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HYPER-SOLUBILIZING TRICALCIUM PHOSPHATE MUTANTS OF *Klebsiella* sp. GMD08

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ABSTRACT

Klebsiella sp. GMD08 is a bacteria that can dissolve insoluble inorganic phosphate into soluble phosphate ion through their organic acid production. Transposon is a genetic element agent commonly used to generate mutant through mutagenesis. Thus, it can be used to identify the genetic functions involved in those phosphate solubilizing mechanisms. This research was conducted to identify the genes of *Klebsiella* sp. GMD08 involved in phosphate solubilization through sequence detection obtained from a hyper-solubilizing phosphate mutant library. Mutation was conducted by inserting mini-Tn5 transposon hosted in *Escherichia coli* S17-1/λ*pir* [pBSL202] into *Klebsiella* sp. GMD08 chromosome by the filter mating conjugation method. Trans conjugant mutant candidates were then qualitatively and quantitatively analyzed for their solubilizing ability to dissolve tricalcium phosphate [$Ca_3(PO_4)_2$] using Pikovskaya medium. The organic acid characteristics of transconjugant mutants were detected using High Performance Liquid Chromatography (HPLC). Meanwhile, suspected genes involved in phosphate solubilizing were detected using the sequencing method obtained from the transposon insertion result. Nucleotide Basic Local Alignment Search Tool (nucleotide BLAST) was used to identify the nucleotide base sequence similarity with the database. Two main transconjugant mutants, PB116 and PB122 which had higher tricalcium phosphate dissolving ability, were obtained from the transposon mutagenesis. Gluconic acid was the main organic acid produced by *Klebsiella* sp. GMD08 phosphate solubilizing mechanism. Moreover, arginine repressor (ArgR) and malate dehydrogenase gene (mdh) coding gene were involved in *Klebsiella* sp. GMD08 phosphate solubilizing mechanism.

Keywords: *Klebsiella* sp. GMD08, phosphate solubilization, transposon mutagenesis

INTRODUCTION

Phosphorus (P) is the essential nutrient required for plant growth and development (Lavania & Nautiyal 2013). It exists in soils in both inorganic P (Pi) and organic P (Po) forms, but only the soluble inorganic P form is available for plant uptake (Sharma *et al.* 2013). However, phosphorus can quickly become insoluble and immobile due to high reactivity of soluble P with reactive cations calcium (Ca^{+2}), aluminum (Al^{+3}) and iron (Fe^{+3}) (Gyaneshwar *et al.* 2002). The precipitate reaction with those cations makes the phosphorus concentration in the soil solution rarely exceeds 0.1 mg/kg (Adnan *et al.* 2017).

Phosphate-solubilizing bacteria (PSB) actively participate in soil P cycle by producing and releasing metabolites, such as organic acid which chelate the cations of Ca, Al and Fe, bind the phosphates through their hydroxyl and carboxyl groups and convert P into soluble forms (Vassilev *et al.* 2012; Chen *et al.* 2016). Among the commonly studied organic acids, gluconic and keto-gluconic acids are considered the most effective chelating agents in the mineral phosphate solubilization mechanism (mps). These acids are produced in the periplasm of

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many gram-negative bacteria through a direct oxidation pathway of glucose (DOPG, non-phosphorylating oxidation) via the membrane-bound glucose dehydrogenase (GDH) enzyme which is also dependent on pyrroloquinoline quinone (PQQ) as an enzymatic cofactor (Pérez *et al.* 2007; de Werra *et al.* 2009).

The production of organic acids is considered as the key principal process for the mineral phosphate solubilization mechanism in bacteria. Several genes are involved in the mineral phosphate solubilization and they have been cloned from a number of bacteria mainly related to *glucose dehydrogenase* (GDH), *gluconate dehydrogenase* (GADH), and pyrroloquinoline quinone (PQQ) biosynthesis (Sashidhar & Podile 2010). Besides, a *gabY* gene cloned from *Pseudomonas cepacia* in *E. coli* was shown to be involved in mineral phosphate solubilization as an alternative gene (Babu-Khan *et al.* 1995; Chhabra *et al.* 2013).

Previous genetic studies involving mineral phosphate solubilization of gram-negative bacteria had used the mutagenesis method. The open reading frame of *gabY* gene was disrupted from *Pseudomonas cepacia* via site-directed mutagenesis which resulted in defective phosphate solubilization phenotype and also eliminated the gluconic acid production (Babu-Khan *et al.* 1995). A transposon mutant library of *Gluconacetobacter diazotrophicus* mps gene was constructed and resulted in a defective phosphate solubilization phenotype (Intorne *et al.* 2009). This research showed that gluconic acid production was correlated with phosphate solubilization. A random transposon mutagenesis mutant library was constructed to identify novel genes involved in phosphate solubilization from *Enterobacter* sp. Wi28, *Pseudomonas* sp. Ha200 and *Burkholderia* sp. Ha185 (Hsu (2014). This research showed that *hemX* gene was involved in the 2-keto-gluconic acid production.

Klebsiella sp., mainly *K. pneumonia* is a *plant growth-promoting rhizobacteria* (PGPR) that has inorganic phosphorus solubilizing capability (Li *et al.* 2011). Furthermore, in *K. pneumonia*, six genes constituting the *pqqABCDEF* operon, which are required for the synthesis of the cofactor PQQ associated with mineral phosphate solubilization, were cloned and identified (Meulenberg *et al.* 1990; Velterop *et al.* 1995; Han *et al.* 2008; Naveed *et al.* 2016).

This study aims to identify the genes of *Klebsiella* sp. GMD08 involved in the phosphate solubilization through sequence detection obtained from transposon insertion. A mini-Tn5 derivative transposon mutant library was constructed and analyzed to identify the hyper-solubilizing phosphate mutants. Then, the interrupted genes were identified, allowing the investigation of the mechanisms involved in the solubilization of phosphorus.

MATERIALS AND METHODS

Bacterial Strains and Medium Preparation

The wild-type *Klebsiella* sp. GMD08 strains were used for the construction of the transposon mutant library. *Klebsiella* sp. GMD08 strains were grown at 37 °C in Luria-Bertani (LB) medium at a g/L composition of 10 triptone, 5 yeast extract and NaCl 10 (Meulenberg *et al.* 1990).

Klebsiella sp. GMD08 was spontaneously mutagenesized by rifampicin antibiotics. The mutation result (WT Rif +) was then used as the recipient in the conjugation step (Lorenzo & Timmis 1994; Sasaki & Kurusu 2004).

Escherichia coli S17-1/λ*pir*[pBSL202] strains harboring the mini-Tn5 derivative transposon plasmid constructed by Alexeyev *et al.* (1995) were used for the transposon mutagenesis. *E. coli* S17-1/λ*pir* [pBSL202] strains were grown at 37 °C in LB medium supplemented with gentamycin antibiotic at 50 μg/mL concentration.

The phosphate-solubilizing capability analysis was performed using the Pikovskaya medium following the Prijambada *et al.* (2009) method, and was composed of: $Ca_3(PO_4)_2$ (5.0 g), $(NH_4)_2SO_4$ (0.5 g), NaCl (0.5 g), $MgSO_4.7H_2O$ (0.1 g), KCl (0.2 g), glucose (10.0 g), yeast extract (0.5 g), $FeSO_4$ (trace), $MnSO_4$ (trace) and agar (15.0 g).

Transposon Mutagenesis

Transposon mutagenesis was performed following the conjugation method of de Lorenzo *et al.* (1990) and Martínez-García *et al.* (2014). The recipient (*Klebsiella* sp. GMD08 WT Rif +) and donor (*E. coli* S17-1/λ*pir*[pBSL202])

strains were grown overnight in LB with the appropriate antibiotics. After incubation, 0.7 mL of recipient was mixed with 0.3 mL of donor. Cells were then collected by centrifugation at 13,000 rpm for 1 min. The pellet was washed twice with 10 mM $MgSO_4$ and was then vortexed and centrifuged again. The pellet was resuspended in 200 µL of 10 mM $MgSO_4$, filtered with a sterile filter *cellulose acetate filter* 0.45 µm in a 13 mm polycarbonate syringe filter holder and then spotted on an LB plate. After an overnight incubation at 37 °C, the cells were scraped off from the plate and resuspended in 500 µL of 10 mM $MgSO_4$. The dilution were placed on an LB plate which contained 50 µg/mL rifampicin and 5 µg/mL gentamycin antibiotics.

Mutant Screening

The transformants obtained from conjugation results were grown overnight in LB broth medium at 37 °C with constant shaking at 100/min. To screen for the hyper-mutants in phosphorus solubilization, the 10 µL of the culture results were inoculated on a paperdisk of 0.6 cm diameter in a Pikovskaya plate medium. After 3 days of incubation at 37 °C, the solubilizing zone (halozone) was observed around the colony. The mutants that formed a larger zone than the wild-type, were then selected for identification.

Phosphate Solubilization in Liquid Medium

Briefly, the selected mutants were used to measure the phosphate solubilization in a liquid medium. The isolates were grown in Pikovskaya broth medium for 4 days at 37 °C with continuous agitation at 100 rpm. Some 100 mL Erlenmeyer flasks containing 50 mL of Pikovskaya broth medium were inoculated with 500 µL of bacterial suspension with OD = 0.4 ± 2.1 × 10^9 cfu/mL, except the control flasks which were not inoculated. During some interval points of incubation (at the beginning of 0, 24, 48, 72 and 96 hours), culture aliquots were aseptically taken from each flask to follow the bacterial growth, the medium pH, as well as the concentration of released soluble phosphorus. All experiments were performed in triplicates.

Cell growth was estimated colometrically by measuring the absorbance at 600 nm (OD_{600}). The pH changes of the broth culture were recorded by pH meter equipped with a glass electrode, while analysis of the concentration of released soluble phosphorus was performed by the vanadate-molybdate method of Yoshida *et al.* (1976).

Organic Acid Analysis by High Performance Liquid Chromatography (HPLC)

HPLC was used for the analysis of organic acids produced by bacterial strains in Pikovskaya broth medium. Supernatant was taken from 10,000 g bacterial cultures that had been centrifuged at -4 °C for 10 min. Thereafter, the samples were filtered through 0.22 µm Sartorius filter. A total of 20 µL filtrates were injected to a Phenomenex bond clone C18 HPLC in a 300 x 3.9 mm column equipped with a 210 nm UV-Vis detector. The operating conditions consisted of 2.5% acetonitrile at 0.05 M NaH_2PO_4 as mobile phase at a constant flow rate of 1.2 mL/min and the column were operated at 40 °C. The detected organic acids were identified by comparing their retention times and the peak areas of their chromatograms with those of the standards.

Transposon Flanking DNA Analysis

Genomic DNA from the selected mutants was isolated using DNA Purification Kit (PureLink Genomic, Invitrogen), following the manufacturer's protocol. Transposon insertion sites were determined by arbitrary PCR, followed by sequencing, which was determined by the arbitrary PCR according to Espinosa-Urgel *et al.* (2000). The first round of amplification was done by using the chromosomal DNA of the mutants as a template with an arbitrary primer (ARB1; 5,-GGCACGCGTCGACTAGTACNNNNNN NNNNGATAT-3') and an internal primer of mini-Tn5 that is unique for the right end (TNEXT; 5'-TGATGAATGTTCCGTTGCGC TGCC-3'). The first round was as follows: 3 min at 95 °C; 6 cycles of 30 sec at 95 °C; 30 sec at 30 °C; and 1 min of 72 °C; 30 cycles of 30 sec at 95 °C; 30 sec at 50 °C; and 1 min at 72 °C; and an extension period of 7 min at 72 °C. The second round of amplification was done with the first-round reaction as the template as follows: 3 min at 95 °C; 35 cycles of 30 sec at

95 °C; 30 sec at 57 °C; and 1 min at 72 °C; and an extension period of 7 min at 72 °C. Primers used for the second round were those corresponding to the conserved region of ARB1 (ARB2; 5'-GGCACGCGTCGACTAGTAC-3') and a second internal primer of mini-Tn5, closer to the end (TNINT; 5'-GACCTGCAGGCATG CAAGCTCGGC-3').

The PCR amplification results were electrophoresed at 0.8% agarose and visualized on UV Transilluminator. Selected DNA bands were then purified and sequenced using ARB2 and TNIT primers. The sequences were analyzed and compared with the GeneBank database by using the nucleotide basic local alignment search tool (nucleotide BLAST) program available at the NCBI website (http://www.ncbi.nlm.nih.gov/blast/ Blast.cgi).

RESULTS AND DISCUSSION

Screening Mutants with Altered Solubilization Zones

Transposon mutant libraries were constructed by random transposon mutagenesis where the *Klebsiella* sp. GMD08 rifampicin mutant, as a recipient, was conjugated with *E. coli* S17-1/λ*pir*[pBSL202] harboring the mini-Tn5 derivative transposon. Mutants were initially selected for rifampicin-gentamycin resistance on an LB plate indicating the presence of mini-Tn5 derivative transposon which carried the gentamycin resistant genes.

The insertion of mutant library was used to search for the hyper-phosphate solubilization mutant. In the phosphorus solubilization screening, the 340 transformants were put in Pikovskaya medium containing $Ca_3(PO_4)_2$. After 3 days of culture, two hyperquality mutants (PB116 and PB122), were identified as producing halozone around the colony. These two mutants showed enhanced phosphate solubilization ability on Pikovskaya plates, where the ratio of the halo size to the colony size was greater than that of the wild-type of *Klebsiella* sp. GMD08 (Fig. 1a).

These mutants were selected and the phosphorus solubilization assay was repeated three times. The phosphate efficiency measurement using solubilization index (SI) was calculated as the ratio of the total diameter (colony + halozone) to the colony diameter according to Mardad *et al.* (2013) and Pande *et al.* (2017). Results showed that PB116 mutant had the highest index at SI of 3.125 ± 0.33, followed by PB122 at SI of 2.833 ± 0.19 (Fig. 1b).

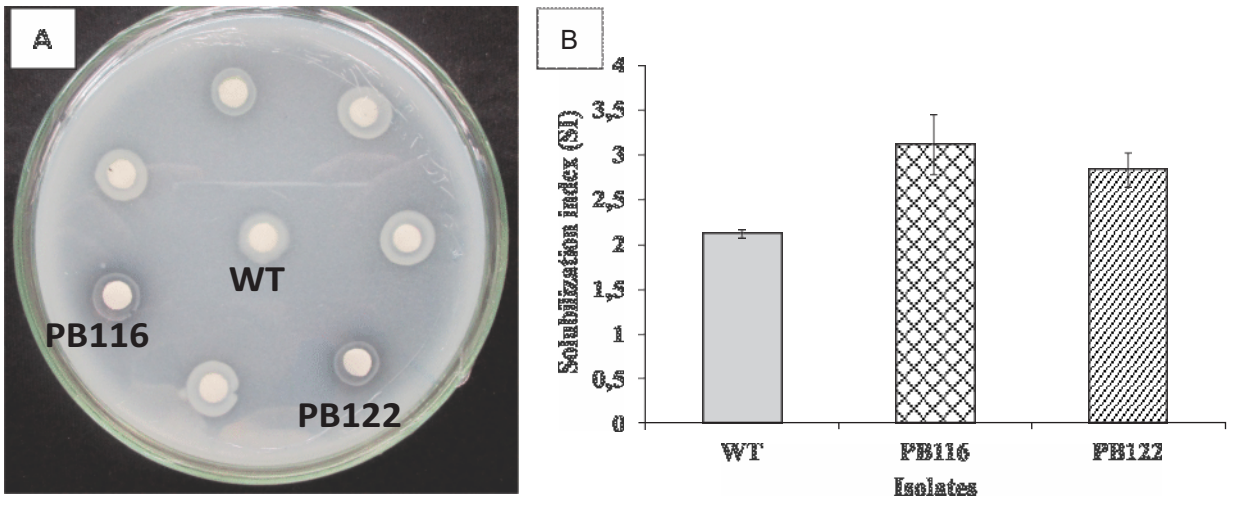

Figure 1 Mini-Tn5 derivative transposon mutants of *Klebsiella* sp. GMD08 screened assays on Pikovskaya plate medium containing the tricalcium phosphate, after 3 days incubation at 37 °C

Notes: A = Solubilization halos by the three isolated bacterial strains (wild-type, PB116, and PB122) on Pikovskaya plate medium; B = solubilization index (SI) of the selected isolated bacterial strains. Solubilization index for each isolate based on colony diameter and clear zones formed by solubilizing suspended tricalcium phosphate; SI = (colony diameter+halozone diameter)/colony diameter.

Pikovskaya plate medium is routinely used to show a halozone (clear-zone) around the colony of bacterial growth as an indicator for phosphate solubilization (Nautiyal 1999; Pande *et al.* 2017). The halozone formation is due to the production of organic acids or to the production of polysaccharides or to the activity of phosphatase enzymes that convert tricalcium phosphate in the medium from insoluble to soluble forms (Paul & Sinha 2017; Pande *et al.* 2017).

However, the qualitative method of assay screening by measuring the halozone around the colony to assess the phosphate-solubilizing ability (the halo-based technique) is yet to be well established (Baig *et al.* 2010; de Bolle *et al.* 2013). Therefore, an additional test in liquid media to assay phosphate dissolution has to be performed (Bashan *et al.* 2013). Quantitative method reflects the amount of soluble phosphate released from insoluble substrate as a result of microbial activity.

Phosphate Solubilization by Wild Type and Mutants

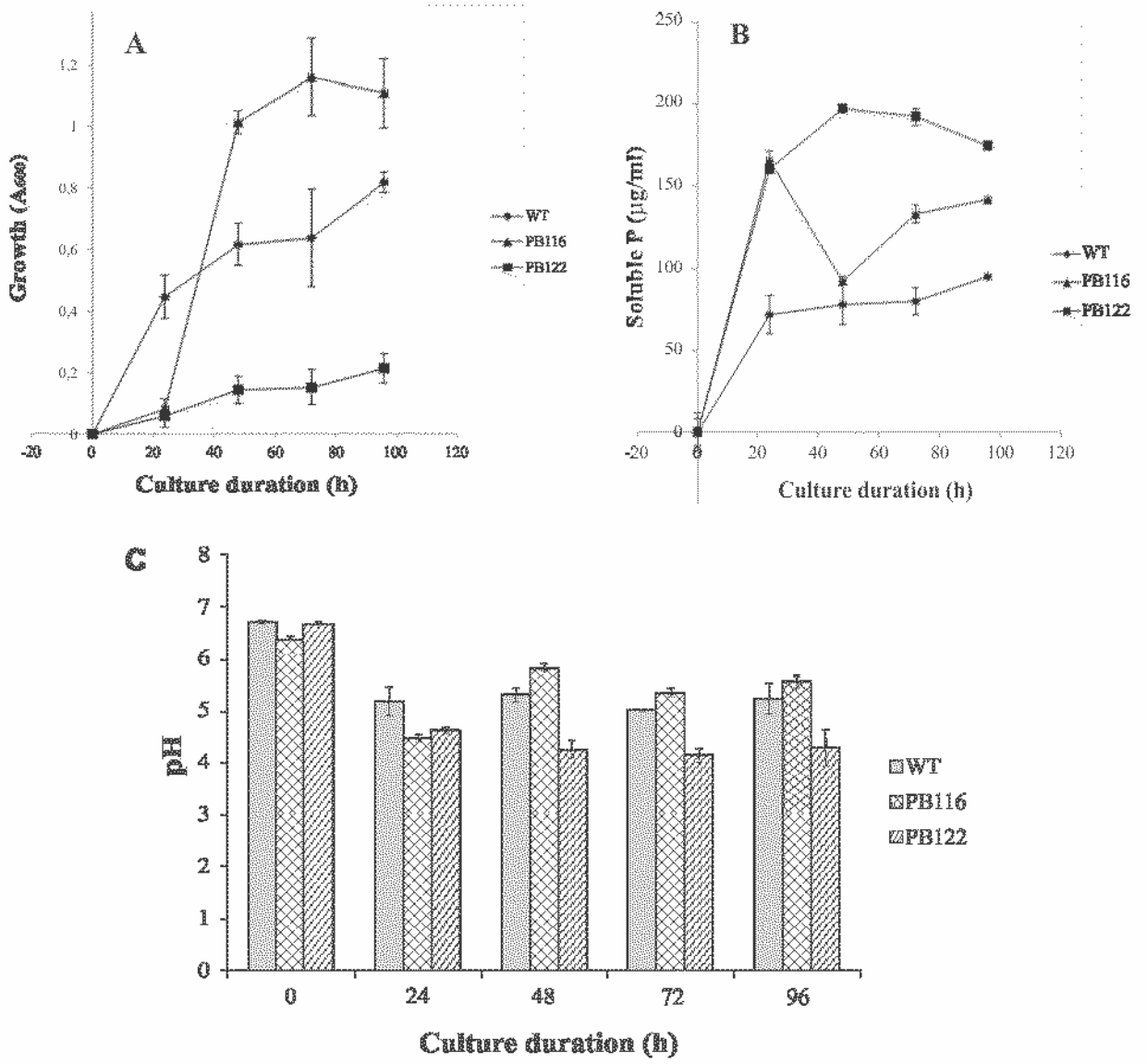

Figure 2 Changes in (A) microbial cell growth, (B) soluble phosphate concentration, and (C) medium pH of *Klebsiella* sp. GMD08 wild-type and two selected mutants (PB116 and PB122) in Pikovskaya broth medium containing the tricalcium phosphate for 96 h incubation

Notes: Each value is the mean of three replicates; the bars indicate the standard error; The soluble phosphate concentration was measured by the vanado-molybdate colorimetric method; Colorimetric change from colorless to yellow corresponds to the concentration of soluble phosphate.

In this study, the wild-type and mutants started to grow exponentially when inoculated in Pikovskaya broth medium containing $Ca_3(PO_4)_2$ as the only source of phosphate. PB116 mutant had the highest cell growth rate with an average of 1.014 ± 0.036 detected at 48 h as compared with *Klebsiella* sp. GMD08 wild-type of 0.618 ± 0.068 and with the PB122 mutant of 0.144 ± 0.045 (Fig. 2a).

The mutant soluble phosphates that had increased rapidly after inoculation, reaching a maximum of 165.86 ± 7.939 µg/mL (PB116) and 160.22 ± 11.411 µg/mL (PB122), was detected at 24 h. However, the ability of PB116 mutant to solubilize phosphate significantly decreased to 92.397 ± 5.514 µg/mL at 48 h (Fig. 2b).

The pH of the Pikovskaya broth medium also started to drop immediately after the cells were inoculated. The significant decrease in pH was recorded at 24 h to 4.48 ± 0.07 pH in PB116 and 4.63 ± 0.05 pH in PB122. However, the pH of PB116 significantly increased to 5.83 ± 0.08 at 48 h (Fig. 2c).

The pH condition was correlated with an increase or decrease in the soluble phosphates concentration in the Pikovskaya broth medium. The soluble phosphates of the mutants had increased rapidly after inoculation, reaching a maximum of 165.86 ± 7.939 µg/mL (PB116) and 160.22 ± 11.411 µg/mL (PB122) detected at 24 h. However, the PB116 mutant ability to solubilize phosphate significantly decreased to 92.397 ± 5.514 µg/mL at 48 h.

The acidification which affected the pH is attributed to the consumption of the glucose from the growth media and the production of organic acids during the bacterial growth (Mardad *et al.* 2013). During the culture, the increased bacterial growth with decreasing pH values and production of organic acids resulted in considerable amount of phosphates solubilized, indicating that phosphate solubilization occurs at low pH (Panhwar *et al.* 2009).

The decrease of soluble phosphate was probably caused by the decrease in the production of organic acids once the free phosphate was released into the medium (Tripura *et al.* 2007). A series of organic acids have various acidity constants which determine their ability to change the acidity of the environments. However, the environmental effect of acidity on bacterial activity is not yet well documented (Li *et al.* 2016).

Reduction in the quantity of soluble phosphate and the increase in the pH can be explained as an auto-consumption of available free phosphate for metabolism of the growing bacterial population (Seshadri *et al.* 2000; Crespo *et al.* 2011). In addition, the bacterial growth may have also utilized for metabolism the organic acid secretion product when a major carbon source is not available (Seshadri *et al.* 2000; Tripura *et al.* 2007).

Organic Acid Production

Organic acids produced by the wild-type and the mutants in the Pikovskaya broth culture were investigated at 24 h of growth. HPLC analysis of the culture filtrate revealed two major peaks of *Klebsiella* sp. GMD08 wild-type. One of the peaks was identified as gluconic acid by comparing the retention times with the standard ones, while the other one was an unknown acid. The mutant isolates shown had produced only gluconic acid with high concentration (Fig. 3).

The quantity of gluconic acid, the main organic acid produced by *Klebsiella* sp. GMD08 wild-type and mutants, was further analyzed by comparing their concentration with the gluconic acid standard. The results showed that the PB116 and PB122 mutants produced twice higher gluconic acid concentration (114. 618 mM and 114. 742 mM, respectively) than that of *Klebsiella* sp. GMD08 wild-type (Fig. 4).

The gluconic acid was the main organic acid produced by *Klebsiella* sp. GMD08 phosphate solubilizing mechanism. Based on this research, the bacteria that produce high concentrations of gluconic acid have high levels of soluble phosphate during acidification in the medium. Therefore, the gluconic acid concentration played a major role in the phosphate-solubilizing mechanism.

These results confirmed those of several previous studies that gluconic acid was the major organic acid produced by phosphate-solubilizing bacteria (Lin *et al.* 2006; Delvasto *et al.* 2008; Song *et al.* 2008; Stella & Halimi 2015). The gram-negative bacteria may mobilize insoluble phosphates very efficiently as a consequence of the production of gluconic acid, which resulted from the extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase (Pérez *et al.* 2007; An & Moe 2016).

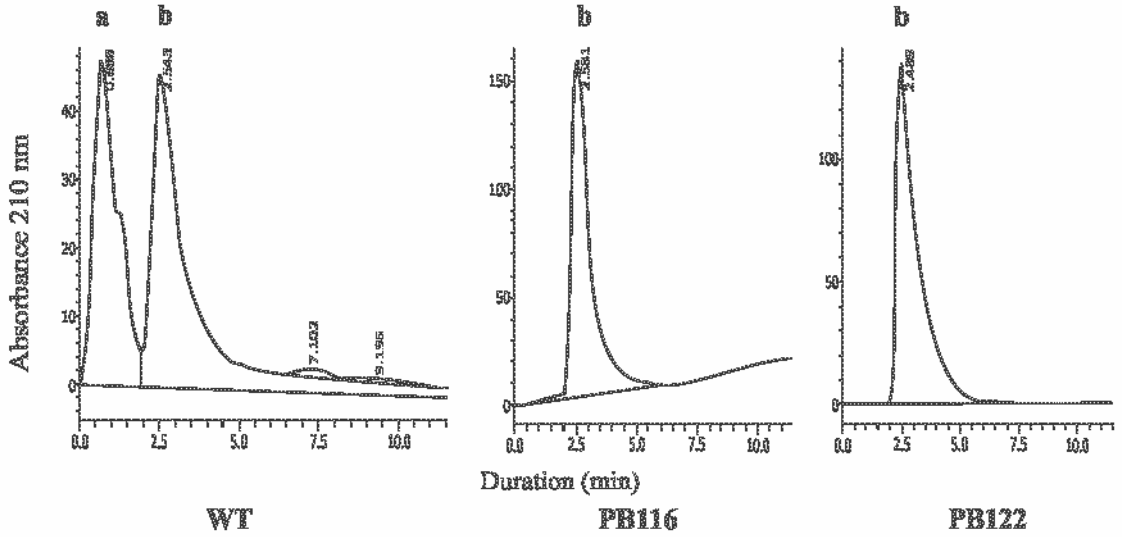

Figure 3 High-performance liquid chromatogram of organic acids detected in the culture of *Klebsiella* sp. GMD08 wild-type and two selected mutants (PB116 and PB122) in Pikovskaya broth medium containing tricalcium phosphate at 24 h incubation
Notes: a = unknown acid, b = gluconic acid).

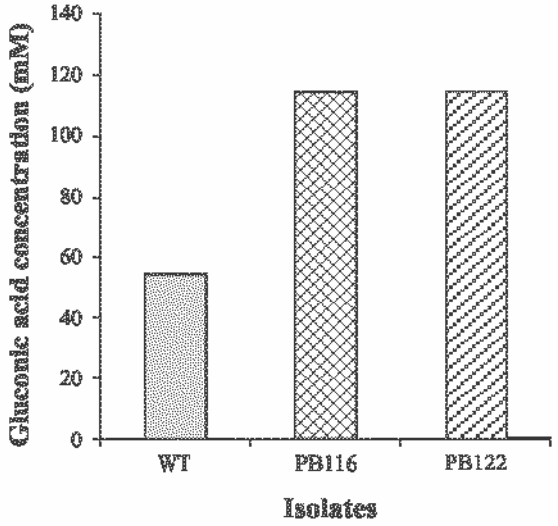

Figure 4 Concentration of gluconic acid secreted by *Klebsiella* sp. GMD08 wild-type and two selected mutants (PB116 and PB122) in Pikovskaya broth medium containing tricalcium phosphate at 24 h incubation

Sequence Analysis of Genes Involved in Phosphate Solubilization of Mutants

To impair the ability of *Klebsiella* sp. GMD08 to solubilize insoluble mineral-phosphate, the Mini-Tn5 derivative transposon random mutagenesis was used to create a mutant library of strains with mutation in genes. In order to identify the gene interrupted by the transposon insertion of the mutants, the flanking regions of the transposon insertion points were analyzed by sequencing. The mini-Tn5 insertion sites were identified from the resulting sequences of arbitrary PCR fragments by analysis with the BLAST tool.

The PB116 mutant produce two main bands of 800 bp and 600 bp, whereas PB122 mutant produce three main bands of 600 bp, 500 bp, and 150 bp (Fig. 5). Using the nucleotide BLAST program, the transposon insertion sequence of 600 bp size of PB116 and 500 bp size of PB122 showed a high similarity with arginine repressor encoding gene (ArgR) (MN167465 and MN231301), whereas the 600 bp size of PB122 showed a similar sequence with malate dehydrogenase encoding gene (mdh) (MN179490). Both encoded genes were mostly similar with *Klebsiella quasipneumoniae* strain ATCC 700603 (CP014696.2).

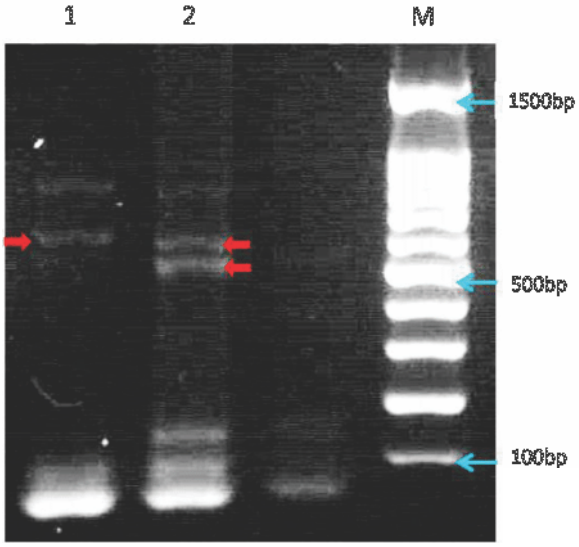

Figure 5. The second round of Arbitrary PCR results with ARB2 and TNIT primers
Notes: 1 = PB116 mutant; 2 = PB122 mutant; M = 100 kb *ladder*.

In another study, the *gdh* gene disruption in *Rahnella aquatilis* strain HX2 was partially responsible for its abolished antibacterial phenotype and impaired bio-control phenotype (Guo *et al.* 2009). Thus, it was suggested that gene disruption would produce non-functional genes. Therefore, in this study, the introduction of mini-Tn5 derived transposon elements to ArgR and mdh genes of *Klebsiella* sp. GMD08 had probably produced a non-functional gene which affected their expression.

ArgR is also essential for arcABC operon which enables *Streptococcus suis* to survive in an acidic environment (Fulde *et al.* 2011). Therefore, the non-functional ArgR gene may be related with the disruption of the negative expression. This condition resulted in the absence of down-regulators that will interact with the operon promoters involved in the gluconic acid production mechanism. As a result, gluconic acid was overproduced because of the inactivity of negative regulators as those found in PB116 and PB122 mutants.

CONCLUSION

The introduction of mini-Tn5 derived transposon elements to *Klebsiella* sp. GMD08 has obtained two main transconjugant mutants PB116 and PB122 which have higher tricalcium phosphate dissolving ability. The gluconic acid was the main organic acid produced by the *Klebsiella* sp. GMD08 phosphate solubilizing mechanism demonstrating that arginine repressor (ArgR) and malate dehydrogenase (mdh) encoding gene were involved in the phosphate solubilizing mechanism.

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