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Screening of microbes for novel acidic cutinases and cloning and expression of an acidic cutinase from *Aspergillus niger* CBS 513.88

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ABSTRACT

Isolates from gardening waste compost and 38 culture collection microbes were grown on agar plates at pH 4.0 with the cutinase model substrate polycaprolactone as a carbon source. The strains showing polycaprolactone hydrolysis were cultivated in liquid at acidic pH and the cultivations were monitored by assaying the p-nitrophenyl butyrate esterase activities. Culture supernatants of four strains were analyzed for the hydrolysis of tritiated apple cutin at different pHs. Highest amounts of radioactive hydrolysis products were detected at pHs below 5. The hydrolysis of apple cutin by the culture supernatants at acidic pH was further confirmed by GC–MS analysis of the hydrolysis products. On the basis of screening, the acidic cutinase from *Aspergillus niger* CBS 513.88 was chosen for heterogeneous production in *Pichia pastoris* and for analysis of the effects of pH on activity and stability. The recombinant enzyme showed activity over a broad range of pHs with maximal activity between pH 5.0 and 6.5. Activity could be detected still at pH 3.5.

1. Introduction

The cuticle is a layer that protects the aerial parts of plants from microbial infections, desiccation and loss of solutes [1,2]. The major constituent of the cuticle is cutin, which is composed of variably substituted fatty acids interlinked with ester bonds. The main structural components of cutin are hydroxyl and epoxy substituted ω-hydroxy fatty acids with C16 and C18 carbon chain lengths [3].

Many micro-organisms produce cutinases (EC 3.1.1.74), which catalyze the hydrolysis of the ester bonds of cutin. Cutinases are especially common for fungal plant pathogens (*e.g. Fusaria*, *Botrytis cineria* and *Venturia inaequalis*) and it is believed that cutinase catalyzed degradation of the cuticular layer enables the fungi to penetrate the surfaces of plants [4–6]. Prokaryotic cutinase producers such as *Thermobifida fusca* [7] and various *Pseudomonas* strains [8,9] are also known. All characterized cutinases are serine esterases, which contain the catalytic triad Ser, His, and Asp, common also for serine proteases and lipases [10]. The pH optima reported for the cutinases are almost exclusively either in the neutral or alkaline range.

A variety of applications have been suggested for cutinolytic enzymes. Cutinases could be used in detergents for dishwashing and laundry applications. For use in the textile industry, a bioscouring method utilizing cutinases for the removal of the waxy layer present in cotton has been developed [11]. Cutinases have also been tested for modification of the surfaces of polyester fibers [12]. The use of cutinases for the detoxification of feed products contaminated by the heat-stable mycotoxin zearalenone has been patented [13].

Processing of fruits, vegetables and berries generates millions of tons of acidic by-products annually. These cutin rich materials are mainly disposed of as waste or used as animal feed [14–16]. Cutinases could therefore be used in facilitating the release of bioactive compounds from these materials. Many cuticular fatty acids show interesting functionalities, which could be utilized in the production of for example lubricants and binders [17]. In addition, cutinases have also been suggested for use in combination with other hydrolytic enzymes, such as cellulases, for the degradation of plant materials [18].

Despite their wide potential, the number of applications for known cutinases is limited, since their cutinolytic activities are either poor or nonexistent at low pH. In particular, plant derived materials are in many cases acidic. Many environmental applications for cutinases would also require functionality at low pH. In the

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current work we describe screening of acidic cutinases and show that cutinolytic enzymes having acidic pH optima (below 5.0) are produced by microbes. Furthermore, we present the heterologous production of an acidic cutinase from *Aspergillus niger* CBS 513.88 with the easily manipulatable and fast-growing *Pichia pastoris* yeast as the host and analyze the effects of pH on the activity and the stability of the enzyme.

2. Materials and methods

2.1. Microbial strains screened, culture media and screening conditions

The 38 VTT culture collection (VTT Technical Research Centre of Finland) strains screened for the production of cutinolytic enzymes at acidic pH were: *Pichia membranifaciens* C-05638T (CBS 244), *Pichia membranifaciens* C-05639T (CBS 184), *Alternaria citri* D-04950 (DSM 62013, Greece), *Aspergillus fumigatus* D-081308 (FGSC A1100), *Aspergillus niger* D-081307 (CBS 513.88), *Aspergillus versicolor* D-77063 (CBS 584.65), *Curvularia inaequalis* D-79116T (CBS 116.22), *Curvularia inaequalis* D-79118, *Curvularia protuberata* D-79119, *Fusarium oxysporum* D-82178, *Gibberella intricans* D-82087, *Gibberella pulicaris* D-82172, *Hypocrea schweinitzii* D-93421, *Hypocrea virens* D-83215, *Mucor circinelloides* D-00820 (Portugal), *Mucor plumbeus* D-071257 (DSM 16513, Portugal), *Neurospora sitophila* D-071258 (DSM 16514, Portugal), *Penicillium chrysogenum* D-88381, *Penicillium glabrum* D-071260 (DSM 16516, Portugal), *Penicillium olsonii* D-071259 (DSM 16515, Portugal), *Peniophora* sp. D-00815 (Portugal), *Peniophora* sp. D-01840 (Portugal), *Phanerochaete chrysosporium* D-85242T, *Phlebia spongipellis* D-00817 (Portugal), *Thermoascus thermophiles* D-96491T, *Trichoderma atroviride* D-00818 (Portugal), *Trichoderma atroviride* D-00821 (Portugal), *Trichoderma harzianum* D-00819 (Portugal), *Trichoderma longibrachiatum* D-071261 (DSM 16517, Portugal), *Wallemia sebi* D-96478 (CBS 411.77), *Burkholderia thailandensis* E-082813 (DSM 13276, Thailand), *Pectobacterium carotuvorum* E-981115T (DSM 30168, Denmark), *Pseudonocardia sulfidoxydans* E-073032T (DSM 44248), *Streptomyces griseus* E-072713T (DSM 40395), *Streptomyces luridiscabiei* E-072710T (LMG 21390, South-Korea), *Streptomyces puniciscabiei* E-072791T (LMG 21391, South-Korea), *Streptomyces sampsonii* E-073026T (DSM 40394), *Streptomyces scabiei* E-011978T (DSM 41658). (The countries of origin are given for the strains that are under the Convention of Biological Diversity, 1993.) In addition to the culture collection strains, microbes from a gardening waste compost were screened.

2.2. Culture conditions

The mineral medium described previously [19] was supplemented with 0.5 g l^{-1} of yeast extract, 0.5 g l^{-1} of tryptone and 1.0 g l^{-1} of polycaprolactone (Sigma) and used for screening at pH 4.0 on agar plates. For liquid cultivations of the polycaprolactone hydrolyzing strains the mineral medium was supplemented with 0.25 g l^{-1} of apple cutin [20] and 0.25 g l^{-1} of the cutinase inducer 16-hydroxyhexadecanoic acid (Aldrich). The strains isolated from the gardening waste were grown at 30 °C and the VTT culture collection strains at the recommended temperatures (25 °C or 30 °C). The cells were cultivated aerobically and 5 ml samples were taken daily or every other day, depending on the strain, for activity assays. Triton X-100 (Fluka) was added at the final concentration of 0.05% (w v^{-1}) to the cultivation samples. After a 30 min incubation at room temperature the samples were centrifuged (15 000 × *g*, 10 min) and the supernatants stored as frozen.

2.3. Enzyme activity assays

The previously purified neutral cutinase from *Coprinus cinereus* (VTT Biotechnology, Finland) was used as the positive control in some of the activity assays [17].

The reaction mixtures for the *p*-nitrophenyl butyrate (*p*NP-butyrate) esterase assays comprised 2 mmol l^{-1} *p*NP-butyrate (Sigma); 0.75% (w v^{-1}) Triton X-100 (Fluka); 1× McIllvaine buffer, pH 3.5; and the enzyme sample. *p*NP-butyrate hydrolysis was followed at room temperature by measuring the absorbance change of the reaction mixtures at 340 nm, which indicates the release of *p*-nitrophenol. The assays were carried out in microtiter plate format at 0.3 ml. The method used is a modification of a previously reported procedure [21].

Tritiated apple cutin (VTT Biotechnology, Finland) used in the cutinase assays was prepared with minor modifications as previously described [22]. The labeled cutin (specific activity approximately 1.4 μCi mg^{-1}) was mixed with unlabelled cutin [20] to obtain a suitable specific activity for radioactivity measurements and the mixture was finely powdered using a ball mill (Mixer Mill, MM301, Retsch, Haan, Germany). The cutin stock solution was prepared in water and supplemented with 0.1 mg ml^{-1} sodium azide and 100 mg ml^{-1} to prevent bacterial growth.

The reaction mixture (0.3 ml) consisted of 1× McIllvaine buffer, pH 3.5; 5 mg ml^{-1} tritiated cutin; 0.05% (w v^{-1}) Triton X-100 (Fluka) and the enzyme sample. After incubation with shaking at 40 °C, 45 μl aliquots of the reaction mixtures were centrifuged and the supernatants analyzed for radioactivity by liquid scintillation counting (model 1410; Wallac, Turku, Finland). Values measured from blank samples (reaction mixtures with water instead of enzyme) were subtracted from each value measured from the reaction mixtures.

For determining the pH profiles of the cutinolytic activities of the culture supernatants, McIllvaine buffer was used at pHs ranging from 2 to 8. The reaction mixtures were incubated for 24 h at 40 °C. Since it may be possible that pH affects the adsorption of the released cutinase mono- and oligomers on unhydrolyzed cutin, 0.35 ml of 0.5 mol l^{-1} Tris–HCl, pH 9.0 was added to the reaction mixtures (0.3 ml) after the incubation. The samples were centrifuged and the supernatants analyzed for radioactivity as above.

Lipase activities of the culture supernatants were determined using olive oil (Bertolli, Italy) as the substrate and the released fatty acids were determined colorimetrically using the Free Fatty Acids, Half Micro Test by Roche as previously described [23].

Protein concentration was determined using the BioRad DC Protein Assay kit (500-0112) according to the instructions by the manufacturer. Bovine serum albumin (Sigma) was used as the protein standard.

2.4. GC–MS analysis of cutin hydrolysis products

To remove released fatty acids, cutin monomers and Triton X-100, the culture supernatants were filter sterilized, diluted fourfold with McIllvaine buffer (pH 3.5) and concentrated by ultrafiltration to one quarter (Vivaspin 20, cut-off 5 kDa, Sartorius AG, Goettingen, Germany), diluted again and concentrated to approximately 1/16 of the original volume. Apple cutin was finely powdered using the ball mill and 150 mg of cutin was suspended into 1.5 ml of the enzyme sample supplemented with 10 μg ml^{-1} of the surface active agent hydrophobin II (VTT Biotechnology, Finland) [17,24].

The reaction mixtures were incubated for 40 h at pH 3.5 and at 40 °C with shaking, after which 390 μl methanol and 120 μl 2 mol l^{-1} H_2SO_4 were added. The cutin mono- and oligomers were recovered by extraction with chloroform, derivatized by silylation and analyzed by GC–MS as described previously [25].

As controls, cutin suspended in McIllvaine buffer without the enzyme sample and enzyme samples without cutin were incubated and analyzed as described above.

2.5. Fractionation of *A. niger* CBS 513.88 culture medium for acidic cutinase activity and cutinase identification

A. niger CBS 513.88 was cultivated aerobically in the mineral medium (pH 4.0) [19] supplemented with 1 g l^{-1} powdered apple cutin (VTT Biotechnology), 5 g l^{-1} of polycaprolactone (Sigma) and 250 mg l^{-1} of 16-hydroxyhexadecanoic acid (Aldrich) at 25 °C for two weeks. Triton X-100 (Fluka) was added at 0.5% (v v^{-1}) to the cultures to prevent adsorption of the cutinases onto the cell surfaces. The cells were removed by centrifugation at 5500 × *g* for 15 min and the supernatants collected.

Polycaprolactone (Sigma) was powdered by acetone treatment [19] and added at 75 g l^{-1} to the sterile filtered *A. niger* growth supernatant at pH 3.5. The suspension was incubated at room temperature under magnetic stirring for 2 h. Polycaprolactone was recovered by centrifugation for 5 min at 3200 × *g* and washed twice by suspending it into McIllvaine buffer, pH 3.5, using 5 ml of buffer per 1 g of polycaprolactone. Esterases were extracted from the polycaprolactone by suspending the pellet into McIllvaine buffer, pH 3.5, supplemented with 0.5% (v v^{-1}) Triton X-100 (Fluka) at a ratio of 1.3 ml of buffer per 1 g of polycaprolactone. The extraction was repeated thrice and the extracts combined.

The extract was concentrated to 0.6 ml, 5% (v v^{-1}) of glycerol was added and the sample was subjected to fractionation by gel filtration using a HiLoad 16/60 Superdex 200 column (GE Healthcare) according to the instructions by the manufacturer. Diluted (1:9) McIllvaine buffer, pH 3.5, with 5% (v v^{-1}) of glycerol was used for elution. The fractions were analyzed for *p*NP-butyrate esterase activity at pH 3.5. The active fractions were pooled and subjected to peptide mass fingerprint analysis, carried out by mass spectrometry at the Institute of Biotechnology (Helsinki, Finland).

2.6. Cloning of *A. niger* cutinase CBS 513.88 gene into an expression vector and transformation of the construct into *Pichia pastoris*

The *A. niger* cutinase gene was codon optimized for expression in *P. pastoris* and synthesized by GeneArt (Germany). The synthetic gene fragment contained *Eco*RI and *Not*I restriction sites at the 5′ and 3′ ends, respectively, and a sequence encoding a C-terminal His-tag. The sequence for the original secretion signal was omitted from the gene. The gene fragment was ligated into the *Eco*RI and *Not*I sites of the expression vector pPicZα-A (Invitrogen), in which it was transcriptionally fused to an α-factor signal peptide. The resulting plasmid was transformed into chemically competent *Escherichia coli* XL1-Blue MRF′ Supercompetent cells (Stratagene) by the protocol described by the manufacturer. Cells from the transformant colonies were suspended into 100 μl of H_2O and the suspensions were used as templates for colony PCR. The transformants were screened for the cutinase insert using the primers 5′AOX1 (5′-GAC TGG TTC CAA TTG ACA AGC-3′) and 3′AOX1 (5′-GCA AAT GGC ATT CTG ACA TCC-3′), which hybridize to the sites flanking the coding region. DNAs were isolated from five positive transformants and the identities of the coding regions were established by sequencing.

Electrocompetent *P. pastoris* X-33 (Invitrogen) cells were prepared as described by the manufacturer by growing the cells aerobically in Yeast Extract Peptone