# Isolation, phylogeny and characterisation of proteases and *p*-hydroxyphenylacetic acid hydroxylase from thermophilic *Geobacillus* strains from Buranga Hot Springs in Uganda

by

**JOSEPH HAWUMBA**

Submitted in partial fulfilment of the requirements of the degree
Philosophiae Doctor
in the Faculty of Natural and Agricultural Sciences
University of Pretoria
Pretoria

**August 2003**

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## DECLARATION

I declare that the thesis, which I hereby submit for the degree, Philosophiae Doctor (Microbiology) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another University.

Signed: ..... Date: 05/08/2003

## DEDICATION

To my parents; Pr. W.W. Hawumba and Suzan Hawumba, Charles Mukiibi and Kezia Mukiibi, Mr.and Mrs. Esau Kato, brothers and sisters, and, above all, my lord God.

## SUMMARY

# Isolation, phylogeny and characterisation of proteases and *p*-hydroxyphenylacetic acid hydroxylase from thermophilic *Geobacillus* strains from Buranga Hot Springs in Uganda

by

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Enzymatic processes that can be run at high temperatures are attractive, as the reaction rates and the substrate solution is often increased. Consequently, there is a continuous search for new thermostable enzymes with the required technological properties. In this study, two thermophilic bacterial isolates, *Geobacillus* PA-9 and PA-5, obtained from the Buranga hot springs in western Uganda, were characterised with the specific aim of isolating and characterising genes encoding novel enzymes.

Both bacterial isolates grew at an optimum temperature and pH of 60°C and 7.5-8.5, respectively, and zymogram analyses indicated that the isolates produced two (isolate PA-5) or more (isolate PA-9) extracellular protease enzymes. The optimum temperature and pH for casein-degrading activity were 70°C, pH 6.5 for isolate PA-9, but caseinolytic activity could also be observed at 2°C. Isolate PA-9 was thus selected for further characterisation. Although various strategies were used to isolate the protease-encoding genes, including enzyme purification and functional screening of a constructed genomic DNA library in *Bacillus megaterium* and in *Escherichia coli*, none resulted in the isolation of the desired

genes. The inability to purify the protease(s) may suggest that low amounts of the protease(s) are being produced or that the protease(s) may be distinct from other characterised proteases.

A clone containing the gene encoding the hydroxylase involved in the degradation of 4-hydroxylphenylacetic acid was, however, isolated from the *Geobacillus* sp. PA-9 genomic DNA library. Sequence analysis indicated the presence of three novel open reading frames (ORFs) of which *PheH* exhibited homology to several 4-hydroxyphenylacetate 3-hydroxylases (4-HPA hydroxylase), *PheH2* appeared to be unique and *PheC* exhibited homology to 2,3-dioxygenases. The 4-HPA hydroxylase has an optimum pH and temperature of 9.0 and 50°C, respectively. Purified PheH did not display hydroxylase activity, suggesting that the 4-HPA 3-hydroxylase from *Geobacillus* isolate PA-9 is composed of two proteins with PheH being the hydroxylase and PheH2 serving as a helper protein required for efficient substrate hydroxylation.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| ABC | ATP-binding cassette |
| Ala | alanine |
| $amp^r$ | ampicillin resistance |
| Arg | arginine |
| ATP | adenosine trisphosphate |
| bp | base pair |
| *ca.* | approximately |
| CO | carbon monoxide |
| $CO_2$ | carbon dioxide |
| Cys | cystein |
| °C | degrees Celsius |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside-5'-triphosphate |
| EARS | East African Rift valley Systems |
| *e.g.* | for example |
| EtOH | ethanol |
| 4-HPA | 4-hydroxyphenylacetic acid |
| FAD | flavin adenine dinucleotide |
| Fe | iron |
| Fig. | figure |
| Gly | glycine |
| h | hour |
| $H_2$ | hydrogen |
| His | Histidine |
| $H_2S$ | hydrogen sulphide |
| IPTG | isopropyl $\beta$-D-thiogalactoside |
| kb | kilobase pairs |
| kDa | kilodalton |
| l | litre |
| *lacZ* | $\beta$-galactosidase gene |
| LB-broth | Luria-Bertani broth |
| Lys | lysine |
| M | molar |
| mA | milliampere |
| min | minute |

| | |
|---|---|
| ml | millilitre |
| mM | millimolar |
| Mn | manganese |
| $NAD^+$ | nicotinic adenine dinucleotide |
| nm | nanometer |
| nt | nucleotide |
| OD | optical density |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| pmol | picomole |
| Pro | proline |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| rpm | revolutions per minute |
| s | second |
| SDS | sodium dodecyl sulphate |
| Ser | serine |
| 3,4-DHPA | 3,4-dihydroxyphenylacetic acid |
| TE | Tris-EDTA |
| $tet^r$ | tetracycline resistance |
| Tyr | tyrosine |
| U | units |
| µg | microgram |
| µl | microlitre |
| V | volts |
| v/v | volume per volume |
| w/v | weight per volume |
| X-gal | 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside |

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# RESEARCH COMMUNICATIONS

**Papers published:**

1. **Hawumba, J.F.**, Theron, J. and Brözel, V.S. (2002). Thermophilic protease-producing *Geobacillus* from Buranga hot springs in western Uganda. Current Microbiology 45: 144-150

2. **Hawumba, J.F.**, Theron, J. and Brözel, V.S. (2001). Thermophilic protease-producing *Bacillus* from Buranga hot spring in western Uganda. Proceedings of the 8th NAPRECA Symposium, Nairobi, Kenya.

**Papers submitted:**

1. **Hawumba, J.F.**, Brözel, V.S. and Theron, J. (2003). Nucleotide sequence and biochemical characterization of a 4-hydroxyphenylacetic acid 3-hydroxylase from thermophilic *Geobacillus* sp. PA-9. Submitted for publication: *Applied and Environmental Microbiology*.

**Conference contributions:**

**National conferences:**

1. **J.F. Hawumba**, J. Theron and V.S. Brözel. Characterisation of three protease-secreting thermophilic bacteria from hot springs in western Uganda. BioY2K Combined Millennium Meeting, Grahamstown, South Africa, January 2000 (Poster).

2. **J.F. Hawumba**, J. Theron and V.S. Brözel. Characterisation of extracellular proteases produced by extremely thermophilic *Bacillus* isolates. BioY2K Combined Millennium Meeting, Grahamstown, South Africa, January 2000 (Poster).

**International conferences:**

1. **J.F. Hawumba**, J. Theron and V.S. Brözel. Isolation of thermophilic *Bacillus* producing small proteases active over a wide temperature range from a Ugandan hot spring. American Society for Microbiology Meeting, Orlando, USA, May 2001 (Poster).

2. **J.F. Hawumba**, J. Theron and V.S. Brözel. Thermophilic protease-producing *Bacillus* from Buranga hot spring in Western Uganda. NAPRECA Symposium, Nairobi, Kenya, 28 - 31 August 2001 (Paper presentation and poster).