VTT BIOTECHNOLOGY

A HIGH THROUGHPUT PROFILING METHOD FOR CUTINOLYTIC ESTERASES

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INTRODUCTION

Cutinases are esterases that can degrade the protective surface polymer (cutin) of aerial parts of plants [1]. They are secreted mostly by phytopathogenic fungi [2, 3], and the major cutinase from *Fusarium solani* f. sp. *pisi* has been thoroughly characterized [4]. Cutinases have been shown to be promising catalysts in a variety of chemical and biotechnological applications due to their ability to operate both in aqueous and nonaqueous environments [5]. Here we describe a novel method for quick detection of cutinolytic esterases for potential utilization.

PLATE SCREENS

The primary screening of cutinolytic esterases from 38 micro-organisms was done on agar plates. Cutin, or a cutin analog, polycaprolactone (PCL), were supplied as the sole carbon source to follow microbial growth and esterase activity. Cutin hydrolysis was monitored via a pH change as a proton is released into the medium during an ester bond hydrolysis (Figure 1). Cutinase is also a PCL depolymerase [6], and a clear halo could be observed upon polymer degradation on PCL plates (Figure 1).

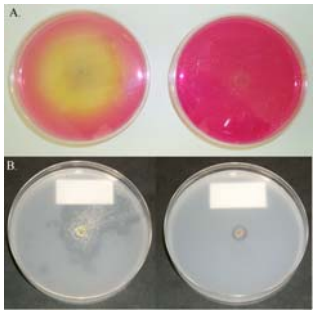

Figure 1. A plate test for cutinases. A. pH drop is followed with Phenol Red (red to yellow) B. PCL degradation (halo) enables strong fungal growth (left) compared to lipolytic yeast (right).

ROBOTIC SCREENS

The automated activity assay method was optimised for a 96-well microtiter plate format allowing high throughput. A common procedure for fast activity screening on ORCA (Optimized Robot for Chemical Analysis) was developed (Figure 2). First, a set of progeny plates are transferred to the substrate dispensing station (Multidrop 1). The reaction is followed at the reading station, after which a stopping solution is added on the reaction plate (Multidrop 2). The plate is transferred again to the reading station, back to the microtiter plate rack, and finally, a new progeny plate is taken for the next operational cycle.

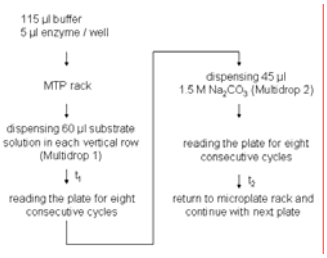

Figure 2. The procedure for automated profiling.

ESTEROLYTIC FINGERPRINTING

p-nitrophenyl (pNP) fatty acids (carbon chain 2 - 16 atoms) were used in robotic screening to explore substrate and pH dependence of enzymes secreted in liquid media by selected micro-organisms. pH dependent isoenzymes were observed at different stages of cultivation, as the action by neutral esterases induced the production of alkaline esterases (Figure 3).

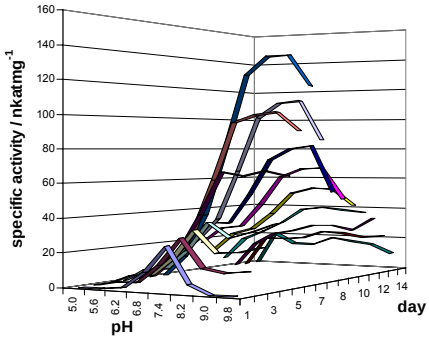

Figure 3. pH profile of pNP-butyrate hydrolysis by *A. alternata* growth media during a 14 day cultivation.

The use of rapid non-specific screens with large variability in the reaction parameters (eg. type of substrate, pH) will result in superior identification and selection of enzymes with desired properties than could be otherwise obtained by any individual screen *per se*. Using this approach, cutinase activity was observed to correlate with the C_4 / C_{16} activity ratio (Figure 4).

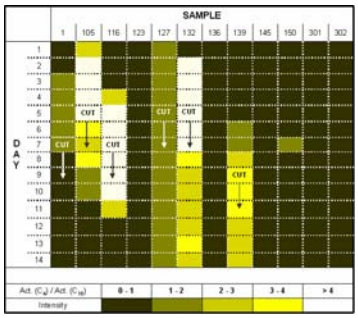

Figure 4. A correlation between the ratio of hydrolysis rates of pNP-butyrate (C_4) and pNP-palmitate (C_{16}) and the onset of cutinase activity.

The semi-continuous detection mode (allowing *true* initial rate determination) gives far more reliable and informative results than any end-point assay (eg. > 26 000 data points / day). Additionally, the time spent for esterolytic profiling of growth media with p-nitrophenyl fatty acids as substrates (and indicators of cutinase activity) is reduced to only a few hours instead of days.

CUTIN HYDROLYSIS

^{3}H-labelled apple cutin was used as substrate for the selected esterolytic preparations from the automated screens to verify their real cutinase activity (Figure 5). Interestingly, cutin degradation was observed to be biphasic. Black currant skin was also incubated with the growth media and fluorescently labelled to visualize and verify cutinolytic degradation by fluorescence microscopy (Figure 6).

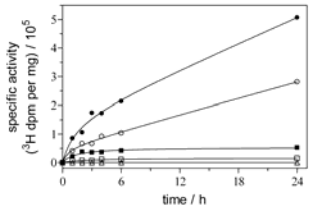

Figure 5. ^{3}H-cutin hydrolysis by cutinase in cultivation media. ●, *G. intricans* growth media; ○, *F. oxysporum* growth media; ■, *M. albomyces* steryl esterase; □, *M. albomyces* growth media; Δ, cellulase

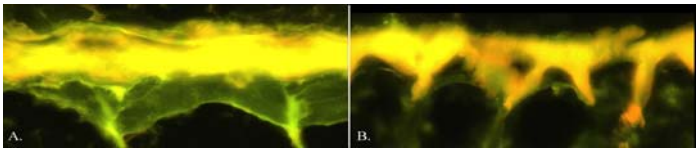

Figure 6. The degradation of black currant cutin by *F. oxysporum* growth media. The fluorescence emitted by the cutin bound Oil Red dye is significantly quenched in the enzyme treated sample (B) compared to reference (A).

CONCLUSIONS

- A novel procedure for profiling and identification of cutinolytic esterases was developed by combining plate screens with a robotic workstation:
  1. microbial growth and extracellular esterase activity are first assessed qualitatively on cutin and PCL plates
  2. an automated activity assay in a 96-well MTP format is used for esterolytic fingerprinting of different stages of microbial cultivation
  3. ^{3}H-cutin and fluorescence microscopy are used for hit verification
- The induction of esterase isoforms, and their substrate specificity and pH dependence can be monitored and quantitatively determined
- Eventually, novel cutinolytic activities with desired properties can be found

REFERENCES

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