

**CHARACTERIZATION OF NEWLY ISOLATED  
PROTEASE PRODUCING MARINE BACTERIA  
AND EXPRESSION OF A NEUTRAL PROTEASE  
FROM *Bacillus* sp PPB15 ISOLATED FROM  
MANGROVES IN SABAH**



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UNIVERSITI MALAYSIA SABAH

**BIOTECHNOLOGY RESEARCH INSTITUTE  
UNIVERSITI MALAYSIA SABAH  
2012**

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**THIS THESIS SUBMITTED IN FULLFILLMENT  
FOR THE DEGREE OF DOCTOR OF  
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## DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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Shuhadah bte Mustapha  
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## ABSTRACT

### CHARACTERIZATION OF NEWLY ISOLATED PROTEASE PRODUCING MARINE BACTERIA AND EXPRESSION OF NEUTRAL PROTEASE FROM *Bacillus* sp PPB15 ISOLATED FROM MANGROVES IN SABAH

A total of 112 species of marine bacteria were isolated from the mangrove habitats along the east coast of Sabah, East Malaysia. Eighteen of these isolates were protease producing bacteria (PPB). Molecular identification of these protease producing bacteria based on 16S rDNA was carried out in order to facilitate the identification of the bacterial strains. PPB1, PPB6, PPB11 and PPB13 were identified as *Bacillus cereus* with 99% similarity, whereas PPB3 with 99% similarity with *Proteus mirabilis* H4320. Strain PPB2, PPB4 and PPB10 have shown similarity with *Bacillus* GIDM. Strain PPB5, PPB7 and PPB18 were identified as *Bacillus megaterium* whereas PPB9 and PPB14 were classified as *Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305. Strain PPB16 and PPB17 have shown that these species were 99% similar to *Bacillus* sp CNJ845PL04. Strain PPB8, PPB12 and PPB15 have shown 99% similarity with *Bacillus* sp 41 KBZ.

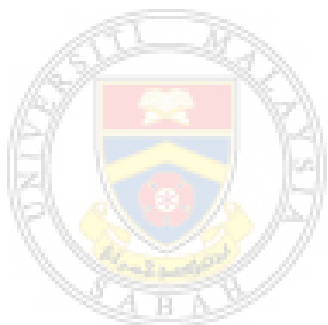
Assays for total protein and proteases activity of these isolated PPBs were conducted. Results on the protease activity study showed that *Proteus mirabilis* PPB3, *Bacillus* sp PPB8, *Bacillus* sp PPB15 and *Bacillus megaterium* PPB18 exhibited the highest protease activity with reading of 0.63, 0.61, 0.64 and 0.62 U/ml respectively. These strains grew up to 50°C with a broad pH range between 5 to 7.5. The optimal temperature and pH for growth were 35°C and 5.0 respectively.

A study to determine the effect of various protease inhibitors, namely phenylmethylsulfonyl fluoride (PMSF), Pepstatin A, E-64 (trans-epoxysuccinyl-Leucylamido(4-guanidino) butane) and EDTA on the activity of these proteases clearly indicated the compound E-64 inhibited the protease activity of isolates from *Bacillus* sp strain PPB15 to a significant degree. EDTA and Pepstatin inhibited the protease isolated from *Bacillus megaterium* strain PPB 18. PMSF had no significant effect on the proteases derived from all PPBs. These results implied that the proteases derived from bacterial *Bacillus* sp PPB12, *Bacillus* sp PPB15 and *Bacillus megaterium* PPB18 can be categorized as belonging to the family of acidic mesophilic proteases and metalloprotease mesophilic proteases.

Further characterization of the proteases was carried out by utilising seven different types of *p*-nitroanilide synthetic substrates. Results have shown that the amino acids in the position P<sub>1</sub> have a strong influence on the catalytic activity of proteases. The neutral protease derived from *Bacillus* sp PPB15 indicated preference for Leucine, phenylalanine and arginine at position P<sub>1</sub> and exhibited high activity for Sar-Pro-Arg-pNA dihydrochloride (1.05 Units/ml enzyme) L-Leucine-pNA (0.74 Units/ml enzyme), *N*-Suc-Ala-Ala-Pro-Leu-pNA (0.83 Units/ml enzyme), and *N*-Suc-Gly-Gly-phe-pNA (0.44 Units/ml enzyme). Lower activity was observed when Ala or Gly was the amino acids residues at position P<sub>1</sub>, notably the *N*-Suc-gly-gly-gly-pNA or *N*-Suc-Ala-Ala-Ala-pNA. The K<sub>m</sub> value for L-

Leucine-pNA and N-Suc-gly-gly-gly-pNA as substrate were 3.31 $\mu$ m and 18.50 $\mu$ m, respectively. The corresponding  $V_{max}$  value were 78.31 $\mu$ M/min and 3.58 $\mu$ M/min, respectively.

Two pairs of gene specific primers were designed to target the neutral protease genes of *Bacillus* sp PPB15 and *Bacillus* sp PPB12. PCR generated an amplicon of around 1638bp, which confirmed the identity of a neutral protease B in the genome of *Bacillus* sp PPB15. The protease gene was cloned in to pEXP5-NT vector which was expressed in *E.coli* BL21(DE3) under control of T7 promoter. SDS-PAGE analysis showed a strong neutral protease gene expression after induction by 1 mM IPTG for 5 hrs at 37°C with molecular mass approximately of 62 kDa. Further investigation on the activity of purified protease from recombinant protein (pEXP5NT-*NptB*) indicated that the protease activity was at 1.3U with the concentration of 0.625 ug.



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## ABSTRAK

Sebanyak 112 spesis bakteria marin telah dipencilkan daripada habitat ekosistem paya-bakau di sepanjang Pantai Timur Sabah, Malaysia Timur. Lapanbelas daripada isolat merupakan bakteria pengeluar protease. Identifikasi secara molikul terhadap bakteria penghasil protease berdasarkan jujukan 16S rDNA telah dijalankan bagi memudahkan pengenalanpastian setiap strain bakteria. PPB1, PPB6, PPB11 and PPB13 telah dikenalpasti sebagai *Bacillus cereus* dengan 99 % kesamaan, manakala strain PPB3 mempunyai 99% kesamaan dengan strain *Proteus mirabilis* H4320. Strain PPB2, PPB4 dan PPB10 menunjukkan kesamaan dengan *Bacillus* sp. *GIDM*. Tiga strain iaitu PPB5, PPB7 dan PPB18 telah dikenalpasti sebagai *Bacillus megaterium* manakala strain yang lain seperti PPB9 dan PPB14 dikelaskan dengan *Staphylococcus saprophyticus* subsp. *Saprophyticus* ATCC 15305. Strain PPB16 dan PPB17 menunjukkan spesis ini hampir sama dengan homologi sebanyak 99% kepada *Bacillus* sp CNJ845PL04. Strain PPB8, PPB12 dan PPB15 menunjukkan 99% kesamaan dengan *Bacillus* sp 41 KBZ.

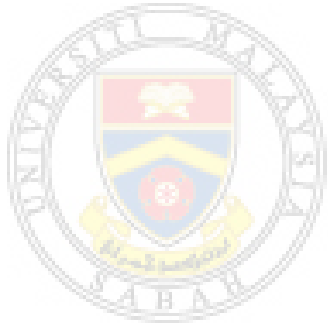
Analisis jumlah protin dan aktiviti protease telah dijalankan keatas kesemua strain PPB. Hasil kajian ke atas *Proteus mirabilis* PPB3, *Bacillus* sp PPB8, *Bacillus* sp PPB15 dan *Bacillus megaterium* PPB18 terhadap aktiviti protease menunjukkan aktiviti protease yang tertinggi dengan bacaan masing-masing 0.63, 0.61, 0.64 dan 0.62 U/ml. Kesemua strain ini dapat tumbuh sehingga suhu 50°C dan dengan pH di antara 5.0 ke 7.5. Suhu dan pH yang optima bagi tumbesaran adalah masing-masing pada 35°C dan 5.0.

Kajian kesan beberapa jenis perencat protease seperti phenylmethylsulfonylflouride (PMSF), Pepstatin A, E-64 (trans-epoxysuccinyl-Leucylamido(4-guanidino) butane dan EDTA ke atas aktiviti protease ini, menunjukkan bahawa E-64 didapati merencat aktiviti protease *Bacillus* sp strain PPB15 pada paras yang signifikan. EDTA merencat protease pada strain *Bacillus megaterium* PPB18. PMSF tidak ada kesan perencatan yang nyata terhadap aktiviti protease daripada kesemua PPB. Hasil kajian ini menunjukkan bahawa protease daripada *Bacillus* sp PPB12, *Bacillus* sp PPB15 dan *Bacillus megaterium* PPB18 dapat di kategorikan berasal daripada keluarga protease mesofilik asidik dan protease mesofilik metalloprotease.

Kajian pencirian lanjut ke atas protease yang dihasilkan telah dijalankan dengan menggunakan tujuh jenis substrat sintetik daripada *p*-nitroanilide. Hasil kajian telah menunjukkan bahawa asid amino pada kedudukan P<sub>1</sub> mempunyai pengaruh yang kuat terhadap aktiviti katalitik protease. Protease neutral daripada strain *Bacillus* sp PPB15 menunjukkan kecenderungan kepada substrat Leucine, Phenylalanine dan Arginine asid amino pada kedudukan P<sub>1</sub> dan menghasilkan aktiviti tertinggi untuk Sar-Pro-Arg-pNA dihydrochloride (1.05 Units/ml enzim) L-Leucine-pNA (0.74 Units/ml enzim), N-Suc-Ala-Ala-Pro-Leu-pNA (0.83 Units/ml enzim), dan N-Suc-Gly-Gly-phe-pNA (0.44 Units/ml enzim). Aktiviti rendah diperhatikan apabila Ala atau Gly sebagai residu asid amino pada kedudukan P<sub>1</sub>, ini terutamanya bagi N-Suc-gly-gly-gly-pNA atau N-Suc-Ala-Ala-pNA. Nilai K<sub>m</sub> adalah masing-masing 3.31µm dan 18.50µm dengan L-Leucine-pNA dan N-Suc-gly-gly-gly-pNA sebagai substrat, manakala nilai V<sub>max</sub> adalah masing-masing

*78.31µM/min dan 3.58µM/min dengan L-Leucine-pNA dan N-Suc-gly-gly-gly-pNA sebagai substrat.*

*Dua pasang primer spesifik gen telah direkabentuk bagi memilih gen protease neutral daripada Bacillus sp PPB15. PCR mengamplifikasi amplicon pada saiz 1638bp, dimana pengesahan identiti protease B neutral di dalam genom Bacillus sp PPB15. Gen protease diklonkan dalam vektor pengklonan TOPO (pEXP5-NT) dizahirkan dalam E.coli DE3 dibawah kawalan promoter T7. Analisa SDS-PAGE menunjukkan gen protease neutral dizahirkan amat ketara dengan jisim molekul bersaiz 62 kDa setelah induksi dengan 1 mM IPTG selama 5 jam pada suhu 37°C. Protease rekombinan ini ditulenkan, hasil analisa menunjukkan bahawa protease rekombinan menghasilkan aktiviti protease pada 1.3U dengan kepekatan 0.625 ug.*



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