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Bacterial biofilm removal using fungal enzymes

Belén Orgaz [a], Juliana Kives [a], Ana M. Pedregosa [b], Inmaculada F. Monistrol [b], Fernando Laborda [b], Carmen SanJosé [a,*]

[a] *Department of Nutrition, Food Science and Technology, Fac. Veterinaria, University Complutense of Madrid, 28040 Madrid, Spain*
[b] *Department of Microbiology, Fac. Farmacia, University Alcala de Henares, 28801 Alcala, Spain*

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Abstract

Three fungal strains of *Aspergillus niger*, *Trichoderma viride* and *Penicillium* spp., were grown on four alternative plant polysaccharides as C-sources, to induce enzymes able to degrade the bacterial biofilm matrix, for industrial cleaning purposes. Gum arabic and pectin were the C-sources providing supernatants with better *Pseudomonas fluorescens* biofilm removal ability. Comparable efficiencies, however, could be achieved with different enzymes, suggesting attacks to alternative points or structures of the biofilm matrix.

Keywords: Biofilm removal; EPS degradation; Polysaccharidases; *A. niger*

1. Introduction

When bacterial cells approach inert surfaces, they first get bound to the substratum by relatively weak forces involving their external structures (flagella, fimbriae and/or capsular components). When the cells remain thus attached for some time, they secrete sticky extracellular substances (EPS) forming a matrix gel that embeds several layers of cells, as the biofilm matures. This matrix is known to include mainly polysaccharides, besides of proteins and nucleic acids; it may presumably contain lipids, mineral ions and various cellular debris as well [1]. Composition of those biofilm polysaccharides is still insufficiently known, but available data on planktonic EPS and a few biofilm EPS, suggest that some of their monomers are identical or similar to those in plant cell-wall materials [2]. In fact, some bacterial exopolysaccharides have been reported to be acceptable substrates for enzyme mixtures from non-bacterial sources [3].

Many fungi can degrade complex plant cell-wall material, by secreting a large variety of enzymes. This versatility makes commercial polysaccharide-degrading enzyme mixtures to have a widespread use in very different fields, such as fruit processing [4] or wastewater treatment [5]. They could possibly be used as well to degrade bacterial biofilm matrices. There are some precedents on the use of commercial enzyme preparations for biofilm removal [6,7]. Preparations with different polysaccharide-degrading activity profiles, can be obtained by induction of different enzymes with appropriate culture substrates [8], a practice that can increase those enzyme yields by several fold [9–12]. The aim of this work was to obtain supernatants from fungal cultures in which enzyme induction was attempted with different plant polysaccharides, that could somewhat resemble those in biofilm EPS from *Pseudomonas*. Several carbohydrate-degrading enzyme activities were determined in those supernatants, to find out which of them were better associated with biofilm removal efficiency.

2. Material and methods

2.1. Microorganisms and culture conditions

Aspergillus niger CECT 2574 and strains of *Trichoderma viride* and *Penicillium* spp. H18, this one isolated from brown algae, belong to the collection of the Department of Microbiology of the University of Alcalá de Henares, Madrid, Spain, and were maintained in dextrose and potato agar, containing: potato infusion (4 g/L), dextrose (20 g/L), agar (15 g/L), where they were incubated at 28 °C until adequate sporulation; filtered spores were stored at 5 °C. *Pseudomonas fluorescens* B52, originally isolated from refrigerated raw milk, was kept at −25 °C in Trypticase Soy Broth containing 15% glycerol.

For fungal growth, the following minimum medium [13] was used: $NaNO_3$ (6.0 g/L), KCl (0.52 g/L), KH_2PO_4 (1.52 g/L) and $MgSO_4 \cdot 7H_2O$ (0.5 g/L), which was supplemented with a trace mineral solution [14]

* Corresponding author. Tel.: +34 91 3943746; fax: +34 91 3943743.
E-mail address: serran@vet.ucm.es (C. SanJosé).

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containing: $FeSO_2{\cdot}7H_2O$ (1.0 mg/L), $ZnSO_4{\cdot}H_2O$ (8.8 mg/L), $CuSO_4{\cdot}5H_2O$ (0.4 mg/L), $MnSO_4{\cdot}4H_2O$ (0.15 mg/L), $NaB_4O_7{\cdot}10H_2O$ (0.1 mg/L) and $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (0.05 mg/L). pH was adjusted to 6.5. Two C-sources were added: 0.2% glucose as growth initiator and 0.8% of one of the following polysaccharides: pectin from apples, low viscosity sodium alginate from brown algae, gum arabic from acacia tree and xylan from oat spelts, all from Sigma. A spore suspension to reach a final concentration of 10^6 spore/mL, was added to each 100 mL shake flask containing 20 mL of medium. Cultures were incubated at 28 °C under shaking (200 rpm). Aliquot samples were withdrawn daily and the present mycelium was separated by filtration in Whatmant paper no. 1; its weight, obtained by drying it at 90 °C for 48 h, was used to measure growth. Supernatants were frozen until use. All cultures were run in duplicate.

2.2. Enzyme activities

All enzymatic activities in culture supernatants were determined at 30 °C. All assays were run in triplicate, standard deviations being below 5%.

Pectin esterase (PE) activity was determined according to Ref. [15]. Reaction mixtures contained 2.95 mL of 0.1% (w/v) apple pectin solution and 0.068% (w/v) bromocresol green, mixed in a proportion 10:1 with pH adjusted to 5.1. The reaction was started with 50 μL of the enzyme source. After 30 min incubation, absorption was measured at 617 nm; D-galacturonic acid (GA) was used as a standard. One PE unit releases 1 μmol de GA/min from apple pectin.

For determination of polygalacturonase (PG) activity [16], samples were diluted in 0.05 M sodium acetate buffer (pH 5.5). Reaction mixtures, consisting of 500 μL enzyme source and 500 μL of 0.5% (w/v) polygalacturonic acid solution, were incubated for 1 h. The hydrolysis product was quantified by the increase in reducing end groups [17], using GA as standard. One PG releases 1 μmol of GA/min, from polygalacturonic acid.

Pectin lyase (PL) activity was determined according to Ref. [18], with 0.5% apple pectin solution in phosphate-citrate buffer pH 5.2 (0.1 M dibasic potassium phosphate and 0.1 M citric acid) as substrate. Reaction mixtures with 2 mL of substrate solution and 0.1 mL of enzyme source were incubated and the absorption was monitored at 235 nm for 30 min. One PL unit produces an increase of 0.555 absorption units 235 nm/min.

To assay cellulase (CEL) activity, 1 g of carboximethyl cellulose dissolved in 100 mL of 100 mM citrate buffer (pH 6.0) was used as substrate [19]. The reaction was started by mixing 0.2 mL of enzyme source with 0.3 mL of substrate solution, and was stopped after 15 min, by addition of 1 mL of a color reagent (0.1 M *p*-hydroxybenzoic acid hydrazide, 0.1 M sodium sulphite, 0.02 M calcium chloride, 0.05 M trisodium citrate and 0.5 M sodium hydroxide, all from Sigma) and immersion of the test tubes in a boiling water bath for 6 min. After cooling in tap water, absorption was measured at 420 nm, with glucose being used as standard. One CEL unit releases 1 μmol of glucose/min.

Arabinase activity (ARA) was assayed according to Ref. [20], using 0.5% arabinogalactane in 0.2 M acetate buffer pH 6.0, as substrate. Reaction mixtures, contained 3.0 mL of the substrate solution, 1.0 mL of enzyme source and two drops of toluene; the 30 min incubation was stopped by adding 4 mL of 0.2 M NaOH. Hydrolysis products were quantified by the increase in reducing end groups [17], using arabinose as standard. One ARA unit generates 1 μmol of arabinose/min, from arabinogalactane.

For determination of alginate lyase (AL) activity, 2 mL of a substrate solution, prepared by dissolving 0.6 g of sodium alginate in 120 mL of 1 M Tris–HCl buffer pH 7.5, were mixed with 3 mL of enzyme source [21]. Absorption at 230 nm was monitored every 5 min during incubation, for 30 min. One AL unit produces an increase of 0.178 absorption units at 230 nm/min.

To determine proteinase activity, the fluorescamine assay was used [22]. One percent bovine seroalbumine in 0.1 M Tris–HCl buffer pH 7.6 was used as a substrate. 2.3 mL of this solution was mixed with 200 μL of enzyme source. After 20 min of incubation, the reaction was stopped by using trichloroacetic acid (5%, v/v, in the final mixture). To complete the precipitation of TCA-insoluble products, tubes were held for 1 h before being centrifuged for 10 min at 7500 rpm. 100 μL of the clear supernatant were mixed with 900 μL of the buffer and 50 μL of the fluorescamine solution (3.4 mg/mL in dry acetone). Fluorescence intensity was immediately measured, with an excitation wavelength of 390 and 475 nm of emission. Alanine was used as standard. One proteolytic unit produces 1 μmol of alanine/min.

2.3. P. fluorescens biofilm formation and removal

P. fluorescens B52 was cultured in mineral medium PMS_7-Ca, which contained: *N,N*-bis-(2-hydroxymetyl)-2-aminoethane sulphonic acid (BES) (10.7 g/L); sodium pyruvate (11.00 g/L); dibasic potassium phosphate (0.86 g/L); ammonium chloride (0.65 g/L); magnesium sulphate (0.20 g/L); this was adjusted to pH 7.0 and, after autoclaving, supplemented with 0.111 g/L of filter-sterilised calcium chloride. Standard biofilms were obtained by attachment of bacterial cells on sterile glass coupons (22 mm × 22 mm), clamped vertically to a Teflon carousel, which was placed inside a beaker almost filled with the culture medium and covered by a lid. Washed cells to reach 10^7 CFU/mL were inoculated; after 24 h culture at 21 °C, the coupons were withdrawn and dipped into saline (0.9%, w/v) to discard loosely attached cells, before being used as standard biofilm samples in removal tests.

To assay the biofilm removal efficiency, an standarized *P. fluorescens* biofilm coupon was submerged into a Falcon tube with 12 mL of the corresponding fungal culture supernatant, to be incubated for 1 h at 30 °C without shaking. The coupons were then soaked for 1 min in saline (0.9%, w/v), before being dried, fixed for 30 min with formaldehyde and stained for 2 min in Coomassie Blue. Stained coupons were scanned in a Bio-Rad GS-690 densitometer, using Molecular Analyst software (Bio-Rad Inc.) for image analysis. Biofilm biomass removal assays were run in quadruplicate, standard deviations being below 10%.

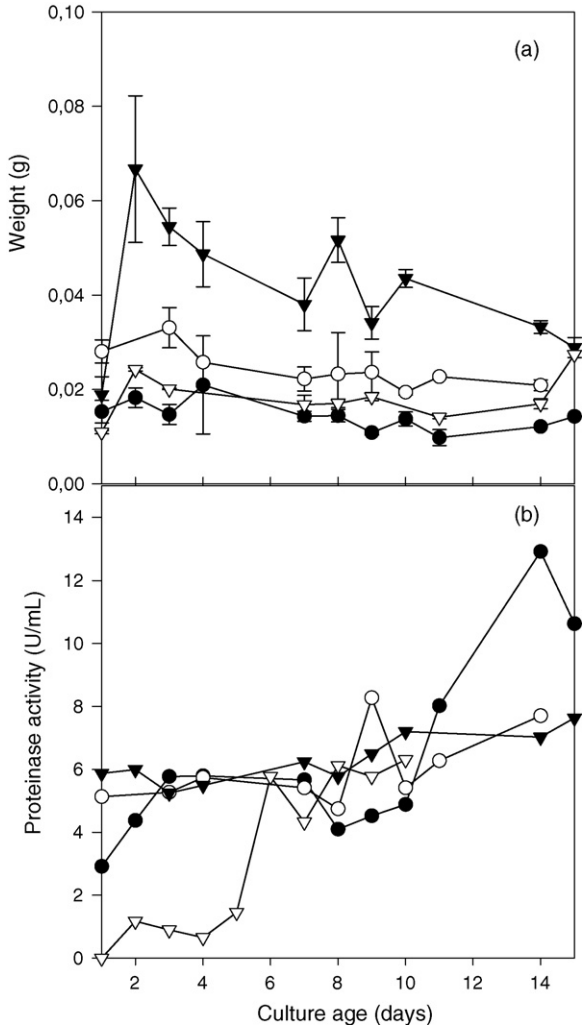

Fig. 1. Growth (a) and proteinase activity (b) in supernatant from *Aspergillus niger* cultured on: ● gum arabic, ○ alginate, ▼ xylan or ▽ apple pectin.