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

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ABSTRACT

*Klebsiella* sp. GMD08 is one of the bacteria that has the capability to dissolve insoluble inorganic phosphate into soluble phosphate ion through their organic acid production. Transposon is a genetic element agent usually used to generate mutants 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>)pir [pBSL202] into *Klebsiella* sp. GMD08 chromosome by the filter mating conjugation method. Transconjugant 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. The results showed that PB116 and PB122 were the two main transconjugant mutants obtained from transposon mutagenesis which had higher tricalcium phosphate dissolving ability. 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: phosphate solubilization, *Klebsiella* sp. GMD08, transposon mutagenesis

INTRODUCTION

Phosphorus (P) is one of the essential nutrients required for the growth and development of plants (Lavania and Nautiyal, 2013). Phosphorus exists in soils in both inorganic P (Pi) and organic P (Po) forms but only a soluble inorganic P form is available for plant uptake (Sharma et al., 2013). However, phosphorus can be rapidly being insoluble and immobile due to high reactivity soluble P with reactive cations like calcium (Ca$^{2+}$), aluminum (Al$^{3+}$) and iron (Fe$^{3+}$) (Gyaneshwar et al.,...
The precipitation reaction with those cations makes the concentration of phosphorus 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, bound to phosphates through their hydroxyl and carboxyl groups and converted into soluble forms (Vassilev et al., 2012; Chen et al., 2016). Among all the organic acids which have been studied, gluconic and keto-gluconic acids seem to be the most effective chelating agents in the mineral phosphate solubilization mechanism (mps). These acids are produced in the periplasm of many gram-negative bacteria through a direct oxidation pathway of glucose (DOPG, non-phosphorylating oxidation) via membrane-bound glucose dehydrogenase (GDH) enzyme which is 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 proposed for the mineral phosphate solubilization mechanism in bacteria. There are several genes 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 and 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).

Study of genes involved in mineral phosphate solubilization of gram-negative bacteria using mutagenesis method was done in previous research. Babu-Khan et al (1995) have disrupted the open reading frame of gabY gene from Pseudomonas cepacia via site-directed mutagenesis which resulted in defective phosphate solubilization phenotype and also eliminated gluconic acid production. Intorne et al (2009) have constructed a transposon mutant library of Gluconacetobacter diazotrophicus mps gene that demonstrated defective phosphate solubilization phenotype. This research showed that gluconic acid production was correlated with phosphate solubilization. Recently, Hsu (2014) has constructed a random transposon mutagenesis mutant library to identify novel genes involved in phosphate solubilization from Enterobacter sp. Wi28, Pseudomonas sp. Ha200 and Burkholderia sp. Ha185. This research showed that hemX gene was involved in 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, have been 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 evaluation 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. According to Meulenberg *et al* (1990), *Klebsiella* sp. GMD08 strains were grown at 37°C in Luria-Bertani (LB) medium (g L⁻¹ composition: triptone 10, yeast extract 5 and NaCl 10).

According to Lorenzo and Timmis (1994) and Sasaki and Kurusu (2004), *Klebsiella* sp. GMD08 was spontaneous mutagenesis by rifampicin antibiotics. The mutation result (WT Rif +) was then used as the recipient in the conjugation step.

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

Phosphate-solubilizing capability analysis was performed using Pikovskaya medium in accordance to Prijambada *et al* (2009), composed of: Ca₃(PO₄)₂ (5.0 g), (NH₄)₂SO₄ (0.5 g), NaCl (0.5 g), MgSO₄·7H₂O (0.1 g), KCl (0.2 g), glucose (10.0 g), yeast extract (0.5 g), FeSO₄ (trace), and MnSO₄ (trace), and agar (15.0 g).

**Transposon mutagenesis**

Transposon mutagenesis was performed by the conjugation method according to de Lorenzo *et al* (1990) and Martínez-García *et al* (2014). The recipient (*Klebsiella* sp. GMD08 WT Rif +) and donor (*E. coli* S17-1/λpir[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 13000 rpm for 1 minute. The pellet was washed twice with 10 mM MgSO₄ and was then vortexed and centrifuged again. The pellet was re-suspended in 200 μl of 10 mM MgSO₄, 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, cells were scraped off the plate and resuspended in 500 μl of 10 mM MgSO₄. The dilution
were plated on an LB plate which contained rifampicin (50 μg/ml) and gentamycin (5 μg/ml) antibiotics.

**Mutant screening**

The transformants obtained from conjugation results were grown in LB broth medium at 37°C for overnight with constant shaking (100 min⁻¹). To screen for the hyper-mutants in phosphorus solubilization, the 10 μl of the culture results were inoculated on a paperdisk (0.6 cm diameter) in a pikovskaya plate medium. After 3 days of incubation at 37°C, it was possible to observe whether a solubilizing zone (halozone) was formed around the colony. Mutants that formed a larger zone than the wild-type, were then selected.

**Phosphate solubilization in liquid medium**

In brief, 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 of 100 rpm. Erlenmeyer flasks (100 ml) containing 50 ml of Pikovskaya broth medium were inoculated with 500 μl of bacterial suspension (OD= 0. 4 ; ± 2. 1×10⁹ CFU/ml). However, control flasks were not inoculated. During some interval points of incubation (at every hour of 0, 24, 48, 72, and 96), aliquots of cultures were aseptically taken from each flask to follow the bacterial growth, the pH of the medium as well as the concentration of released soluble phosphorus. All experiments were performed in triplicates.

Cell growth was estimated colometrically by the measurement of the absorbance at 600 nm (OD₆₀₀). The changes of pH of the broth culture were recorded by pH meter equipped with a glass electrode. The analysis of the concentration of released soluble phosphorus was performed by the

**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 minutes. After that, samples were filtered through 0, 22 μm filter (Sartorius). A total of 20 μl filtrates were injected to a Phenomenex bond clone C18 HPLC column (300 x 3.9 mm in size) equipped with a 210 nm UV-Vis detector. The operating conditions consisting of 2.5% acetonitrile at 0.05 M NaH₂PO₄ 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 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 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'-GGCACGCCTCGACTAGTACNNNNNNNGATAT-3') and an internal primer of mini-Tn5 that is unique for the right end (TNEXT; 5'-TGATGAATGTCCGTGTGCTG-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'-GGCACGCCTCGACTAGTAC-3') and a second internal primer of mini-Tn5, closer to the end (TNINT; 5'-GACCTGCAGGATGCAAGCTG-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. 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 rifampicin mutant of Klebsiella sp. GMD08 as a recipient was conjugated with E. coli S17-1/κpir[pBSL202] harboring the mini-Tn5 derivative transposon. Mutants were initially selected for rifampicin-gentamycin resistance on a LB plate indicating the presence of mini-Tn5 derivative transposon which carried the gentamycin resistance genes.

The insertion of mutant library was used to search for hyper-phosphate solubilization mutant. In the phosphorus solubilization screening, the 340 transformants were plated in Pikovskaya medium containing Ca₃(PO₄)₂. After 3 days of culture, two mutants (PB116 and PB122), which had hyperquality in producing halozone around the colony were identified. These
two mutants were 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 result of the phosphate efficiency measurement using solubilization index (SI). 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), showed that PB116 mutant had the highest index with a SI of 3.125 ± 0.33, followed by PB122 with 2.833 ± 0.19 (Fig. 1b).

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

\[ \text{SI} = \frac{\text{colony diameter} + \text{halozone diameter}}{\text{colony diameter}} \]

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 could be due to the production of organic acids or due to the production of polysaccharides or due to the activity of phosphatase enzymes that convert tricalcium phosphate in the medium from insoluble to soluble forms (Paul and Sinha, 2017; Pande *et al*., 2017).

However, the qualitative method by screening assay of measuring the halozone around the colony to assess the phosphate-solubilizing ability (the halo-based technique) has 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 should 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**

![Graphs showing microbial cell growth, soluble phosphate concentration, and medium pH over time.](image)

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 tricalcium phosphate during 96 h incubation. (Each value was the mean of three replicates; the bars indicate the standard error. The soluble phosphate concentration measured by the vanado-molybdate colorimetric method. Colorimetric change from colourless to yellow corresponding 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. The results showed that PB116 mutant had the highest of the growth rate cell with an average of 1.014 ± 0.036 detected at 48 h compared with Klebsiella sp. GMD08 wild-type (0.618 ± 0.068) as well as the PB122 mutants (0.144 ± 0.045) (Fig. 2a).

The soluble phosphates of the mutants had increased rapidly after inoculation which reached a maximum of 165.86 ± 7.939 µg/ml (PB116) and 160.22 ± 11.411 µg/ml (PB122) detected at 24 h. However, the ability of PB116 mutant to solubilize phosphate significantly decreased to 92.397 ± 5.514 µg/ml which was detected 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 in PB116 and 4.63 ± 0.05 in PB122. However, the pH of PB116 significantly increased to 5.83 ± 0.08 at 48 h (Fig. 2c).

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

The acidification which affects the pH could be attributed to the consumption of the glucose from the growth media and the production of organic acids during 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 (Panwar et al., 2009).

The decrease of soluble phosphate could have been caused by the decrease in the production of organic acids once the free phosphate was released into the medium (Tripura et al., 2007). A serial of organic acids have various acidity constants, which determine their ability in changing acidity of the environments. However, that how the acidity in the environment affect bacterial activity has not been well documented (Li et al., 2016).

The 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 by the growing bacterial population (Seshadri et al., 2000; Crespo et al., 2011). In addition, the bacterial growth could also have utilized the organic acid secretion product for metabolism 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 were investigated in Pikovskaya broth culture 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 other one was an unknown acid. The mutants isolates shown produced only gluconic acid with high concentration (Fig. 3).

![Figure 3](image-url)

**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 (a: unknown acid, b: gluconic acid)

![Figure 4](image-url)

**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
The quantity of gluconic acid, which was the main organic acid produced by *Klebsiella* sp. GMD08 wild-type and mutants, was further analyzed by determining their concentration by comparing the area values with the gluconic acid standard. The analysis on the result of gluconate acid concentration showed that the PB116 and PB122 mutants produced twice higher gluconic acid concentration than that of *Klebsiella* sp. GMD08 wild-type. There were 114.618 mM and 114.742 mM (Fig. 4).

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

These results are also consistent with several previous studies which reported 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 and Halimi*, 2015). It was also reported that the gram-negative bacteria may mobilize insoluble phosphates very efficiently as a consequence of the production of gluconic acid, which was resulted from the extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase (*Pérez et al.*, 2007; *An and Moe*, 2016).

### Sequence analysis of genes involved in phosphate solubilization of mutants

Mini-Tn5 derivative transposon random mutagenesis was used to create a mutant library of strains with mutation in genes so that the ability of *Klebsiella* sp. GMD08 to solubilize insoluble mineral-phosphate was impaired. 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 arbitrary PCR results as observed in Fig. 5 showed that 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. Using the nucleotide BLAST program, we identified that 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 the similar sequence with malate dehydrogenase encoding gene (*mdh*) (MN179490). Both encoded genes were mostly similar with *Klebsiella quasipneumoniae* strain ATCC 700603 (CP014696.2).
Another previous work has also shown that the *gdh* gene disruption in *Rahnella aquatilis* strain HX2 was partially responsible for its abolished antibacterial phenotype and impaired biocontrol 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 might produce a non-functional gene which affects their expression.

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

**CONCLUSION**

In general, 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. This research also reported that gluconic acid was the main organic acid which was produced by *Klebsiella* sp. GMD08 phosphate solubilizing mechanism. This research demonstrated that arginine repressor (*ArgR*) and malate dehydrogenase (*mdh*) encoding gene were involved in *Klebsiella* sp. GMD08 phosphate solubilizing mechanism.
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