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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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 phenylmethylsulfonylflouride (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 P1 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 P1 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 P1, notably the N-Suc-gly-gly-gly-pNA or N-Suc-Ala-Ala-Ala-pNA. The K_m value for L-
Leucine-pNA and N-Suc-gly-gly-gly-pNA as substrate were 3.31μm and 18.50μm, respectively. The corresponding $V_{\text{max}}$ value were 78.31μM/min and 3.58μM/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-NprB) indicated that the protease activity was at 1.3U with the concentration of 0.625 ug.
ABSTRAK

Sebanyak 112 spesis bakteria marin telah dipencilkkan 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 pengenalpastian setiap strain bakteria. PPB1, PPB6, PPB11 dan 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 phenylmethylsulfonylfouride (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 P₁ 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 P₁ 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 P₁, ini terutamanya bagi N-Suc-gly-gly-gly-pNA atau N-Suc-Ala-Ala-Ala-pNA. Nilai $K_m$ adalah masing-masing 3.31μm dan 18.50μm dengan L-Leucine-pNA dan N-Suc-gly-gly-gly-pNA sebagai substrat, manakala nilai $V_{max}$ 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 amplikon pada saiz 1638bp, dimana pengesahan identiti protease B neutral di dalam genom Bacillus sp PPB15. Gen protease dikeluarkan 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 ditularkan, hasil analisa menunjukkan bahawa protease rekombinan menghasilkan aktiviti protease pada 1.3U dengan kepekatan 0.625 ug.
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Enzymes are undoubtedly the most efficient and environmentally-friendly protein catalyst known to have ever been synthesized by living systems. Their significant advantages clearly overrides the chemical catalysts in terms of specificity, high catalytic activity, ability to work at moderate temperatures, and the ability to produce in large amounts.

The current demand for better utilization of renewable resources and pressure exerted on certain industries to operate within environmentally compatible limits led to the onset of the inevitably new enzyme-catalyzed industrial processes (Barredo, 2005). Worldwide sales of industrial enzymes are estimated to be approximately $USD 4.8 billion with an annual growth of about 6.5 to 10% of which 75% are hydrolytic enzymes (Shikha and Darmwal, 2007). The Proteases represent one of the three largest groups of industrial enzymes and account for about 65% of the total worldwide sales of enzymes (Shikha and Darmwal, 2007).

Executing a large variety of functions, the proteases activities extend far beyond the cellular level to the organ and organism level with the sole purpose of producing cascade systems such as homeostasis and inflammation. They are particularly responsible for the complex processes involved in the normal physiology of cells as well as in the somewhat abnormal pathophysiological conditions. Their involvement in the life cycle of disease-causing organisms have ultimately led them to become a visibly potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS.

In the food industry, the proteases portray a major role in nutritional processes due to their predominantly unusual ability in activities involving depolymerization. Additionally, their outstanding applications in the detergent industries have never seized to be overlooked. Similar applications of proteases in the leather industry for the dehairing and bathing purposes are clearly preferred as a substitute to the currently used toxic chemicals. Lately however, there has been
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