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Surface hydrolysis of polyacrylonitrile with nitrile hydrolysing enzymes from *Micrococcus luteus* BST20

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Abstract

A new *Micrococcus luteus* strain *BST20* was isolated with ability to metabolize PAN polymers as sole carbon source. Out of seven synthetized PAN copolymers containing different moieties of acrylic acid and/or vinyl acetate the polymer with lowest crystallinity (PAN with 5% vinyl acetate) was most easily metabolized. ^{13}C labelled PAN was completely converted to the acrylic acid by this strain. *M. luteus BST20* produced membrane-bound nitrile hydrolysing enzymes able to convert nitrile groups on PAN powder surface to the corresponding acids. Similarly, nitrile groups on PAN fabrics were transformed to the corresponding acid as indicated by an *K/S* increased after dying with Methylene blue and the released ammonia. On small soluble substrates the enzyme system showed a preference for aliphatic and aromatic substituted aliphatic nitriles.

Keywords: *Micrococcus luteus*; Nitrilase; Polyacrylonitrile; Surface modification

1. Introduction

Worldwide, 2.73 million tons of polyacrylonitrile (PAN) per year are produced (Saurer Management, 2004). PAN often contains vinyl acetate (5–10 mol%) which disrupts the high regularity of the PAN affecting the crystallinity of the polymer and increasing its elasticity, which is required for textile processing (Wade and Knorr, 1995; Geller, 2002). There is an industrial demand to improve moisture uptake, dyeability with ionic dyes, and the fastness of special functional finishes on PAN, while the good mechanical properties of the polymer shall be maintained. Nitrile hydrolysing enzymes may have a potential to specifically modify the PAN surface by hydrolysing nitrile groups into the corresponding amides or acids. Such enzymes have

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been used for organic synthesis, especially for chiral synthesis (Mylerova and Martinkova, 2003), for the degradation of environmental toxic chemicals in industrial wastes (Kobayashi and Shimizu, 2000), as well as for industrial processes such as large-scale production of acrylamide (Watanabe et al., 1985; Nagasawa et al., 1993).

Nitrilases (EC 3.5.5.1) catalyze the hydrolysis of a nitrile directly to the corresponding acid, most probably forming an acylenzyme as reaction intermediate (Brenner, 2002) while nitrile hydratase (EC 4.2.1.84)/amidase (EC 3.5.1.4) enzyme systems catalyze the hydrolysis of nitriles in a two step reaction to the corresponding acid. In this case, the corresponding amide formed by the nitrile hydratase can be obtained.

Previously, we have shown that nitrile hydrolysing enzymes from a *Rhodococcus rhodochrous* strain were able to modify the surface of PAN (Tauber et al., 2000). Similarly, treatment of PAN with nitrile hydratases from *Brevibacterium imperiale*, *Corynebacterium nitrilophilus* and *Arthrobacter* sp. *ECU1101* resulted in an increase of amide groups on the PAN surface. This change was monitored by XPS surface analysis and lead to properties like increased hydrophilicity, measured by the contact angle method, and dyeability with acid dyes coupling to the protonated amide nitrogen atoms (Battistel et al., 2000; Wang et al., 2004).

In this study, we performed a wide screening for microorganisms degrading PAN resulting in the isolation of a new strain of *Micrococcus luteus*. To our best knowledge this is the first report on nitrile hydrolysing enzyme activity in general and hydrolysis of PAN reported for this organism.

2. Materials and methods

2.1. Screening for microorganisms and culture conditions

In order to find new microorganisms with potential enzymes to modify the polyacrylonitrile (PAN) surface, a screening was carried out. Soil samples from different landfills in the surroundings of Graz, Austria and samples from a sewage composting plant (Hartberg, Austria) served as inocula. The screening was carried out using a PAN medium consisting of 10 g L^{-1} PAN polymer powder obtained from Fisipe containing 5% vinyl acetate (Lisbon, Portugal), in a phosphate buffer containing mineral salts (2.5 g L^{-1} KH_2PO_4, 3 g L^{-1} K_2HPO_4, 2 g L^{-1} NH_4Cl, 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$ in tap water). Five milliliters per liter of a trace element solution according to Stelkes-Ritter were added and the pH was adjusted to 7.0 (Stelkes-Ritter et al., 1995). All chemicals were p.a. grade and obtained from Merck (Darmstadt, Germany). Soil samples (10 g) were added to 100 mL of this medium in 250 mL Erlenmeyer flasks and incubated at 30 °C, 50% humidity and 125 rpm on a reciprocal shaker.

After 3 weeks, samples were taken and transferred to rich medium agar plates (Standard I nutrient agar from Merck) and incubated at 30 °C, 50% humidity. Individual colonies growing on these plates were replated on Standard I agar plates as single colonies and subsequently pure strains were re-transferred to PAN containing agar plates. Agar plates were prepared based on the PAN medium described above containing additionally 15 g L^{-1} agar-agar. Cultures with the ability to grow on the polymer were stored at +4 °C on PAN agar plates.

2.2. Production of nitrile hydrolysing enzymes

Isolated strains were transferred on acetonitrile containing agar plates to induce nitrile hydrolysing enzyme activity. The medium consisted of 16 mM acetonitrile (HPLC grade, Carl Roth, Karlsruhe, Germany) as nitrogen source, and as carbon source either 100 mM glycerol (85%, p.a. grade, Merck), or 50 mM glucose (p.a., Fluka Chemie, Buchs, Switzerland), or 90 mM sodium acetate, and 15 g L^{-1} agar-agar in a mineral salt medium (2 g L^{-1} KH_2PO_4, 2 g L^{-1} K_2HPO_4, 1.5 g L^{-1} Na_2HPO_4, 0.036 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.022 g L^{-1} $MgCl_2 \cdot 7H_2O$, 0.25 mg L^{-1} $FeCl_3 \cdot 7H_2O$, and 5 mL L^{-1}) of a trace element solution according to Stelkes-Ritter (Stelkes-Ritter et al., 1995). Growth of each strain indicated the presence of a nitrile hydrolysing activity, different carbon sources as described above were used to optimize growth of each strain.

Positive cultures were stored at +4 °C on the nitrile hydrolysing enzyme inducing agar plates. For production of nitrile hydrolysing enzymes, isolated strains were transferred into the inducing liquid medium, con-

sisting of the same components as the agar plates described above, without the agar-agar.

Alternatively, in case of slow biomass production, strains were cultivated in a rich liquid medium (Standard I nutrient broth, Merck) containing 16 mM acetonitrile for 24 h, followed by shifting the biomass into the nitrile hydrolysing enzyme inducing liquid medium described above. Incubation was done at 30 °C, 50% humidity and 125 rpm on a reciprocal shaker.

2.3. Isolation and stabilization of membrane-bound nitrile hydrolysing enzymes from Micrococcus luteus

M. luteus isolated from a sewage composting plant (Hartberg, Austria) was cultivated in a nitrile hydrolysing enzymes inducing medium as described above, and cells were harvested by centrifugation (20 min at 4000 × *g*) and washed twice with a 50 mM phosphate buffer pH 7. Cells were treated with lysozyme (8.3 mkat mL^{-1}, 15 min on ice) and the resulting preparation was separated by centrifugation (34,000 × *g*, 30 min). The pellet was resuspended in a nitrilase buffer as described below, pH 7 containing 2% Triton X as detergent. This suspension was treated with ultrasound for 25 min on ice, with breaks of 2 min after every 5 min, to avoid heating of the mixture by the ultrasound treatment. The solubilized membrane-bound proteins were separated from the insoluble membrane compounds by centrifugation (34,000 × *g*, 30 min). The resulting enzyme preparation was either used immediately for further investigations or lyophilized and stored at −20 °C.

2.4. Enzyme assay

The assay was based on the release of ammonia by nitrile hydrolysing enzymes (nitrilases or nitrile hydratase/amidase enzyme systems) and modified from Cramp et al. (1997). One hundred and ninety-five microliters of a nitrilase stabilizing buffer (2 g L^{-1} KH_2PO_4, 3.5 g L^{-1} K_2HPO_4, 1 mM dithiothreitol) were mixed with 100 μL of enzyme preparation or whole-cell suspension and 5 μL of a 4.2 M acetonitrile or 25 mg mL^{-1} PAN powder solution (HPLC grade, Roth, diluted with bidistilled water) and incubated at 30 °C and 1400 rpm on an Eppendorf thermomixer® for 60 min and 24 h. After incubation, the reaction was stopped by centrifugation (9500 × *g* for 5 min; in case of whole-cell suspensions) and put on ice. The ammonia resulting from the hydrolysis was measured using an ammonia test kit based on the NADH-dependent reaction of a transglutaminase, which transfers ammonia to 2-oxoglutarate to form L-glutamate (test kit for ammonia quantification from Boehringer). Measurement was performed as described in the instructions from the manufacturer, using a Hitachi U 2001 spectrophotometer for NADH measurement at 340 nm. One nanokatal of enzyme activity was defined as the amount of enzyme necessary to produce 1 nmol of ammonia from acetonitrile per second.

2.5. HPLC-based assay for qualitative activity determination on soluble nitriles

In order to determine the specificity of the nitrile hydrolysing enzymes towards several nitriles, a HPLC assay was performed. Therefore, samples of whole cells were used for 24 h of incubation. Substrates were applied in a concentration of 2.4 mM in the assay and if necessary, the substrate was dissolved in methanol prior to dilution with water. Samples were taken at the beginning of the incubation, after 3 h, and after 24 h. In order to remove the cell material, samples were centrifuged at 4000 × *g* for 20 min and subsequently filtered using a 0.45 μm filter device. Samples were analyzed using a Waters HPLC system with a diode array detector 996. A reversed-phase column was used (Nova-Pak C18, 5 μm, 3.9 mm × 150 mm, Waters), and a bidistilled water/acetonitrile mixture 75/25 (v/v) containing 0.1% H_3PO_4 served as eluent, at a flux of 0.9 mL min^{-1}. Commercial nitrile substrates were of analytical grade, 2-cyano-3-hydroxyhexene was a gift from the SEESIB Laboratory of University of Blaise Pascal, Clermont-Ferrand; 2-hydroxy-4-phenylbutyronitrile was a gift from the department of organic chemistry, TU Graz. Chemicals used for eluent preparation were HPLC gradient grade, obtained from Sigma. Peak detection was performed by recording 3D spectra in the wavelength range of 210–240 nm.

2.6. Growth studies using different PAN polymers

Seven different polymers were investigated; their compositions are given in Table 1.

Table 1
Composition and crystallinity of the PAN polymers investigated for *M. luteus BST20* growth studies

Sample number	% acrylonitrile	% vinyl acetate	% acrylic acid	Crystallinity
1	90	5	5	0.24
6	80	0	20	0.24
0	100	0	0	0.32
4	95	0	5	0.32
2	85	5	10	0.48
5	90	0	10	0.56
7	95	5	0	0.80

The polymers were synthesized with different initial composition of comonomers in a microemulsion polymerization media containing potassium and sodium persulfate as described previously (Sanghvi et al., 2000). *M. luteus BST20* was grown on these polymers using a mineral salt medium as described for the screening procedure, using 2 g L^{-1} of PAN powder and samples were taken at different time intervals. Growth was investigated over 60 days by determining the cell count using an Abbe-Zeiss® cell counting chamber.

The crystallinity of the different polymers was determined by X-ray analysis. The X-ray diagrams were obtained using a Philips Analytical PW1710 diffractometer with an X-ray tube using Ni filtered Cu Kα radiation. The peak areas of the 2θ signals reflection were calculated from the diffractograms and represent the interchain distance. Higher peak areas are related to a decrease of crystallinity.

Furthermore, *M. luteus BST20* was grown on a ^{13}C labelled PAN with the optimum composition resulting from the growth studies described above. Samples were taken in different time intervals and the incubation mixture was analyzed after lyophilization.

The ^{13}C CP/MAS spectra were recorded at 100.62 MHz in a Bruker Avance 400 spectrometer. Chemical shifts are given in ppm from TMS (del Arco et al., 2004).

2.7. Polymer treatment

Fibres and PAN powder were treated with enzyme solutions in nitrilase buffer at 30 °C for 24 h unless otherwise stated. Fibres were obtained from Sattler, Graz Austria, with the company's specification 9007758 317 010 120 1756 1-1. Fibre treatment was performed in glass petri dishes under gentle shaking (70 rpm), using a bath volume of 7 mL. The enzyme activity used was 1.2 nkat mL^{-1} measured on acetonitrile as substrate, using a total of 5 mL for fabric treatment. PAN powder was incubated with the same enzyme solution on an Eppendorf thermomixer®, using a volume of 1 mL. Release of ammonia was determined as described above using the ammonia test kit.

2.8. Fabric dyeing

Enzyme treated fabric samples were dyed using Methylene blue as a basic dye in order to detect COO^- groups on the polymer surface. Dyeing was carried out using the Ahiba Spectradye system from Datacolor International (Lucerne, Switzerland). Two grams of fabric sample and 40 g of Methylene blue were incubated in 40 mL of distilled water at 70 °C. The depth of staining was detected measuring the *K*/*S*-value of the dyed samples on an ACS reflectance spectrometer. Results were expressed as the ratio of total light absorbed (*K*) and scattered (*S*) calculated from the reflectance *R* (%) of a textile sample using the Kubelka–Munk equation:

$$\frac{K}{S} = \frac{(1 - (R/100))^2}{2R}$$

3. Results and discussion

3.1. Screening

The screening process for microorganisms modifying PAN resulted in the isolation of six strains with the ability to grow on the PAN powder from which three produced nitrile hydrolysing enzymes. Microscopic inspection of the cultivation mixtures showed severe damage of fibres followed by complete degradation (Fig. 1).

Among these organisms, the best strain in terms of enzyme activity and growth on the polymer was chosen for characterization and further investigations. This isolate showed the typical morphology (coccoid-shaped bacteria with a diameter of 1.0–1.4 μm, growth in tetrads, gram-positive) for the genus *Micrococcus*. Furthermore, analysis of the profile of fatty acids and 16S rDNA sequence by DSMZ (Braunschweig, Germany)

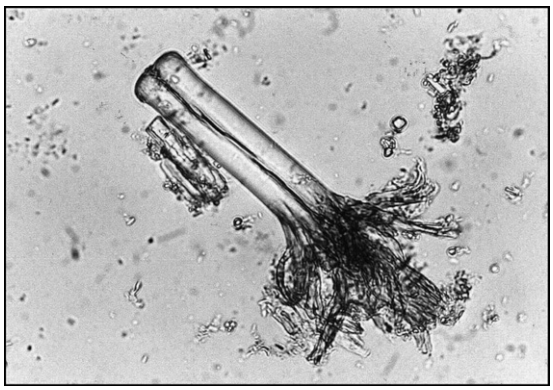

Fig. 1. PAN fibre after 6 weeks incubation with the newly isolated *M. luteus BST20* (1:2000).

lead to the identification as *M. luteus*. It also showed to produce a catalase and a urease, while it was not able to metabolize lactose (absence of β-galactosidase) or to reduce nitrate. Typical mono- and disaccharides, such as glucose, fructose, cellobiose, lactose, saccharose, were not metabolized to acidic compounds, determined by a pH decrease during incubation.

3.2. Growth studies on different PAN polymers

In order to determine the composition of PAN polymers for optimal growth of the *M. luteus BST20* strain, several polymers were produced varying the content of vinyl acetate and acrylic acid as comonomers.

Growth curves are shown in Fig. 2, and crystallinities of the polymers used are shown in Table 1 where high values indicate low crystallinity.

Interestingly, the comonomer content and the crystallinity of the polymers had an influence on growth by *M. luteus BST20*. The minimum comonomer content required for the organisms in order to metabolize the polymer was 5%, based on positive results with samples 1, 4, and 7. An acrylic acid content of 10% and more seemed to have an inhibitory effect on growth. Growth was best in case of PAN No. 7, which did not contain acrylic acid in contrast to samples 1 and 4. Here, the crystallinity was the lowest of all polymers investigated. Therefore, it can be stated that a low crystallinity has a positive effect on growth of *M. luteus BST20*. *M. luteus BST20* was grown on a ^{13}C labelled PAN with the same composition as polymer No. 7 (made by polymerization from ^{13}C labelled acrylonitrile as

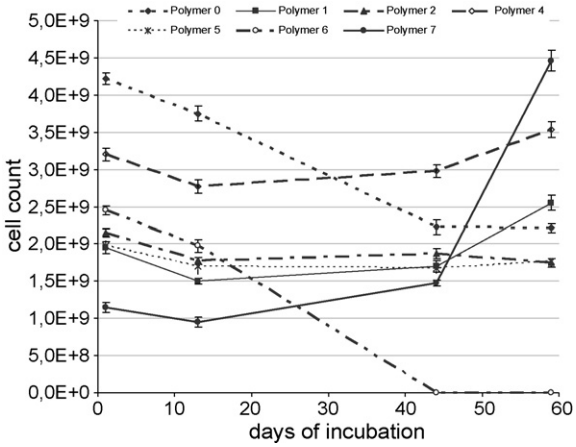

Fig. 2. Cell count curves for growth of *M. luteus BST20* on different PAN polymers.

described above), in order to measure the metabolic products by solid state NMR. It was shown that the PAN was transformed into polyacrylic acid in the first phase of incubation (see Fig. 3).

This and other spectra recorded at earlier stages of the incubation showed the appearance of the COOH peak, while no peak of the corresponding amide appeared at any stage of the fermentation (the amide peak would appear at 150–175 ppm). These measurements indicate that the membrane-bound nitrile hydrolysing enzyme is a nitrilase, hydrolysing the CN groups of the PAN in a one-step reaction or that the hydrolysis of potential amide-intermediates by amidase is much faster.

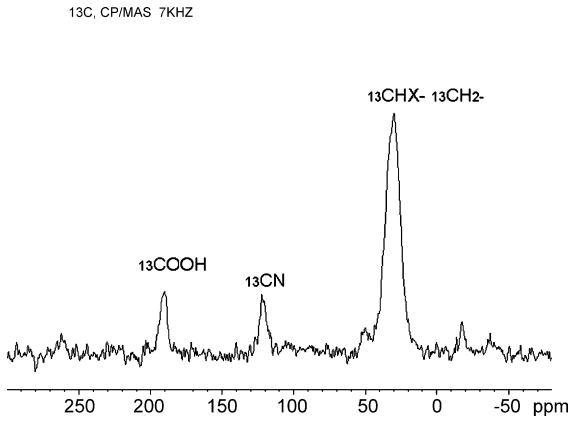

Fig. 3. ^{13}C NMR spectrum from a ^{13}C labelled PAN after a 6-week incubation of *M. luteus BST20*.

3.3. Production of nitrile hydrolysing enzymes

When *M. luteus BST20* was grown on the PAN medium, nitrile hydrolysing enzyme activity was detected. Since growth on PAN took several months with still low enzyme activity, an optimization of enzyme production was necessary. Growth on the rich Standard I medium was fast and colonies with a diameter of 4–5 mm could be obtained after 24 h incubation. Nevertheless, no enzyme activity could be detected in the corresponding cells suspensions. Growth on the nitrilase-inducing media was quite slow, showing colony diameters of 0.5–1 mm after 1 week of incubation. Among different carbon sources tested in this medium, growth was best using glucose, but nitrile hydrolysing enzyme activity was highest when *M. luteus BST20* was grown on glycerol. In order to increase the nitrilase yield in fermentations, *M. luteus BST20* was grown in the Standard I nutrient broth for 24 h and subsequently, the whole biomass was transferred to the glycerol containing nitrilase inducing medium. After 48 h, nitrilase production was at its maximum.

After cells were harvested and cell walls were broken, the nitrile hydrolysing enzyme activity was found in the membrane-containing cell fraction, while neither the intracellular nor the extracellular fraction showed any enzyme activity. Considering the fact that the organism – when grown on PAN – can hydrolyse nitrile groups on the PAN surface, it is not surprising that the enzyme activity is not found intracellularly. However, the isolation of a membrane-bound enzyme requires a technique which maintains a hydrophobic surrounding of the enzyme, so that it will not precipitate. This requirement could be met by adding Triton X above its critical micelle concentration (Hussein and Walter, 1996).

3.4. Substrate specificity of the Micrococcus luteus BST20 nitrile hydrolysing enzymes

The activity of the nitrile hydrolysing enzymes of *M. luteus BST20* was tested towards the substrates shown in Table 2. For reasons of comparability, all substrates were tested using whole cell suspensions of an overnight culture in the nitrilase inducing medium as described above. The nitrile hydrolysing enzymes of *M. luteus BST20* showed a clear preference for aliphatic nitriles (Table 2). 2-Cyano-3-hydroxyhexene was not attacked at all by the *M. luteus* enzymes.

Table 2
Substrate turnover of the nitrile hydrolysing enzymes from *M. luteus BST20*

Substrate	Turnover after 24 h	Amide detected?
Benzonitrile	10	No
Benzyl cyanide	20	Yes
2-Hydroxy-4-phenylbutyronitrile	100	Yes
2-Cyano-3-hydroxyhexene	0	No
Acetonitrile	100	n.d.
Acrylonitrile	100	Yes

The fact that the amide was detected as intermediate of nitrile hydrolysis leads to the conclusion that the soluble nitriles are hydrolysed by a nitrile hydratase/amidase enzyme system.

The activity on PAN powder was tested using solubilized nitrile hydrolysing enzymes in order to avoid a solid–solid enzyme reaction. Also here, incubation was done for 24 h at 30 °C, using 1 mg of PAN powder for 1 mg protein in 1 mL of enzyme preparation. The ammonia released after 24 h was about 5% of the theoretical possible amount of the PAN provided in the incubation assay, accounting for a specific enzyme activity of 22 nkat g^{-1} protein. Taking into account that only a low percentage of the nitrile groups might be accessible to the enzymes this conversion seems reasonable high.

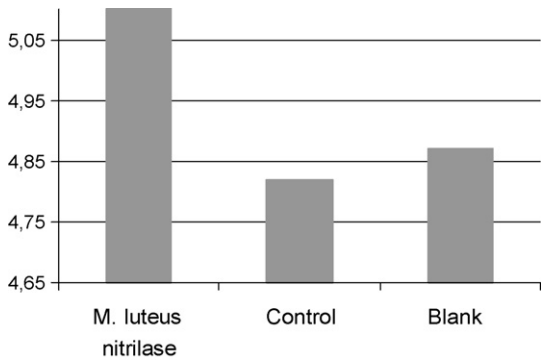

Fig. 4. *K/S* values of PAN fabrics treated with nitrile hydrolysing enzymes from *M. luteus BST20* after dyeing with Methylene blue. *M. luteus* nitrilase: fabric treated with enzyme preparation for 24 h. Control: fabric treated with denatured enzyme preparation from *M. luteus BST20*. Blank: fabric treated with 50 mM phosphate buffer.

3.5. Fabric treatment with the M. luteus BST20 nitrile hydrolysing enzymes

PAN fabrics were treated with the membrane-bound nitrile hydrolysing enzymes from *M. luteus BST20* as described in Section 2. Samples were dyed using Methylene blue, which binds to anionic groups on the polymer surface. The clear increase in the depth of shade indicates an increase in COO^- groups and thus hydrolysis of PAN (Fig. 4). This is an interesting result since all previous studies (Battistel et al., 2000; Tauber et al., 2000; Wang et al., 2004) focused on hydrolysis of PAN fabrics only to the corresponding amides.

4. Conclusions

A new strain of *M. luteus BST20* was isolated which produces membrane-bound nitrile hydrolysing enzymes. The enzymes were shown to hydrolyze the nitrile groups on the PAN surface by determining the NH_3 release from PAN powder, and measuring the depth of shade of enzyme treated fabric after dyeing with a basic dye.

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