicate. After incubation, the diameter of the area of motility of the strain was measured, and the plates were photographed.

Bacterial Growth Measurement. 100 L aliquots of *P. fluorescens* cultures 0.5 OD_{600} (equivalent to 10^7 CFU mL^{-1}) that had previously diluted (2:9) were inoculated into Erlenmeyer flasks containing 13 ml of LB or M9 with glucose. Effect of glucose concentration towards bacterial growth was measured using UV-Vis Spectrophotometer with optical density (OD) at 600 nm (OD_{600}). Measurement was performed every 1 hour with 3 hours of total incubation period. The result was visualized in a bacterial growth curve.

Glucose Utilization during Bacterial Growth. Erlenmeyer flasks containing bacterial cultures from growth measurement activity were centrifuged at 10.000 x g in 30°C for 10 minutes. The supernatant was obtained using a sterile syringe and stored at 4°C until its analysis. Analysis of the amount of glucose consumed by the bacteria was performed using titration method with Benedict Quantitative solution and estimated based on Frais' conversion factor (Frais 1972).

Statistical Analysis. All experiments were performed using a randomized design. For chemotaxis experiment, the data were subjected to one-way analysis of variance (ANOVA) with multiple comparison variables by least significant difference (LSD) test. While the data of bacterial growth measurement were subjected to two-way ANOVA with comparison of means using Tukeys test. Differences between means were considered to be significant at $p \leq 0.05$. Data analysis and graph visualization were presented using software SPSS v.23.

RESULTS

Chemotaxis is a directed movement of bacterial swimming motility towards chemoattractant in growth medium. The movement of bacteria which induced by glucose would create a colony phenotype as seen in halo zones in Fig 2. *P. fluorescens* migrated chemotactically away from the point of inoculation towards another end of the uncolonized agar, which was still rich in nutrients. Optimum concentration of glucose to induce chemotactic motility of *P. fluorescens* was 0.05% with colony diameter 38.3 mm. The colony expansion started significantly decreased as glucose concentration was raised from 0.2 % (34.5 mm), 0.5% (19.3 mm) and 1% (21.2 mm) respectively. The colony diameter in control plate was 37.1 mm. Glucose concentration above 0.05% in LB medium had shown significant effect ($p < 0.05$) in reducing colony diameter of *P. fluorescens* (Fig 4).

While addition of glucose on M9 medium didn't show any significant effect in the reduction of *P. fluorescens* colony diameter (Fig 3). Colony diameter started to decrease in 0.2% concentration, but variation of glucose concentrations didn't show significant differences in each treatment ($p > 0.05$). Mean diameters of swimming motility in M9 medium were 0% (10.7 mm), 0.05% (12.8 mm), 0.2% (10.8 mm), 0.5% (9.6 mm), and 1% (8.3 mm) respectively (Fig 5).

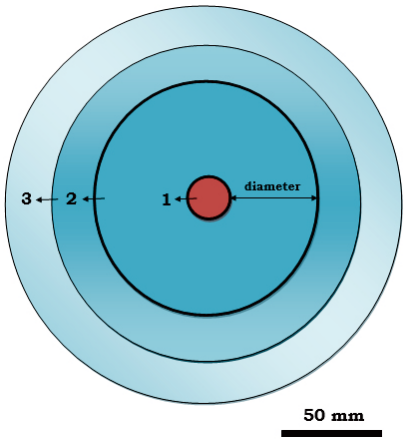

Fig 1 Modified design of chemotaxis assay using *swim plate* method in *Bacillus subtilis* (Kearns and Losick, 2003). 1) inoculation zone, 2) colony expansion zone, 3) uncolonized zone.

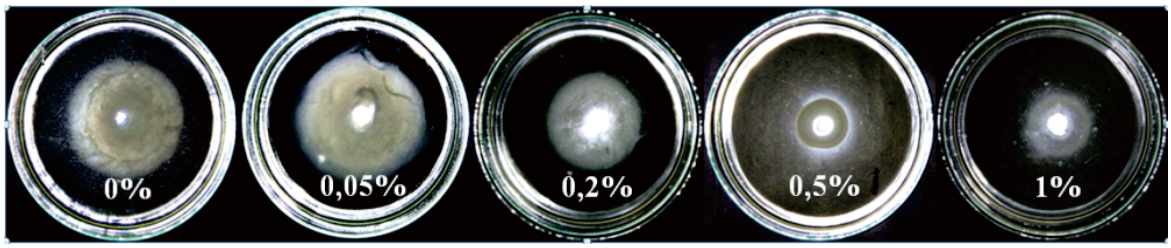

Fig 2 Chemotactic motility of *P. fluorescens* (30ºC) in LB medium after 6 hours incubation.

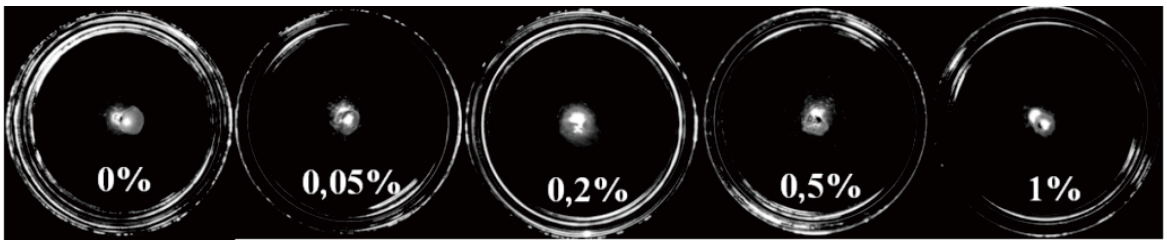

Fig 3 Chemotactic motility of *P. fluorescens* (30 ºC) in M9 medium after 6 hours incubation.

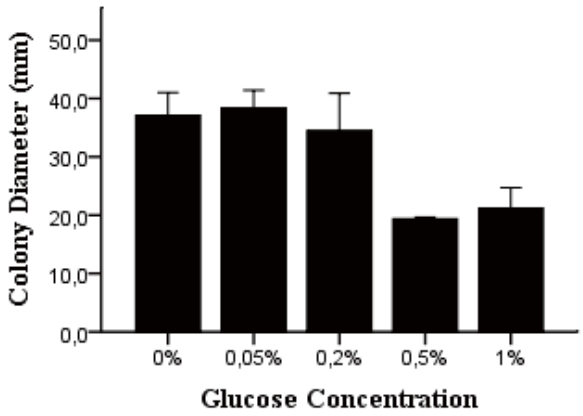

Fig 4 Colony diameter of *P. fluorescens* in LB medium after 6 hours incubation. Values on the histogram are the mean ± standard deviation of the mean of independent experiment.

Addition of glucose as main carbon source in M9 medium could not induce optimum swimming motility of *P. fluorescens,* as the halo diameters were smaller than in LB medium (Fig 6). *P. fluorescens* examined in LB had greater haloes than M9 with average diameter differences 19.64 mm.

Besides chemotaxis, effect of glucose concentration on the growth of *P. fluorescens* was also evaluated in LB and M9 media. Variation of glucose concentration in LB medium showed significant effect ($p < 0.05$) on the growth of *P. fluorescens*. Adding glucose to nutrient-rich medium such as LB caused *P. fluorescens* to have slower growth in its first phase (lag phase). During first and second hour of incubation, LB supplemented with high concentration of glucose (1%) showed lowest growth among other concentrations. Bacteria grown in LB with 1% glucose during last hour of incubation was shown to peak at 0.0169 OD_{600} ($\sim\pm 9.6 \times 10^6$ CFU mL^{-1}). Increased exponential bacterial growth during the third hour was also followed by exponential glucose depletion from 9.5 mg ml^{-1} to 7 mg mL^{-1} which implied that the bacteria had started to consume glucose during that period. However, control condition of *P. fluorescens* showed highest peak at 0.0246 OD_{600} ($\sim\pm 1.44 \times 10^7$ CFU mL^{-1}).

In contrast with LB, adding glucose to M9 medium could increase growth of *P. fluorescens* significantly ($p < 0.05$) higher than control. During third hour of incubation, bacterial growth in M9 broth supplemented with 1% glucose reached highest peak at 0.0227 ($\sim\pm 1.32 \times 10^7$ CFU mL^{-1}). That value surpassed control broth which only reached a peak at 0.0045 ($\sim\pm 2.7 \times 10^6$ CFU mL^{-1}) (Fig 8). Glucose depletion in M9-1% glucose during third hour of incubation was strikingly greater reaching 5 mg mL^{-1} than that in LB-control which only fell to 7 mg mL^{-1}. Meanwhile, *P. fluorescens* growth rate in M9-1% glucose was almost similar with LB-control in which both medium could increase exponential bacterial growth within short period of time (last hour of incubation) (Fig 7-8), faster than *Burkholderia terrae* reported by Yang *et al.* (2017).

DISCUSSION

From the chemotactic motility assay, swimming motility of *P. fluorescens* showed optimum result at 0.05% glucose in LB medium, because low concentration of glucose was more quickly to deplete causing the bacteria to progressively migrate across the end of the agar. This condition was a signal for chemotaxis receptor (MCP) to produce more flagella that enabled the bacteria to form a wider circular band compared to other concentrations. Inhibition of bacteria swimming motility at 0.2% glucose to 1% could be caused by catabolite repression, as downregulated intracellular cyclic-AMP (cAMP) also inhibited biosynthesis of flagella as well as activation of *fleQ* gene. *fleQ* gene is a master regulator of flagellar synthesis (Redondo-Nieto *et al.* 2008; Patrick and Kearns 2012; Alsohim *et al.* 2014). If master regulator could not be activated, the number of flagella synthesized would also decrease.

Previous study (Jahid *et al.* 2013) reported that inhibition of swimming motility of *A. hydrophila* was initially observed at 0.25% glucose and motility was completely inhibited by addition of 1% glucose. While in *P. fluorescens*, 1% glucose concentration could not inhibit the movement of the bacteria thoroughly, though it appeared to be restricted. This strengthen a priori of Knight and Gregory (2014) who had previously mentioned that *P. fluorescens* could survive high osmotic pressure. Inhibition of swimming motility due to increased glucose concentration had also been observed in some gram-negative bacteria such as *E. coli* and *Vibrio vulnificus* (Dobrogosz and Hamilton, 1971; Park *et al.* 2016).

Diameters of the halo of *P. fluorescens* in M9 medium supplemented with glucose appeared to be reduced and smaller than in LB medium. This is not surprising as M9 medium only contained least amount of nutrients that was less sufficient in supporting motility of the bacteria. Nevertheless, Hidalgo *et al.* (2011) showed completely different result in *E. coli* CFT073 which motility appeared to be undisturbed by minimum nutrient composition in the medium. While this explained that M9 was a poor medium for *P. fluorescens* motility expression, it also showed that the bacteria required another carbon source or stimulus to induce a more progressive motility. Some stimulus that could induce bacteria motility in minimal medium such as glycerol, *casamino acids*, *yeast extract* and *tryptone* (Cong *et al.* 2011).

From this observation we could suggest that the type of media used for the assay contributed in determining swimming motility of *P. fluorescens*. LB medium supported chemotactic motility of *P. fluorescens* better than M9 medium due to its energy-rich composition which contained *yeast extract*, *casein enzyme hydrolysate* and *tryptone* as a source of amino acids and protein. In comparison, M9 medium only contained glucose as its main energy source that made the process of flagella synthesis less effective. As a

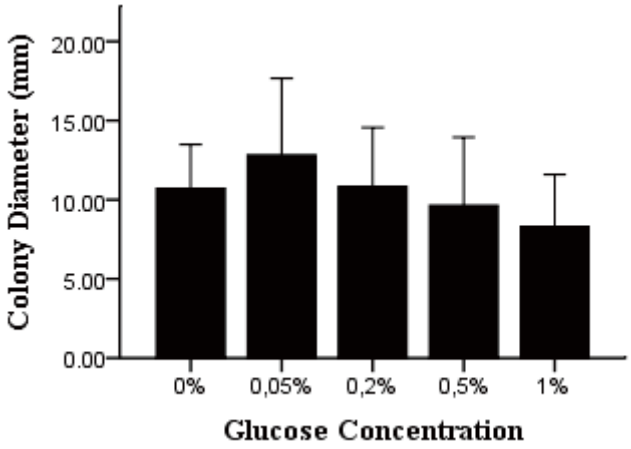

Fig 5 Colony diameter of *P. fluorescens* in M9 medium after 6 hours incubation. Values on the histogram are the mean ± standard deviation of the mean of independent experiment.

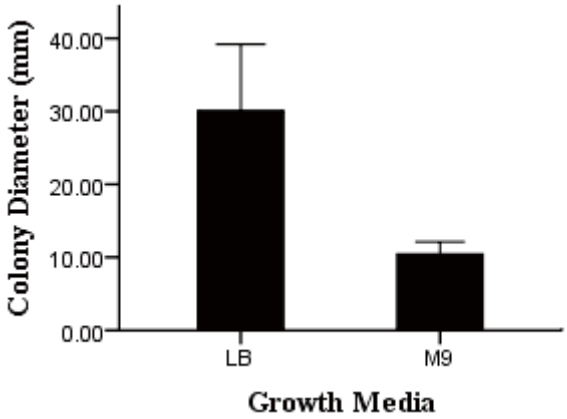

Fig 6 Variation of *P. fluorescens* chemotactic motility in LB and M9 medium. Values on the histogram are the mean ± standard deviation of the mean of two independent experiments.

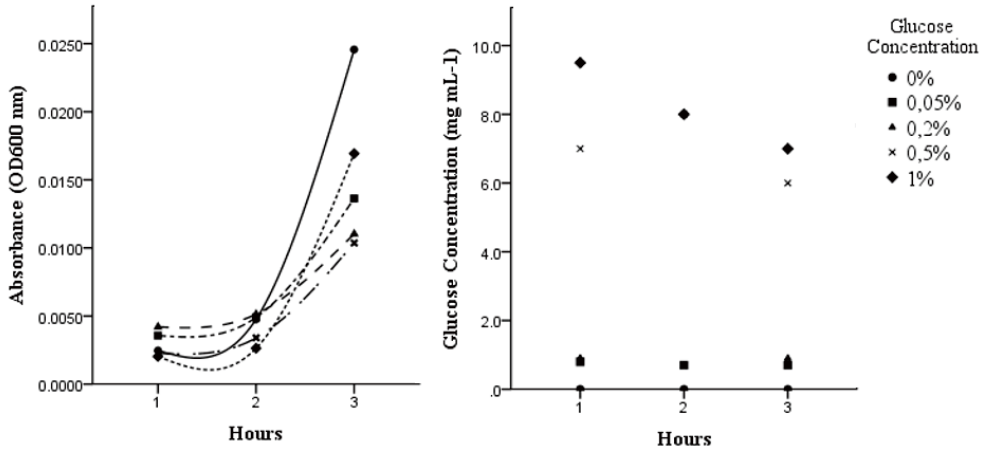

Fig 7 Growth curve of *P. fluorescens* in LB medium towards glucose concentrations was measured using UV-Vis Spectrophotometer OD_{600}. Growth reached highest peak in control condition during last hour of incubation followed by glucose depletion. Statistical analysis was two-way ANOVA with Tukeys test ($p < 0.05$).

result, the cells' ability to migrate across the agar became restricted and diameters of the colony appeared smaller. Previous investigation by Sridhar and Steele-Mortimer (2016) also reported that *Salmonella typhimurium* motility was more invasive when grown in LB than in M9.

Significant effect of glucose concentration was also observed in the first growth phase of *P. fluorescens* in LB medium. Addition of glucose in nutrient-rich LB did not appear to give beneficial effect for growth of *P.*

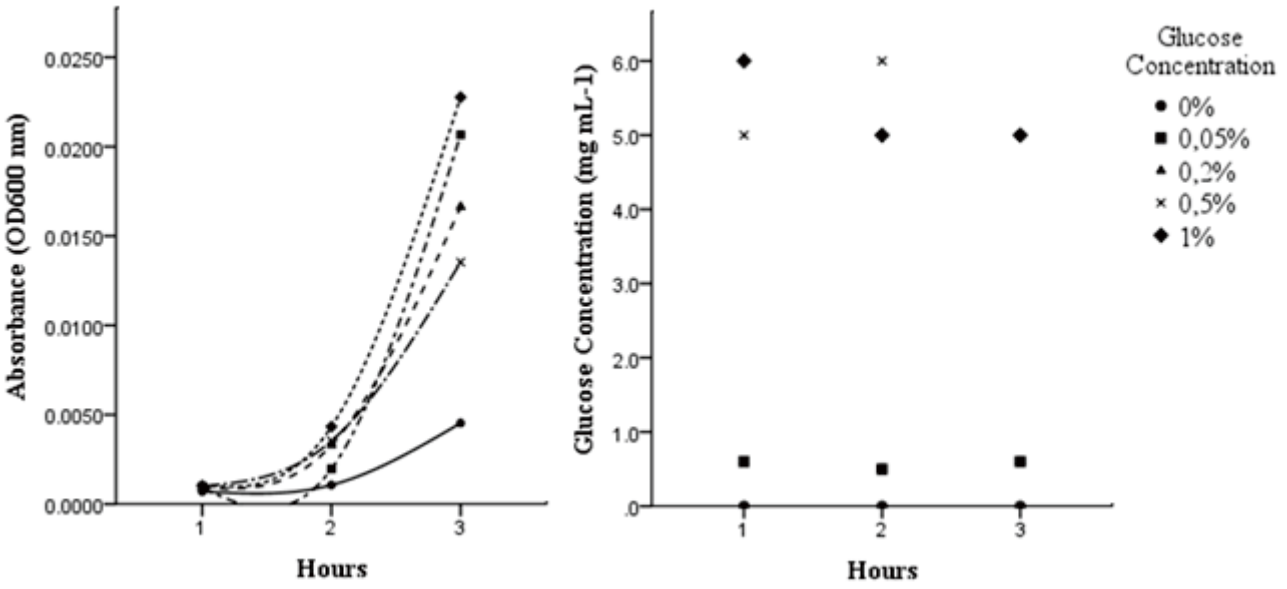

Fig 8 Growth curve of *P. fluorescens* in M9 medium towards glucose concentrations was measured using UV-Vis Spectrophotometer OD_{600}. Growth reached highest peak in 1% glucose during last hour of incubation followed by substantial glucose depletion. Statistical analysis was two-way ANOVA with Tukeys test ($p < 0.05$).

fluorescens. Conversely, glucose presence in LB caused catabolite repression to take place. Although glucose was not the repressing factor in this case, bacterial growth in 1% glucose showed to be slower than control condition due to the interference of utilization of one carbon source. This led the bacteria to undergo increased lag times as well as the diauxic growth. *Pseudomonas* species has reversed regulatory process of carbon catabolite repression and is different from *E. coli*, where the repressing carbon sources are organic acids or amino acids, and not glucose (Rojo 2010). This investigation suggested a priori that glucose could not be consumed optimally to support higher growth as it was repressed by casein enzyme hydrolysate as the preferred carbon source for *P. fluorescens*. Furthermore, Siddique and Zalik (2016) also mentioned that addition of glucose to nutrient-rich medium did not appear to be beneficial for microbial culturing practices if the intent is to increase growth rates.

On the other hand, control condition of *P. fluorescens* in M9 medium was shown to be very low due to the fact that M9 is a minimal media containing only traces of mineral and nitrogen without any carbon sources. Addition of glucose into M9 making glucose as the only carbon source that could be utilized by the bacteria to support growth. Therefore, no additional carbon source would play a role as repressing factor and intervene the utilization of another carbon source. *P. fluorescens* could focus its catabolism process on one carbon source to promote its growth, which in turn made glucose consumption in M9 medium was greater than that in LB. Nevertheless, *P. fluorescens* growth was still seen higher in LB-control as the medium contained casein enzyme hyrolysate as preferred carbon source for the bacteria. Sridhar and Steele-Mortimer (2016) and Yang *et al.* (2017) also had reported that bacterial growth was seen higher in LB medium rather than in M9-glucose.

P. fluorescens is one of the species that has the capability to grow well in minimal media. This catabolic versatility has enabled *P. fluorescens* to survive in extreme environment and to become an important biotechnological organism (Palleroni 1984; Mailloux *et al.* 2011).

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