



**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**



UNIVERSITEIT VAN PRETORIA  
UNIVERSITY OF PRETORIA  
YUNIBESITHI YA PRETORIA

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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: .....

*J. R. Hamman*

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-hydroxyphenylacetic 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 <sup>r</sup>	ampicillin resistance
Arg	arginine
ATP	adenosine triphosphate
bp	base pair
<i>ca.</i>	approximately
CO	carbon monoxide
CO <sub>2</sub>	carbon dioxide
Cys	cystein
°C	degrees Celsius
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside-5'-triphosphate
EARS	East African Rift valley Systems
<i>e.g.</i>	for example
EtOH	ethanol
4-HPA	4-hydroxyphenylacetic acid
FAD	flavin adenine dinucleotide
Fe	iron
Fig.	figure
Gly	glycine
h	hour
H <sub>2</sub>	hydrogen
His	Histidine
H <sub>2</sub> S	hydrogen sulphide
IPTG	isopropyl β-D-thiogalactoside
kb	kilobase pairs
kDa	kilodalton
l	litre
<i>lacZ</i>	β-galactosidase gene
LB-broth	Luria-Bertani broth
Lys	lysine
M	molar
mA	milliampere
min	minute



ml	millilitre
mM	millimolar
Mn	manganese
NAD <sup>+</sup>	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 <sup>r</sup>	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 8<sup>th</sup> NAPRECA Symposium, Nairobi, Kenya.

### Papers submitted:

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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).