A new, sensitive protease-immunoassay tandem assay method for detecting very low levels of microbial activity in water samples.

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Project: Intelligent Dressing for Monitoring Wound Healing

Principal Methods Used in Microbial Detection

Culturing

- Generic media
- Specific media
- Media + enzyme substrates
- Direct sampling
- Concentrated samples by microfiltration

Catalytic (enzymatic)

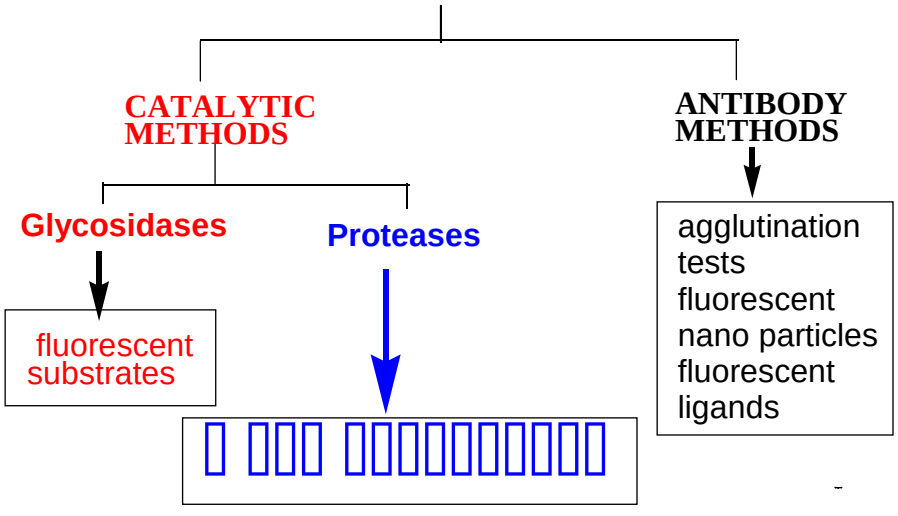
- Substrates for glycosidases
  - Fluorigenic
  - Chromogenic

Antibody-antigen interaction

Nucleic acid probes

Biosensor devices

OPTICALLY-ADDRESSABLE TECHNIQUES FOR RAPID DETECTION OF BACTERIA



Enzymatic (catalytic) methods for detection of bacteria:

History.

Main driving forces:

- Fluorescent synthetic substrates.
- Offer a degree of specificity.

Role in current techniques in water analysis.

Commercial products access.

Advantages.

Catalytic route for detection of bacteria:

- Optically addressable substrates for many enzymes are known.
- In-built amplification of signal.
- Specific enzyme markers for certain organisms.
- Detectable signals, proportional to bacterial load.
- Simpler to couple to bio-sensing physical elements.
- Signal is cumulative with time.

Problems with conventional enzymatic methods in the detection of bacteria and coliforms.

Glycosidases (β-D-galactosidase, β-D-glucuronidase)

Phosphatases

Need to present substrates in excess and in solution.

This has limited the extending the fuller exploitation of the highly sensitive catalytic route.

PROTEASES

- Highly reactive classes of enzymes in all microbes.
- Intracellular and extra-cellular.
- Stable proteins.
- A wide range of suitable fluorescent substrates are available.
- Synthetic & natural protein substrates.
- "*Specific markers*" for certain bacteria ?.
- Level of activity is directly proportional to active bacterial load.

Technical advantages of proteases-dependent detection of bacteria:

- Relatively easy to develop designer substrates.
- Kinetics of protease-substrate action are more favourable to sensor designs.
- Action on soluble and solid phase substrates are similar.

Proteases are classified into four groups on the basis of their catalytic mechanism and the structure of their active site:

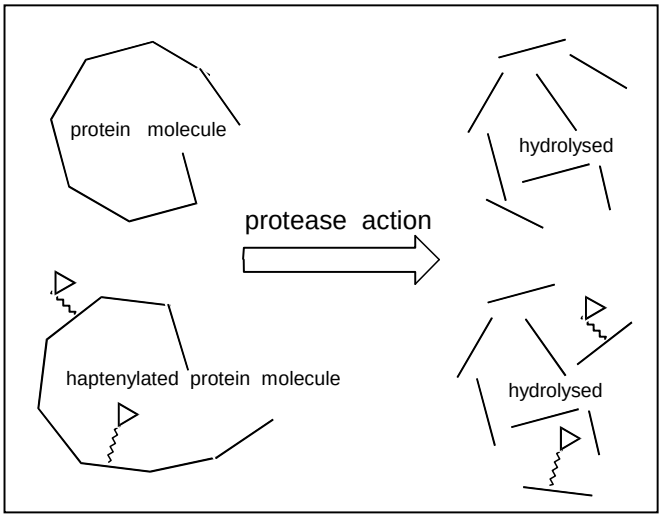
- serine,
- metallo-,
- cysteine, and
- aspartate proteases.

Serine and metalloproteases are most active at neutral pH, and these neutral proteases mediate most extracellular proteolytic events that occur in vivo.

The main role of cysteine and aspartic proteases, which have optimal activity at acidic pH, is in intracellular proteolysis that occurs in the acidic environment of lysosomes.

Our new approach: at this stage

- Haptenylated macromolecule protein substrates.
- Presented as adsorbed on to polystyrene or covalently attached to glass surfaces.
- Protease action or exposure to viable bacterial causes digestion of macromolecule into smaller fragments which detach from surfaces.
- Protease action is detected by probing for attached hapten with anti hapten-enzyme reagents + appropriate substrate.



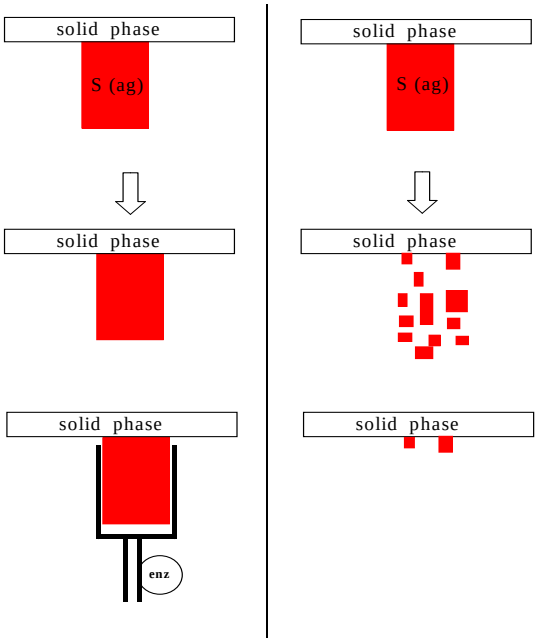
Protein digestion products are taken up by bacteria.

A schematic of assay principle:

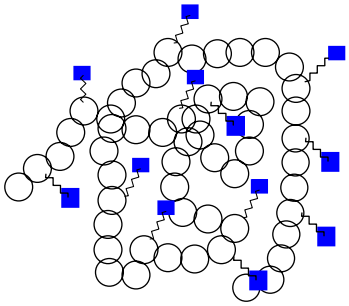
Adsorbed proteins substrate which also presents antigenic moieties (haptens).

Digestion reduces antigen content on surface.

Remainder antigen is measured by labelled antibodies.



Hapten-protein conjugates = protease substrate



Hapten =

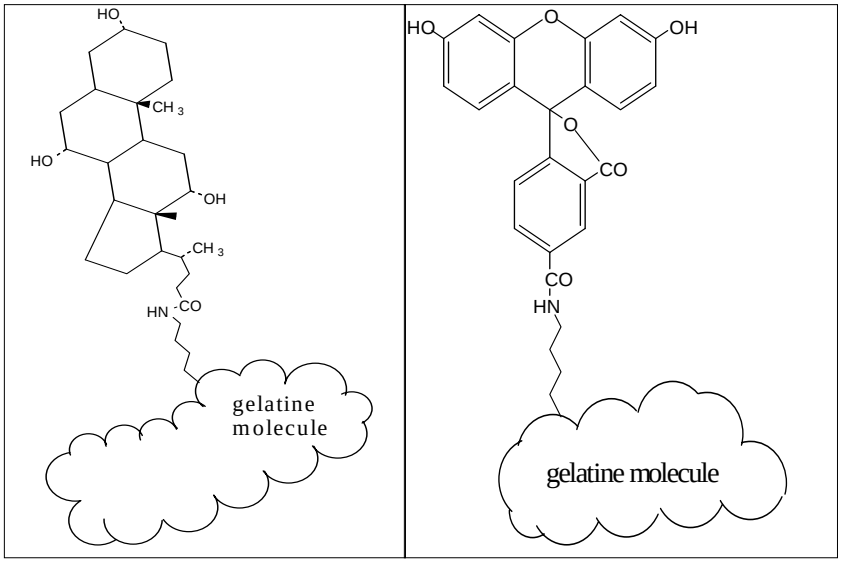
fluorecein

or chloic acid

protein = gelatine

soluble liquid phase presentation

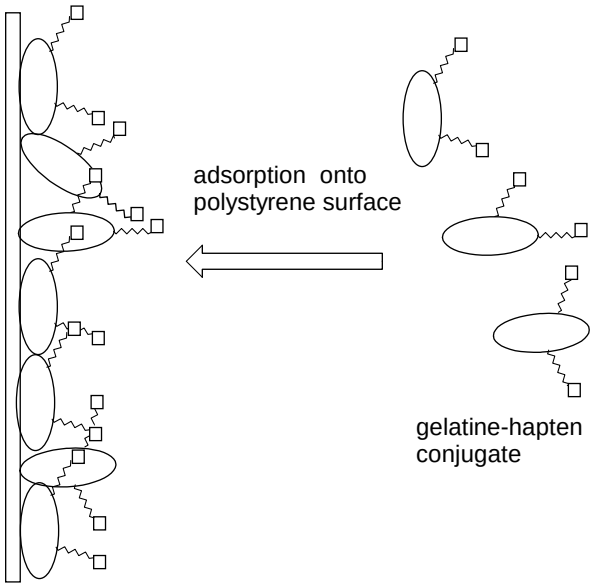
surface-attached solid phase presentation

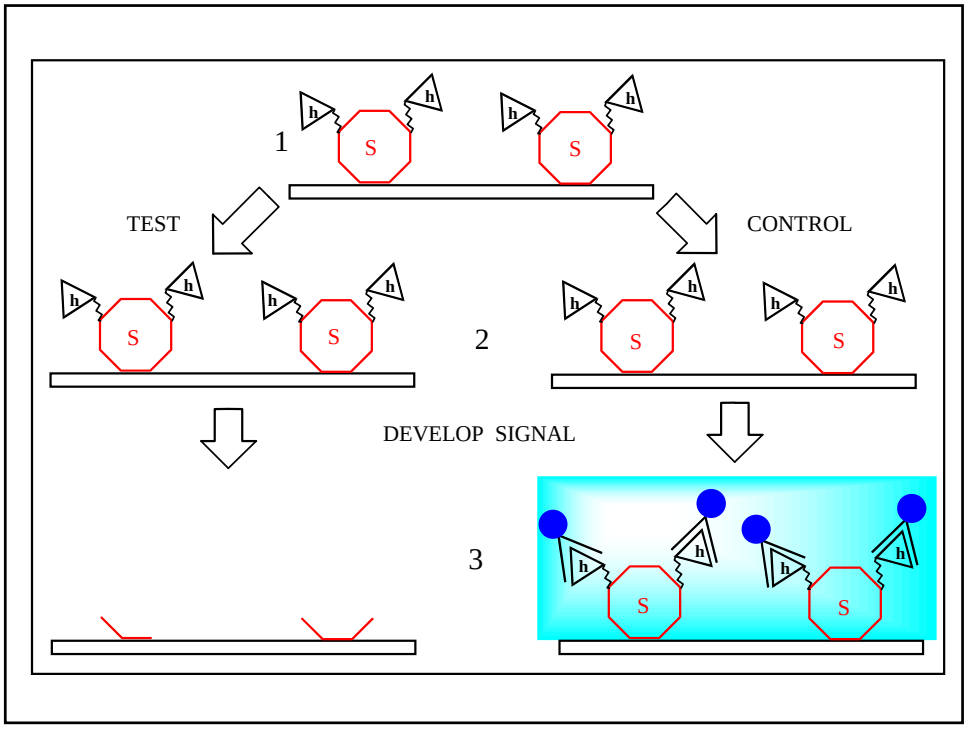


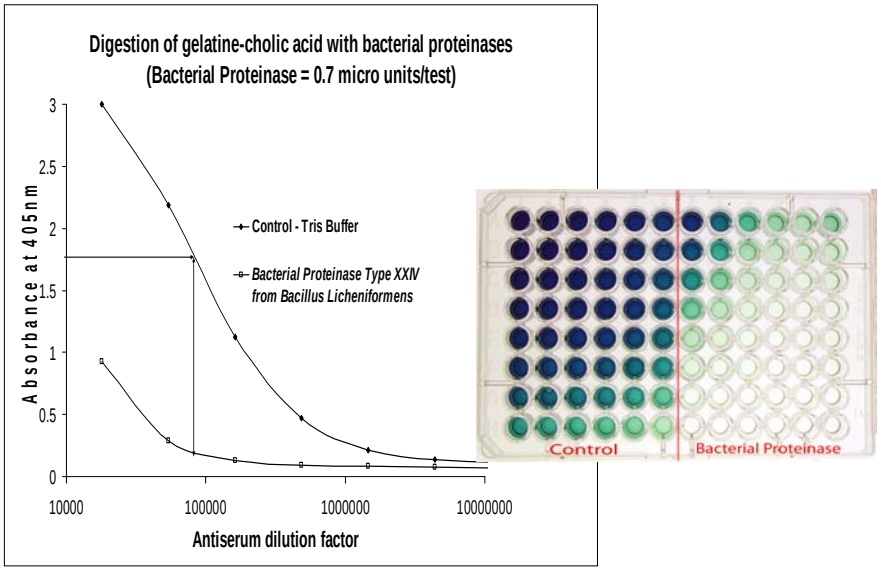
Surface-attached protease substrate.

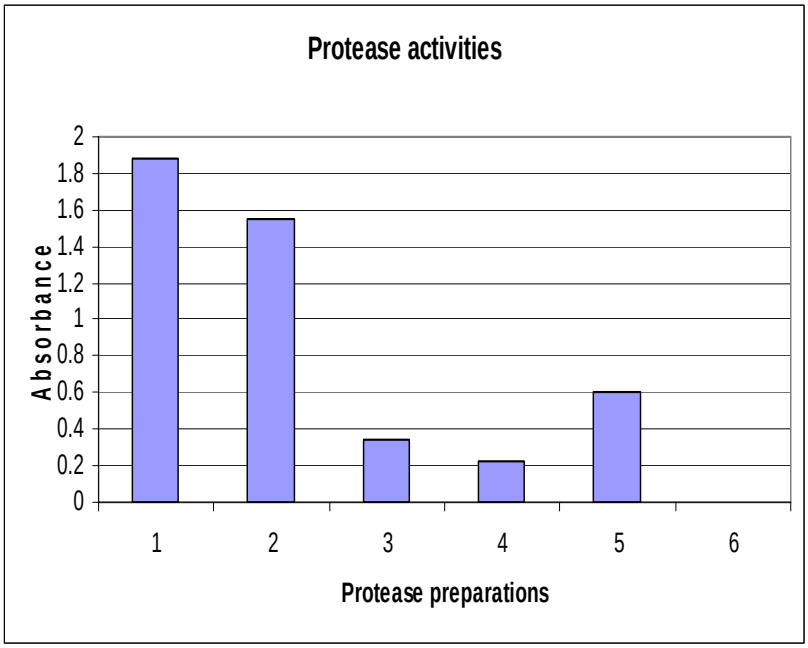
OR

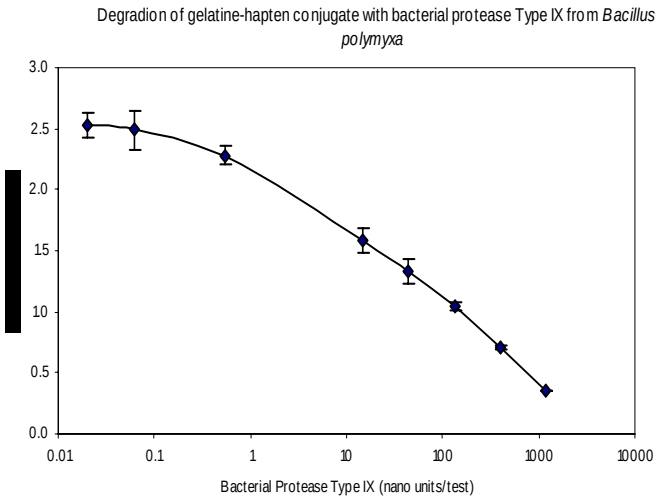
Haptenylated protein.




1
TEST
CONTROL
2
DEVELOP SIGNAL
3
h
S


Digestion of gelatine-cholic acid with bacterial proteinases
(Bacterial Proteinase = 0.7 micro units/test)
Absorbance at 405nm
3
2.5
2
1.5
1
0.5
0
10000
100000
1000000
10000000
Antiserum dilution factor
Control - Tris Buffer
Bacterial Proteinase Type XXIV
from Bacillus Licheniformens
Control
Bacterial Proteinase


Protease activities
Absorbance
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
4
5
6
Protease preparations


Degradion of gelatine-hapten conjugate with bacterial protease Type IX from *Bacillus polymyxa*
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.01
0.1
1
10
100
1000
10000
Bacterial Protease Type IX (nano units/test)

River Samples:

Thames at Waterloo Bridge

London

Tests with samples from The Thames:

Samples collected from river on day of analysis:

Water allowed to settle.

Samples filtered through 0.45 micron filter.

Millipore water used as control samples.

Tap water samples tested at same time.

Coat 1 mL of protein-hapten substrate onto polystyrene tubes

⇩

Add 1 mL of buffered water samples or control solutions

⇩

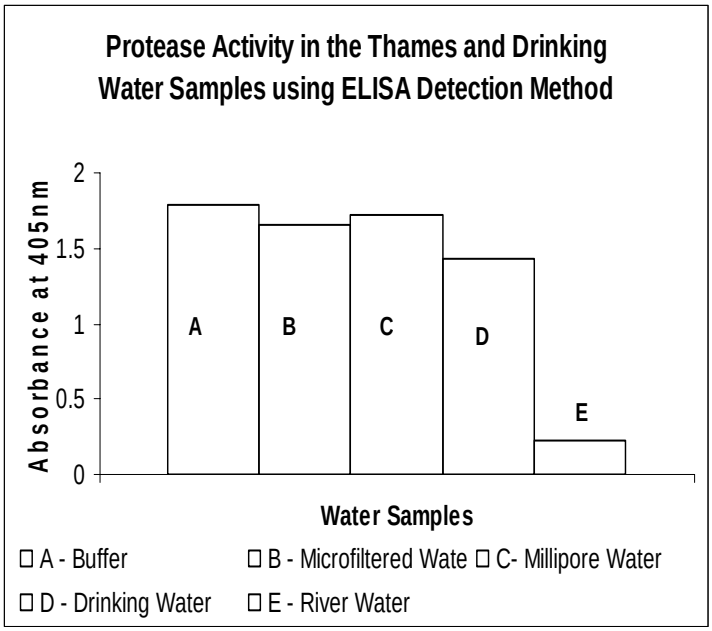
Incubate at 37^{o}C for required period

⇩

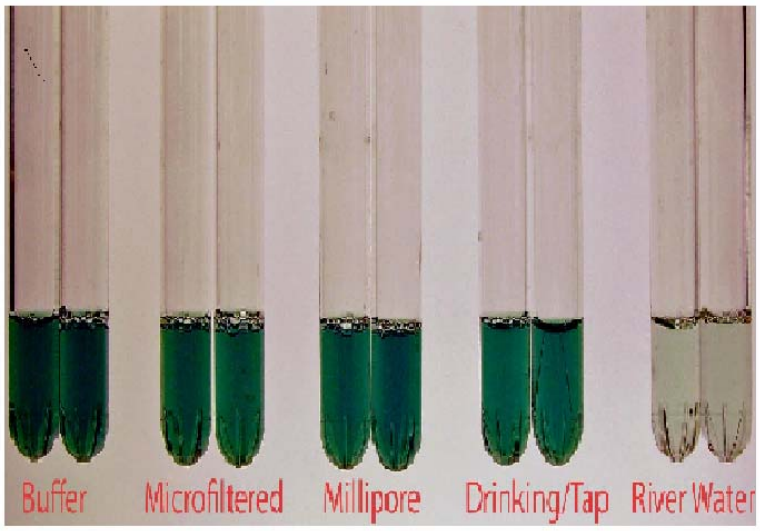
Wash and add enzyme-antibody conjugate and incubate for 1h

⇩

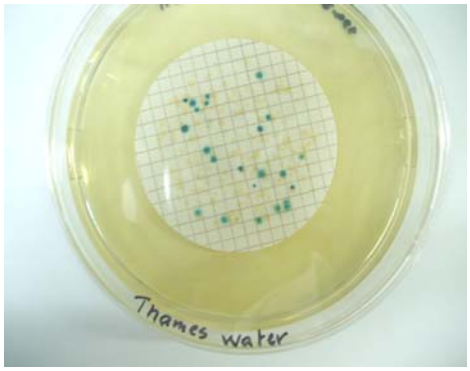
Develop with substrate (20 min) and read absorbance



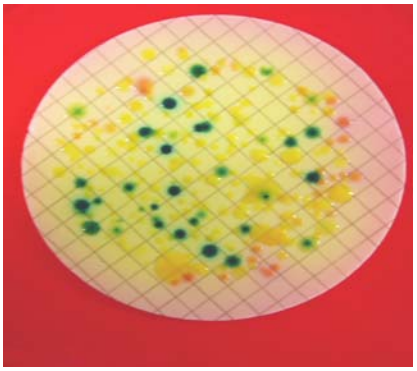
An example of a tube test result



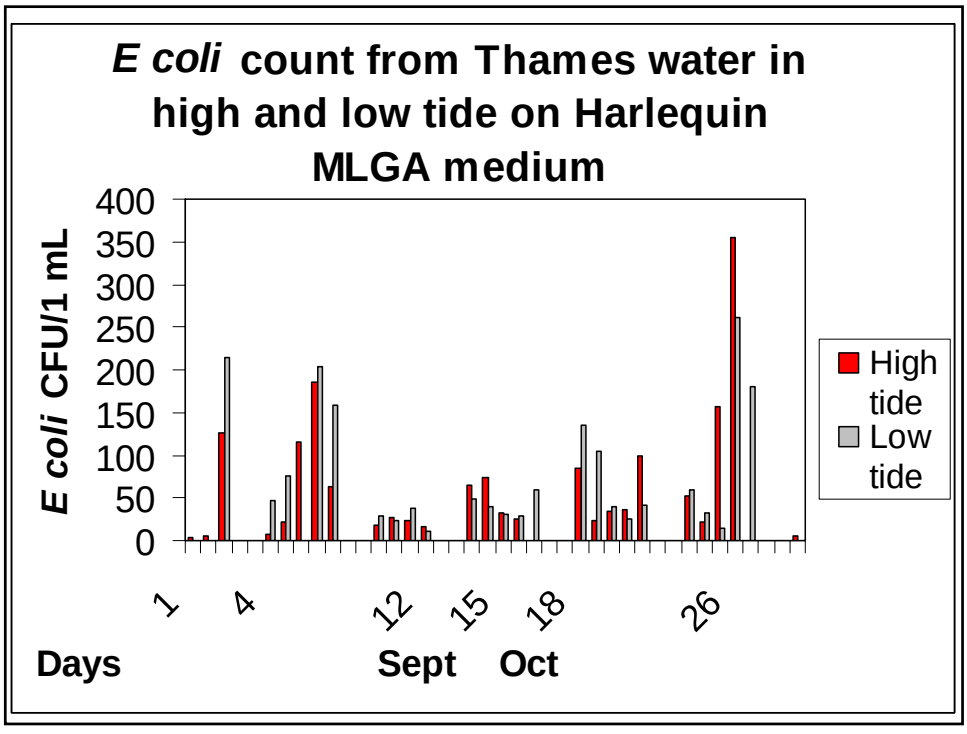
Coliforms/*E. coli* in Thames Water



Chromocult TBX Medium

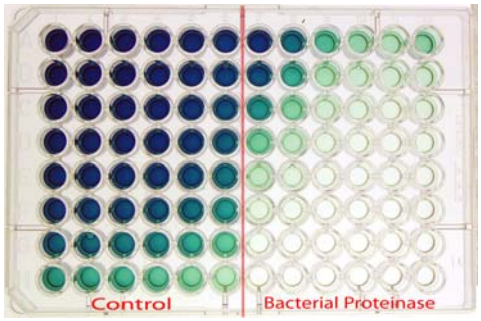


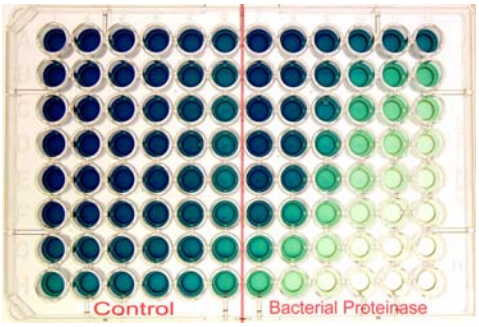
Harlequin mLGA Medium



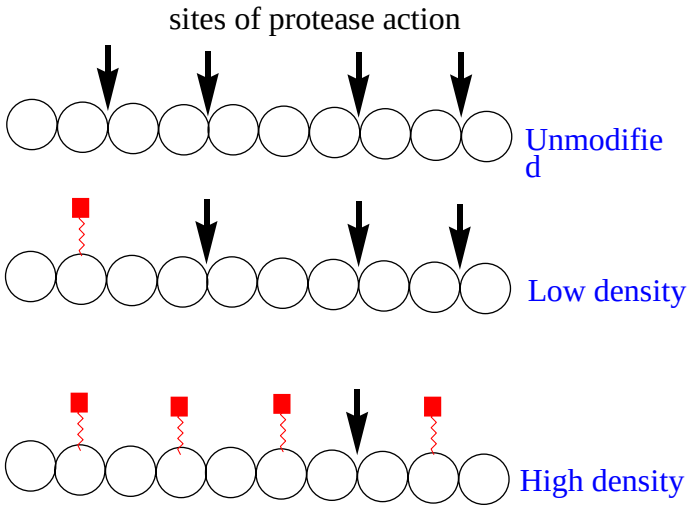
Optimisation of protease substrate structure:

1. Relationship between hapten density in substrate and protease activity.

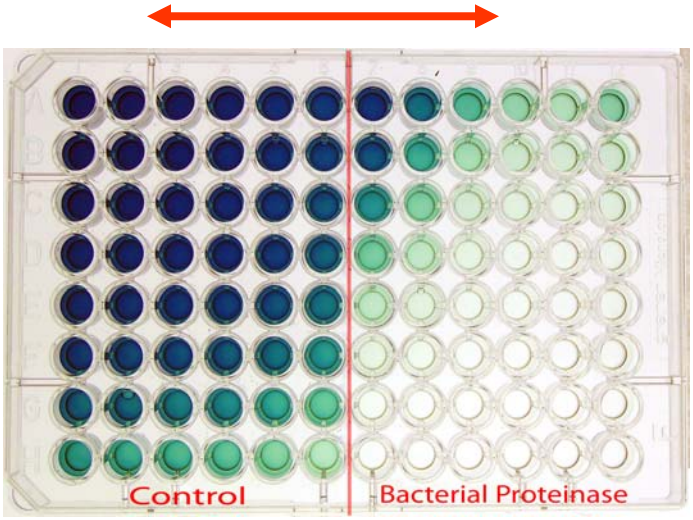




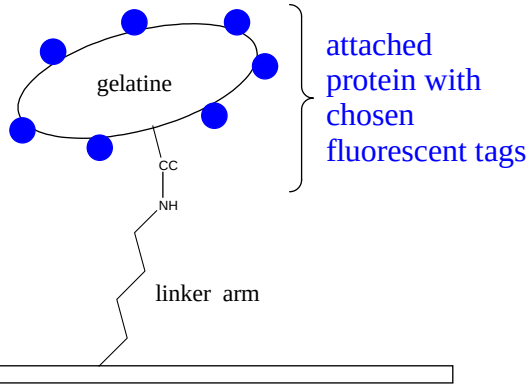
Effect of hapten density



Potential detection sensitivity and test speed:



Ultimate aim: portable or hand-held sensors for rapid detection of viable bacteria in water.



Acknowledgements:

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