ISOLATION AND CHARACTERISATION OF THE ALKALINE PHOSPHATASE FROM PHOSPHATE-SOLUBILISING BACTERIA ISOLATED FROM THE SOIL OF DANUM VALLEY RAINFOREST, SABAH

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BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
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HERMAN UMBAU ANAK LINDANG

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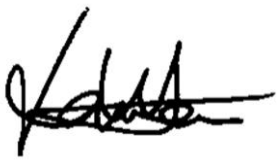

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ABSTRACT

Despite the essential roles of soil phosphate for plant growth, only 0.1% of soil phosphates are available for direct uptake by plants. This leads to the high use of phosphate chemical fertiliser to promote plant growth. The development of bio-fertiliser for replacing phosphate chemical fertiliser requires highly active phosphate-solubilising bacteria (PSB). It is widely reported that soil PSB profiles are closely related to the geological character of the soil. Therefore, the unique PSB is possibly available from the soil with unique characteristics. While the Danum Valley (DV) of Sabah exhibited low base saturation and strongly leached Acrisols soils, it is hypothesised that DV soil may harbour novel microorganisms producing phosphatase that can thrive in the complex soil condition and exhibit unique functional and structural enzyme characteristics. This study is aimed to screen, isolate phosphate-solubilising bacteria (PSB) from DV soil and characterise their alkaline phosphatases. To address, PSB screening was performed using a selective medium, NBRIP agar, which resulted in five PSB isolates displaying remarkable phosphate solubilising activity. Further molecular identification revealed that the five isolates were *Bacillus* sp. PSB01, *Pseudomonas oryzyhabitans* PSB02, *Staphylococcus pasteuri* PSB03, *Paenibacillus* sp. PSB04, and *Staphylococcus pasteuri* PSB05. The combination (consortium) of these PSB was then proven to promote plant growth in *Oryza sativa* and *Brassica rapa* var. *parachinensis* under a sterile soil growth medium. One contributor to the activity of these five PSB to promote plant growth is their phosphatase activity. While the structure and function of phosphatases of *Bacillus*, *Pseudomonas* and *Staphylococcus* were extensively studied, to date, no report on the phosphatase of the *Paenibacillae* family. Accordingly, PSB04 was further studied genomically to identify the genetic regulation behind its phosphatase activity. The whole genome sequence of PSB04 revealed the existence alkaline phosphatase gene (AP-PSB04) but no acid phosphatase, which is predicted to be the main contributor to the phosphatase activity of this strain. Further *in silico* analysis revealed that PSB04 is about 44 kDa in size and secreted through the Sec pathway. The catalytic mechanism of AP-PSB04 is unique due to the absence of canonical Lys residue at the substrate binding cavity. Further, the protein was in dimeric structure both in the structural model and size exclusion chromatography analysis. Recombinant AP-PSB04 was produced using *the E. coli* system and used for further analysis, which revealed that this protein is highly active with a specific activity of 395070.35 U/mg against *the p*NPP substrate. The optimum temperature and pH for the activity of this enzyme were found to be 70 ºC and pH 8.0, respectively. The presence of Zn^{2+} metal ion has remarkably enhanced the activity of AP-PSB04 by 100% and completely abolished by Mn, EDTA or EGTA. The isothermal titration calorimetry (ITC) experiment further revealed that two binding events of metal ions might occur in AP-PSB04, involving six Zn ions per dimeric molecule per event. Further, a unique structural segment (Asp307-Thr405) of AP-PSB04, namely a crown-like domain, was found to play a major role in the metal ion binding preferences. This crown-like domain demonstrated essential roles in the stability of dimerisation and thermal stability with no serious effect on the catalytic activity. Altogether, this study

provides the first-ever PSB isolated from the Sabah rainforest soil, promising to be further applied as a plant growth promoter. Besides, the alkaline phosphatase produced from the PSB04 strain is also promising for further studies and industrial applications.

ABSTRAK

ISOLASI DAN PENCIRIAN ENZIM BAKTERIA PELARUT FOSFAT ALKALI DIPENCILKAN DARIPADA TANAH HUTAN HUJAN TROPIKA LEMBAH DANUM, SABAH

Meskipun nutrient fosfat adalah satu daripada makronutrien yang diperlukan oleh tumbuhan untuk ketumbesaran, hanya 0.1% sahaja fosfat tanah yang mampu di serap secara langsung oleh tumbuhan.Oleh itu, pengunaan baja kimia fosfat yang banyak telah digunakan untuk merangsang pertumbuhan tanaman. Sehubungan dengan itu, pembangunan baja biologi yang terdiri daripada bakteria yang merembes enzim pelarut fosfat yang tinggi kadar aktiviti enzimnya. Mikrobakteria pula lazimnya berkait rapat dengan profil geologi tanah. Bakteria pelarut fosfat tersebut kemungkinan boleh didapati darpada tanah yang mempunyai ciri-ciri yang tersendiri. Hutan Hujan Tropika Lembah Danum (LD) mempunyai tanah Akrisoil yang bersifat ketepuan bes yang rendah dan larut lesap yang kuat. Lantaran itu, LD kebarangkalian mempunyai mikroorganisma yang merembes enzim pelarut fosfat yang novel. Enzim pelarut fosfat tersebut di anggarkan mampu berfungsi dalam ekosistem tanah yang kompleks, serta mempunyai sifat fungsi dan struktur bentuk yang unik. Maka, kajian ini bertujuan untuk memencilkan dan mencirikan enzim pelarut fosfat alkali yang di hasilkan oleh bakteria tersebut. Untuk mengutarakan tujuan yang dinyatakan tersebut, bakteria yang merembeskan enzim pelarut fosfat daripada sampel tanah telah dipencilkan menggunakan medium kultur agar-agar NBRIP dan pencilan molekul telah mencirikan lima pencilan bakteria iaitu Bacillus sp. PSB01, Pseudomonas oryzyhabitans PSB02, Staphylococcus pasteuri PSB03, Paenibacillus sp. PSB04, dan Staphylococcus pasteuri PSB05. Hasil dapatan kajian menunjukkan gabungan kesemua pencilan bakteria tersebut mampu meransang pertumbuhan Oryza sativa dan Brassica rapa var. parachinensis yang di tanam menggunakan tanah yang steril. Salah satu daripada pemangkin kepada kelima-lima pencilan tersebut adalah aktiviti enzim fosfat yang di telah rembeskan oleh bakteria tersebut. Sehingga kini, terdapat hasil kajian sebelum ini yang telah mengesahkan kefungsian dan struktur enzim larut fosfat daripada Bacillus, Pseudomonas dan Staphylococcus tetapi bukan daripada Paenibacillae. Lantaran itu, bakteria PSB04 telah di pilih untuk pencirian regulasi genetic untuk aktiviti enzim fosfat. Penjujukan genom penuh PSB04 adalah bersaiz 7,323,160 pb serta telah mengenal pasti satu gen enzim pelarut fosfat alkali (AP-PSB04) protein bersaiz 44 kDa yang dirembes keluar sel bakteria melalui laluan Sec. Aktiviti mangkinan AP-PSB04 adalah uni kerana ketiadaan kanonika residue Lys pada kaviti ikatan substrat. Struktur model homologi serta kromatografi pengasingan saiz juga telah mengesahkan AP-PSB04 adalah protein homodimer. Protein rekombinan AP-PSB04 telah berjaya di hasilkan melalui sistem E. coli dan aktiviti khusus AP-PSB04 adalah 395070.35 U/mg mengunakan substrat pNPP. Suhu optima dan pH untuk aktiviti enzim AP-PSB04 adalah masing-masing 70 ^{o}C dan pH 8.0. Sehubungan itu, kehadiran ion logam Zn^{2+} juga turut meningkatan aktiviti enzim AP-PSB04 kepada 100% dan kehadiran ion logam Mn, EDTA serta EGTA juga telah memusnahkan aktiviti enzim AP-PSB04 secara kesuluruhan. Analisis

tenaga pengikatan menggunakan kalorimetri titratan isoterma (KTI) menunjukkan 6 ion Zn per dimer. Menariknya, protein AP-PSB04 ini mempunyai struktur enzim yang unik (Asp307 - Thr405) dan bersepadan dengan domain protein mahkota. Domain tersebut adalah penting untuk kestabilan terma dan pendimeran protein serta tiada kesan yang serius terhadap aktiviti enzim AP-PSB04. Tuntasnya, kajian ini menghasilkan bakteria pelarut fosfat yang pertama di isolasi daripada tanah hutan di Sabah, mempunyai potensi untuk dijadikan sebagai pemangkin kadar pertumbuhan tumbuhan serta diaplikasikan kepada industri sedia ada.

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AcP	-	Acid Phosphatase
AP	-	Alkaline Phosphatase
AP-PSB04	-	Alkaline Phosphatase of PSB04 Strain
BLASTn	-	BLAST Nucleotide
CD	-	Circular Dichroism
COGs	-	Cluster of Orthologous Groups
DAI	-	Days After Inoculation
DV	-	Danum Valley
ECAP	-	*Escherichia coli* Alkaline Phosphatase
gDNA	-	Genomic Deoxyribonucleic acid
HI	-	Harvest Index
IAA	-	Indole Acetic-Acid
IAP	-	Intestinal Alkaline Phosphatase
LB	-	Luria Bertani broth
NB	-	Nutrient Broth
NBRIP	-	National Botanical Research Institute Phosphate Growth Media
NCBI	-	National Centre For Biotechnology Information
NSAP	-	Non-specific acid phosphatase
P	-	Phosphorus
PAP∆WL	-	Mutant Variant of AP-PSB04
PCR	-	Polymerase Chain Reaction
PGPB	-	Plant Growth Promoting Bacteria

PGPR	-	Plant Growth Promoting Rhizobacteria
Pi	-	Inorganic Phosphorus
PLAP	-	Placental Alkaline Phosphatase
Po	-	Organic Phosphorus
PSB	-	Phosphate Solubilising Bacteria
RAST	-	Rapid Annotation Using Subsystem technology
SDS-PAGE	-	Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis
SEM	-	Scanning Electron Microscope
SNP	-	Ingle Nucleotide Polymorphism
TB	-	Terrific Broth
VAP	-	*Vibrio* Alkaline Phosphatase
WB	-	Western Blot
WGS	-	Whole Genome Sequence
YASARA	-	Yet Another Scientific Artificial Reality Application
ΔG	-	Gibbs Energy

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