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Development of cellulose-degrading lactic acid bacterium *Lactococcus cremoris* by genetic engineering

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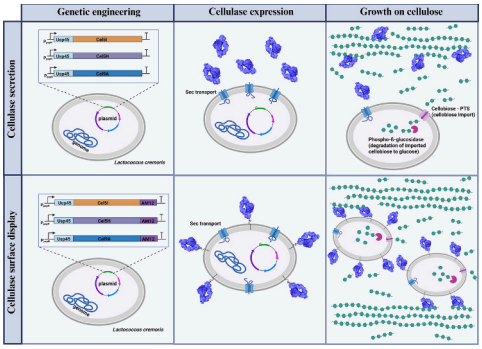
HIGHLIGHTS

- Cel5I, Cel9A and Cel5H cellulases were produced in *Lactococcus cremoris* cells.
- Surface display of cellulases on *L. cremoris* was most effective with cAM12 anchor.
- *L. cremoris* secreting cellulases showed improved growth on amorphous cellulose.
- Cel9A cellulase enabled fastest growth of *L. cremoris* on amorphous cellulose.
- Secreted cellulases promoted better growth than surface-displayed counterparts.

GRAPHICAL ABSTRACT



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ABSTRACT

Cellulose is one of the most abundant potential sources of carbon for sustainable microbial production of biochemicals. Lactic acid bacteria that produce a range of valuable metabolites are generally unable to grow on cellulose. Here, we aimed to develop the lactic acid bacteria *Lactococcus cremoris* for growth on cellulosic substrate. Genes encoding cellulases Cel5I, Cel9A and Cel5H from different cellulolytic bacteria were introduced into *L. cremoris* NZ9000 strain. The genes were designed for constitutive expression, with the produced cellulases being either secreted or displayed on the cell surface. Four promoters for cellulase gene expression and two noncovalent anchors for surface display of cellulases were evaluated using immunoblotting, confocal microscopy and flow cytometry. With the most effective promoter PepN, the cells secreted milligram amounts of cellulases per liter culture. The highest cellulolytic activity on amorphous (phosphoric-acid swollen cellulose, PASC) and microcrystalline (Avicel) cellulose was observed for the secreted Cel5H and Cel5I cellulases, respectively. The major cellodextrin produced by Cel5H and Cel5I was cellobiose, as determined by high performance anion exchange chromatography. The strains secreting cellulases outperformed the corresponding strains displaying the same cellulase on the surface in growth on PASC as the primary carbon source. The fastest growth was observed for the strain secreting Cel9A, followed by strain secreting Cel5H. By demonstrating growth of lactic acid bacteria

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expressing heterologous cellulases on PASC as a major carbon source for the first time, this study presents an important step towards the realization of consolidated bioprocesses involving lactic acid bacteria.

1. Introduction

Lactic acid bacteria (LAB) metabolize carbohydrates to high-value chemicals (e.g. lactic acid, ethanol) that can be used as end products or as precursors and building blocks for sustainable and environmentally-friendly production of other biochemicals and biopolymers. Besides, LAB enable production of many important bioproducts, such as food aromas, vitamins, sweeteners, exopolysaccharides and bacteriocins (Mazzoli et al., 2014; Yankov, 2022; De Groof et al., 2019). However, the carbon sources that are commonly used for growth of lactic acid bacteria are obtained from edible and energy crops (such as sugar cane, sugar beet and corn) (Kim et al., 2022). Since these crops are also used for human and animal nutrition, a more sustainable and cost-effective alternative for LAB growth would be the plant biowaste material from households, food- and agro-industry. This could make the LAB bioprocesses for the production of environmentally friendly biochemicals more economically feasible and potentially facilitate their broader production and use.

The major constituent of plant material is cellulose which is therefore the most easily accessible renewable raw biomaterial in the world. Cellulose is a natural polymer consisting of linear, repeated glucose units and is highly resistant to enzymatic degradation due to its crystalline configuration (Chen, 2014). Although there are some reports of LAB with cellulolytic activity (Zabidi et al., 2020; Román Naranjo et al., 2022, Chatgasem et al., 2023), there are currently no known naturally occurring LAB that can grow independently on cellulose (Román Naranjo et al., 2022). To enable the growth of LAB, this plant biomass must be pretreated with physical, chemical or biological processes, followed by enzymatic treatment, which incurs additional costs and can lead to the formation of inhibitory compounds (Kumar and Sharma, 2017).

To overcome this problem, several efforts to develop genetically engineered LAB with cellulolytic properties to achieve their growth on cellulose have been documented, but without success (Tarraran and Mazzoli, 2018). Several types of enzymes with different modes of action are required for the complete degradation of crystalline cellulose to glucose units: the endoglucanases (hydrolysis within the amorphous regions of cellulose chains, generating cellodextrins of varying lengths), the cellobiohydrolases (release cellobiose from the reducing or non-reducing ends of cellulose chains), and the β-glucosidases (hydrolyze cellobiose to glucose) (Liu et al. 2021). Several studies have indicated that production of heterologous cellulases in bacteria can increase metabolic load and is toxic to cells, making the co-production of multiple cellulases a particular challenge (Mingardon et al., 2011; Liu et al., 2019). Co-expression might also result in lower levels of the co-expressed cellulases, resulting in lower cellulolytic activity on some cellulosic substrates compared to the single-cellulase expression (Wan et al., 2024). The cellulolytic enzyme systems of some bacteria (e.g. *Saccharophagus degradans*) and brown rot fungi do not contain cellobiohydrolase, but only processive endoglucanases (Wu and Wu, 2020). These are functionally equivalent to the combination of non-processive endoglucanases and cellobiohydrolases and enable the growth of *S. degradans* and *Lachnoclostridium phytofermentans* on cellulose substrates (Watson et al., 2009, Tolonen 2009). This type of enzymes could be a solution to reduce the number of cellulases needed for efficient degradation and growth on cellulose. One of the main products of cellulose degradation with processive endoglucanases is normally cellobiose, which can be metabolized by many LAB (Tarraran and Mazzoli, 2018; Linares et al., 2010). Cellulases of cellulolytic bacteria found in nature are either located on the cell surface or secreted into the medium. The exposure of cellulases at the cell surface prevents their diffusion from the cell and ensures the proximity between the cells and the cellulose, allowing a faster uptake of the degradation products by the cell and thus preventing the inhibition of cellulase by its degradation products, resulting in synergy between enzyme and microbe (Lu et al. 2006).

In this study we aimed to develop genetically modified lactic acid bacteria that would be able to degrade cellulose and utilize the cellulose degradation products for growth. *Lactococcus cremoris* NZ9000 was selected as a chassis for constructing the cellulose-degrading LAB as this strain meets the two main requirements to achieve the objective of this study. First, *L. cremoris* is amenable to genetic engineering, necessary to introduce heterologous cellulase genes. Second, the NZ9000 strain is able to utilize cellobiose, as it contains the endogenous cellobiose-specific phosphotransferase transporter and intracellular phospho-β-glucosidase that cleaves cellobiose into glucose and glucose-6-phosphate (Linares et al., 2010; Solopova et al., 2017). For the heterologous expression of cellulases genes in *L. cremoris* NZ9000, two processive (Cel9A and Cel5H) cellulases and one non-processive (Cel5I) cellulase were selected. Cellulase Cel5I is the most active cellulase discovered to date from the cellulolytic bacterium *Ruminiclostridium cellulolyticum*, where it is bound to the surface of the cell wall via the SLH domain (Franche et al., 2016). Cellulase Cel5H is the most abundant secreted endoglucanase from the marine bacterium *S. degradans* and also shows the highest activity on different types of cellulose (Watson et al., 2009). The cellulase Cel9A is produced by the cellulolytic bacterium *L. phytofermentans* and has been shown to be essential for its growth on cellulose (Tolonen et al., 2009). In addition to being crucial for growth of original strains on cellulose and exhibiting high activity, all three cellulases degrade the cellulose to cellobiose (Franche et al., 2016; Vita et al., 2019; Watson et al., 2009), which can be metabolized by the strain *L. cremoris* NZ9000. Heterologous production of cellulases was optimized with regard to their secretion and surface display in *L. cremoris* cells. Additionally, the functionality of produced cellulases and the ability of the engineered *L. cremoris* bacteria to grow on cellulose substrate was evaluated and confirmed.

2. Methods

2.1. Construction of expression plasmids

Genes and other nucleotide sequences were amplified using Phusion High–Fidelity DNA Polymerase (F530S; Thermo Scientific). The amplicons were introduced into the plasmids by cloning with fast digest restriction enzymes (Thermo Scientific) and T4 DNA ligase (M0202S; New England Biolabs). The primers (IDT) and plasmids used are listed in Supplementary material (Table S1). NucleoSpin Gel and PCR Clean-up (740609; Macherey and Nagel) was used to purify the DNA fragments. The nucleotide sequences encoding the cellulases Cel5I, Cel5H and Cel9A (NCBI accession numbers: WP_012634873.1, WP_011469710.1 and WP_312101035.1, respectively) are provided in Supplementary material.

Genes encoding Cel5I, Cel5H and Cel9A were inserted into the plasmid pSD-flagHER (Plavec et al. 2021) between the restriction sites *BamHI/Xba*I (Cel5I, Cel5H) or *BamHI/Hind*III (Cel9A) to fuse the cellulase genes with *N*-terminal secretion signal peptide of the endogenous Usp45 protein (spUsp45), followed by the octapeptide Flag tag (amino acid sequence: DYKDDDDK). For cloning of cellulases in fusion with the C-terminal anchor domain of the endogenous *L. cremoris* autolysin *N*-acetylglucosamidase AcmA, the cellulase genes were inserted into the same plasmid pSD-flagHER between the *BamHI/Eco*RI restriction sites. For cloning of cellulases in fusion with the C-terminal AM12 anchor domain (of the endolysin from lactococcal bacteriophage

AM12), cellulase gene cassettes (containing nisin promoter, signal peptide, Flag tag sequences and cellulase gene) were amplified from the corresponding pNZsp plasmids (described above and in Supplementary material Table S1) and inserted into the pSD-AM12 plasmid (Plavec et. al., 2019) between the *BamHI*/*EcoR*I restriction sites. The constructs obtained encoded the cellulases downstream of the inducible nisin promoter PnisA. To replace the PnisA with constitutive promoters (PepN (NCBI accession number: M87840.1), B6 (Guo et al. 2012), P8 (Zhu et al., 2015) or LDH (NCBI accession number: X70926.1)), the amplified promoter sequences were inserted between the *Bgl*II/*Nco*I restriction sites in the plasmids described above. In selected plasmids with PepN promoter, the Flag tag sequence was removed by amplifying the sequence PepN_spUsp45 and reinserting it between the *Bgl*II/*BamH*I restriction sites in the corresponding plasmid. To achieve the intracellular production of cellulases, the sequence for spUsp45 was removed, while the sequence for the Flag tag was retained. This was achieved by amplifying the cellulase genes with the Flag tag (omitting the spUsp45) and reinserting the amplicons into the same plasmids between the *Nco*I/*Xba*I or *Nco*I/*Hind*III.

All the cloning was performed in *L. cremoris* NZ9000 and all plasmids obtained are listed in Supplementary material (Table S1). Transformation of *L. cremoris* cells with plasmids was performed by electroporation according to the protocol of Holo and Nes (1995) using the BTX Gemini X2 electroporation system. The NucleoSpin Plasmid EasyPure (740727; Macherey and Nagel) kit was used to isolate the plasmid from *L. cremoris* cells, with an additional 30 min treatment with 195,000 U/mL lysozyme (L6876; Sigma-Aldrich) and mutanolysin 62.5 U/mL (M9901; Sigma-Aldrich). The accuracy of the new plasmid sequence was verified by sequencing at Eurofins.

2.2. Bacterial growth and gene expression

Lactococcus cremoris cells were grown in M17 medium (56156; Millipore) supplemented with 5 g/L glucose (Formedium) at 30 °C under microaerophilic conditions without agitation. For bacteria containing plasmids, 10 μg/ml chloramphenicol (CO378; Sigma-Aldrich) was used to maintain selection pressure. For gene expression, the overnight culture was diluted 1:50 in fresh medium. Bacterial cells containing plasmids with a constitutive expression promoter were cultured overnight. Gene expression in bacterial cells containing plasmid with a nisin-inducible promoter was induced with 10 ng/ml or 25 ng/ml nisin (Fluka) at optical density at 600 nm (OD_{600}) of 1 (exponential growth phase). After induction, the bacterial cultures were grown for a further 3 h.

2.3. SDS-PAGE and Western blotting

To evaluate cellulase secretion, proteins in the conditioned medium of the overnight culture were precipitated with 10 % w/v (final concentration) trichloroacetic acid TCA (T6399; Sigma Aldrich). The precipitate was washed with ice-cold acetone and dissolved in 5 × Laemmli sample buffer. Before loading on gel, dithiothreitol (DTT) (R0862; Thermo Fisher Scientific) was added and samples were boiled at 100 °C for 10 min. When cellulases contained an anchor domain (cAM12, cAcmA) for surface display, we analyzed the cell wall fraction of *L. cremoris* cells from the overnight culture. The cell wall fraction was obtained as previously described (Visweswaran et al., 2017). Briefly, the cell pellet from 10 mL overnight culture was resuspended in 400 μL of 50 mM sodium phosphate buffer (pH 6.5) containing 550 mM sucrose, 100 mM NaCl, 5 mg/mL lysozyme and 62.5 U/mL mutanolysin and incubated at 37 °C for 1 h. After incubation, the cell suspension was centrifuged (5000 × *g*, 15 min) and the supernatant containing the cell wall fraction was analyzed by SDS-PAGE electrophoresis. Prior to gel loading, Laemmli sample buffer containing dithiothreitol (DTT) was added to the samples, which were then boiled for 10 min at 100 °C. Proteins from the concentrated conditioned media and from the cell wall fractions were separated on a 10 % Stain-Free gel (1610183; Bio-Rad) at 35 mA. Loading of the samples were normalized to the optical density of the cell culture. After separation, the proteins in the gel were photo-activated under UV light for 1 min and visualized using the ChemiDoc MP Imaging System (Bio-Rad) and ImageLab software (version 5.1, Bio-Rad). To estimate the molecular weights of target proteins, Precision Plus Protein™ All Blue Prestained Protein Standard (1610373; Bio-Rad) was used. Activated proteins from the gels were transferred to a nitrocellulose membrane (1620112; Bio-Rad) for 6 min using the Trans-Blot Turbo System (Bio-Rad). The membrane was blocked with 5 % skim milk in Tris-buffered saline containing 0.05 % Tween-20 (TBST) for 1 h at room temperature (RT). To detect the target proteins, the membrane was incubated overnight at 4 °C with rabbit anti-Flag primary antibodies (20543–1-AP; Proteintech) diluted 1:10,000 in blocking buffer. The membrane was then incubated for 1 h at RT with StarBright IgG Blue 520 fluorescent goat anti-rabbit secondary antibody (12005870; Bio-Rad) diluted 1:10.000 in blocking buffer. After each incubation, the membrane was washed three times with 0.1 %TBST. The ChemiDoc MP Imaging System (Bio-Rad) and ImageLab software (version 5.1, Bio-Rad) were used to acquire and process the images. GelAnalyzer 19.1 software was used for gel analysis.

2.4. Dot blot of bacterial cells

L. cremoris cells were separated from the medium by centrifugation (10 min, 6000 × *g*, 4 °C), washed with PBS and resuspended in PBS to OD_{600} of 4. Cell suspension (2 μl) was spotted onto a nitrocellulose membrane (Bio-Rad). Incubation of the membrane with primary and secondary antibodies and imaging of the membrane were performed as described above.

2.5. Flow cytometry and confocal microscopy

Cells for flow cytometry and confocal microscopy were prepared as described previously (Zadravec et al., 2015) with modifications. 20 μl of overnight cell culture adjusted to the OD_{600} of 4 was washed with 500 μL Tris-buffered saline (TBS) and then resuspended in 250 μL of rabbit anti-Flag primary antibody (20543–1-AP; Proteintech) diluted 1:500 in TBS. The cells were incubated with the primary antibody for 2 h at RT with shaking at 550 rpm. To detect the anti-Flag antibodies, cells were then incubated (2 h, RT, 500 rpm) with goat anti-rabbit antibody conjugated with Alexa Fluor 488 (4412S; Cell Signalling Technology) diluted 1:1000 in TBS. After each incubation, the cells were washed three times with 0.1 % TBST. Centrifugation was performed at 7500 x g, 4 °C for 5 min.

For the flow cytometry, the cells were fixed using paraformaldehyde (PFA). The pelleted cells were resuspended in 400 μL 4 % PFA and incubated for 20 min at RT in the dark. After incubation, the cell suspension in PFA was centrifuged (5000 × *g*, 5 min) and the cells were washed three times with 900 μL TBS. Finally, the cells were resuspended in 400 μL TBS and analyzed using the Attune NxT flow cytometer (Invitrogen) by measuring the geometric mean fluorescence intensity (MFI) of at least 10,000 bacterial cells at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. The data obtained was analyzed using FlowJo V10.10 software.

For confocal microscopy, stained cells were pelleted and resuspended in 300 μL TBS using StatSpin Cytofuge 2 cytocentrifuge (1000 × *g*, 5 min, RT) (Beckman Coulter). The slides were coated with poly-L-lysine (Sigma-Aldrich). An LSM 710 confocal microscope (Carl Zeiss) was used to examine the prepared samples. Samples with the fluorophore Alexa Flour 488 were excited with a laser at a wavelength of 488 nm. The emitted light was filtered using a bandpass filter from 505 nm to 530 nm. All images were taken with the same settings and processed with ImageJ software version 1.53 k (Schroeder et al., 2021).

2.6. Activity of cellulases on carboxymethyl cellulose

To evaluate cellulase activity on carboxymethyl cellulose (CMC) (C4888; Sigma Aldrich), 5 µL of conditioned medium were applied onto 1.5 % agar plates containing 0.5 % CMC and incubated overnight at 30 °C. Afterward, plates were further incubated for 15 min with 0.1 % Congo red (C6277; Sigma Aldrich) solution. Enzymatic activity was subsequently detected after washing the plates with 1 M NaCl solution.

2.7. Activity of cellulases on microcrystalline and amorphous cellulose

The activity of heterologously expressed cellulases, either secreted or displayed on the surface of *L. cremoris* cells, was evaluated using microcrystalline cellulose Avicel (PH 101) (11365; Supelco) or phosphoric acid swollen cellulose (PASC) as substrates. The amorphous cellulose PASC was prepared from microcrystalline cellulose Avicel (PH 101) as previously described (Wood, 1988). To analyze the activity of the secreted cellulases, the medium of overnight culture was separated from the cells by centrifugation (6000 × *g*, 10 min, 4 °C). The supernatant was exchanged with 50 mM potassium phosphate buffer (pH 7.0) and 20 × concentrated using Vivaspin concentrators (VS2002; Sartorius) with 10 kDa molecular weight limit. The 150 µL of the dialyzed concentrate containing cellulase was added to 3 mL substrate solution (3.5 g/L Avicel or PASC in 50 mM potassium phosphate buffer (pH 7.0), 0.01 % sodium azide). The enzymatic reaction mixture was incubated at 30 °C and 70 rpm for 24 h.

To analyze the activity of the surface-displayed cellulases on *L. cremoris* cells, the NZ3900 strain with cellobiose metabolism under catabolic repression was used. The cells were separated from the overnight culture medium by centrifugation (6000 × *g* for 10 min at 4 °C). The cell pellet was washed and then resuspended in 50 mM potassium phosphate buffer (pH 7.0) containing 137 mM NaCl to an OD_{600} of 28. The resulting cell suspension (500 µL) was added to 3 mL substrate solution (4.0 g/L Avicel or PASC in 50 mM potassium phosphate buffer (pH 7.0) with 0.01 % sodium azide). The final OD_{600} value of the cells in this reaction mixture was 4. The cells were incubated with the substrate for 24 h at 30 °C and 70 rpm. After 24 h incubation, the enzymatic reactions were stopped by placing the samples on ice. The insoluble substrate and cells were removed by centrifugation (18,000 × *g*, 10 min, 4 °C). The released soluble reducing sugars present in the supernatants were analyzed using the ferricyanide method of Park and Johnson (1949). The amounts for the released soluble reducing sugars were presented as glucose equivalents.

The proportions of cellodextrins released by the secreted cellulases were determined using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD). HPAEC-PAD analyses of released soluble sugars were performed using a Dionex CarboPac PA1 column (4 × 250 mm) on a Dionex ICS 3000 system (Sunnyvale) equipped with a pulsed amperometric detector as previously described (Ravachol et al., 2014). Standards with known concentrations of glucose (G7528; Sigma), cellobiose (22150; Sigma-Aldrich), cellotriose (O-CTR; Megazyme) and cellotetraose (O-CTE; Megazyme) were used to determine the concentration of released cellodextrins.

2.8. Growth of recombinant L. cremoris on PASC

Overnight cultures of *L. cremoris* cells harboring plasmids for secretion or surface display of cellulases were inoculated at a ratio of 1:50 in 40 mL of M17 containing 1.9 g/L PASC and 10 µg/mL chloramphenicol. The cells were grown for 8 days at 32 °C under microaerophilic conditions. To monitor the growth of genetically modified *L. cremoris* strains producing heterologous cellulases on PASC, total protein content in the cell lysates of the bacteria, the production of metabolic acids and the residual insoluble cellulose content were analyzed during cultivation as described below. At specific time points during the cultivation, 500 µl sample per culture was collected. The samples were centrifuged at 18,000 × *g* for 10 min to obtain the supernatant containing metabolic acids and the pellet containing the cells and insoluble cellulose.

2.9. Analysis of insoluble cellulose content during growth on PASC

Pellets from 500 µL growth samples were resuspended in 500 µL 12 M H_2SO_4 and incubated for 1 h at 37 °C with gentle shaking at 70 rpm. 20 µL of each sample was diluted 12-fold with dH_2O and autoclaved at 120 °C for 1 h. After cooling, 50 µL of 10 M NaOH was added to 240 µL of the sample to neutralize the acid. To remove any particles, the sample was centrifuged (18,000 × *g*, 10 min). Finally, 10 µL of supernatant were mixed with 190 µL of distilled water and 50 µL of 0.5 M NaOH. The glucose obtained by acidic hydrolysis of the insoluble cellulose was quantified with high pressure liquid chromatography (HPLC) using a Dionex CarboPac PA1 column (4 × 250 mm) on a Dionex ICS 3000 system (Sunnyvale), equipped with a pulsed amperometric detector (PAD), as described previously (Fosses et al., 2017).

2.10. Analysis of fermentation products during growth on PASC

To analyze the metabolic acids (lactate, formate, acetate) and ethanol production during growth, 200 µl of culture supernatant was combined with 50 µl of 25 mM H_2SO_4. Samples were analyzed by HPLC on the Aminex HPX87H column as previously described (Borne et al., 2021). The concentration of each fermentation product was determined using standards with known concentrations.

2.11. Analysis of total protein content in the bacterial cell lysates during growth on PASC

The cells in the sample pellet were washed three times with dH_2O to remove the remaining medium. To achieve partial degradation of the cell wall, the cells were resuspended in 200 µL lysis buffer (25 mM Tris-HCl (pH 7.0), 20 % (w/v) sucrose, 0.6 % (w/v) NaCl, 2 g/L lysozyme) and incubated for 30 min at 37 °C with gentle shaking (70 rpm). After incubation, the cells were separated from the lysis buffer by centrifugation (15 min, 5000 × *g*), washed with dH_2O and resuspended in 100 µL of 1 % SDS. The cell suspension was boiled for 15 min. The lysed cell suspension was then centrifuged at 1000 × *g* for 10 min at 4 °C, and the supernatant was collected for further analysis. 12 % stain-free gels (1610185; Bio-Rad) were used for SDS-PAGE electrophoresis to separate and quantify the proteins in the cell lysate as described above. An equal volume of each sample was loaded onto the gel for analysis. After separation, the proteins in the gel were photoactivated under UV for 5 min and visualized with 5 s exposure time for all gels equally using the ChemiDoc MP Imaging System (Bio-Rad) and ImageLab software (version 5.1, Bio-Rad). The gel images were analyzed with the GelAnalyzer software (version 19.1). Raw volumes of SDS-PAGE lanes corresponding to lysate proteins were normalized to raw volume of 1.5 µg bovine serum albumin loaded on the same gel.

2.12. Statistical analysis

Results are presented as average of at least two biological replicates ± standard deviation (SD) or ± standard error (SEM), as stated. GraphPad software (version 10.00) was used for the statistical analysis. The unpaired Student's *t*-test or the one-way ANOVA with Šidák or Dunnett post-hoc tests were used to compare the samples as indicated, with a *p*-value of less than 0.05 indicating significance.

3. Results

3.1. Engineering of L. cremoris for constitutive expression of heterologous cellulase genes

First, we aimed to establish the constitutive expression of a recombinant cellulase gene in *L. cremoris*. Cellulase Cel9A gene was selected as a reference cellulase and its constitutive expression was evaluated using four different constitutive promoters: the LDH promoter (promoter of *Lactiplantibacillus plantarum ldhL* gene) (NCBI accession number: X70926.1), the P8 promoter (promoter of *L. cremoris* phosphopentomutase gene) (Zhu et al., 2015), the B6 promoter (modified promoter of *L. cremoris noxE* gene) (Guo et al. 2012) and the pepN promoter (promoter of *L. cremoris pepN* gene) (NCBI accession number: M87840.1) (Fig. 1a).

The amount of produced Cel9A protein was evaluated by SDS-PAGE (Fig. 1b) using densitometric analysis of SDS-PAGE bands (Fig. 1c). Based on these results the highest constitutive expression of *cel9A* was achieved with PepN and B6 promoters, which yielded considerably higher Cel9A amounts compared to LDH and P8 promoters and similar amounts compared to the nisin-inducible promoter PnisA (Mierau and Kleerebezem, 2005), which is the most commonly used promoter for overexpression of recombinant genes in *L. cremoris*. Similar results were also observed from the activity assay on CMC-agar plates (Fig. 1d). However, as constitutive expression is generally preferred over inducible expression for the genes coding for essential substrate-degrading enzyme, all further constructs for expression of cellulase–encoding genes in this study were designed with the constitutive PepN promoter.

3.2. Cellulase secretion from L. cremoris cells

To evaluate the secretion level of constitutively produced cellulases from *L. cremoris* cells, different genetic constructs for *cel5H*, *cel9A* and *cel5I*, with or without the *N*-terminal Flag tag encoding sequence, were prepared. In all constructs, the cellulase-encoding genes were regulated by the PepN promoter (Fig. 2a). The concentrations of cellulases in extracellular media of overnight cultures were quantified from the intensities of SDS-PAGE bands of secreted cellulases, using the corresponding purified recombinant cellulases with known concentrations as standards (Fig. 2b, S1). The Flag tag significantly reduced the amount of

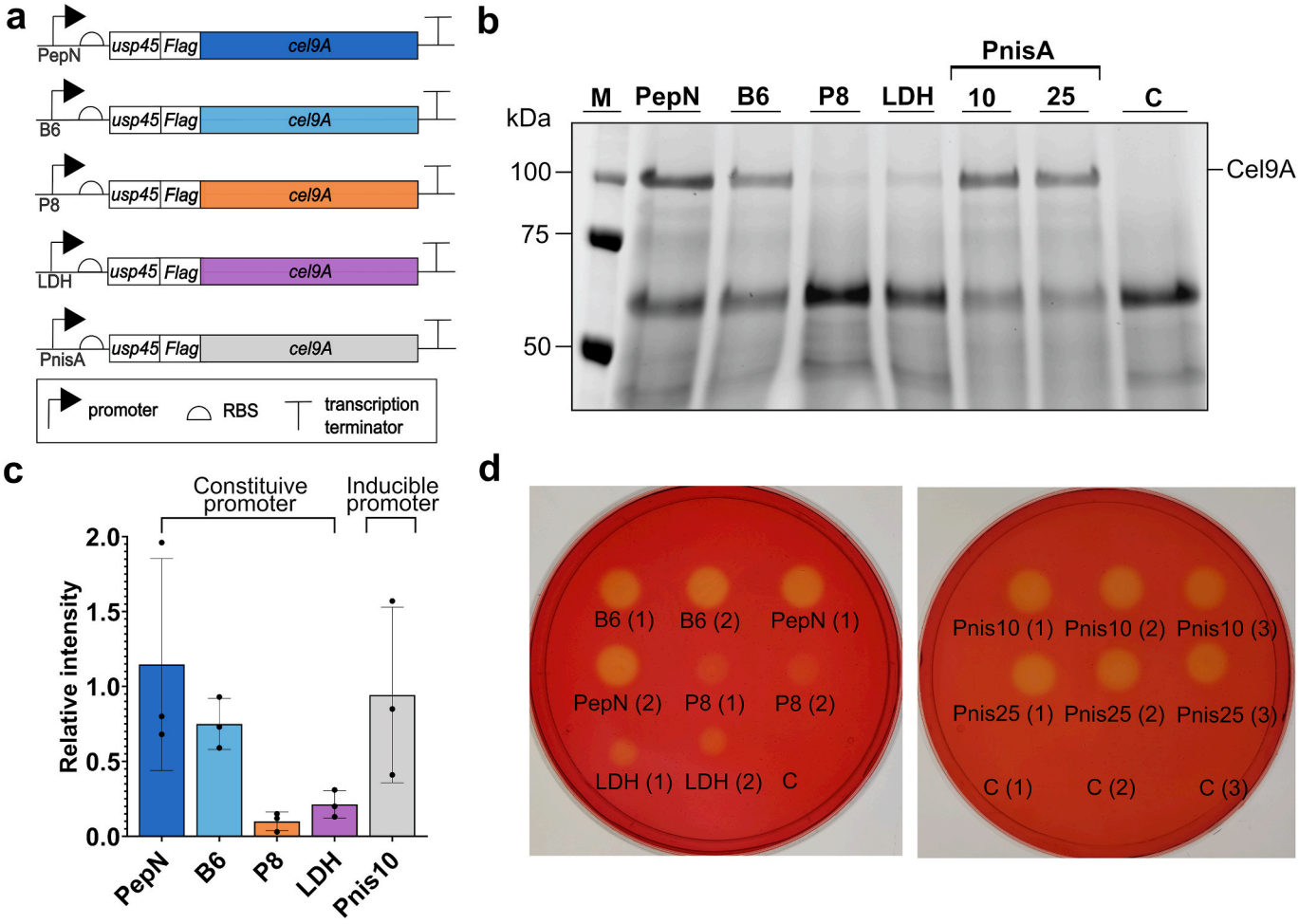

Fig. 1. Expression of the cellulase gene *cel9A* under different promoters. (a) Constructs designed for expression of the *cel9A* cellulase gene using different promoters. Four constitutive promoters (PepN, B6, P8 and LDH) and one inducible promoter (PnisA) were tested. *usp45*: signal peptide for protein secretion. Flag: protein tag. (b) Representative SDS–PAGE gel of concentrated conditioned media of *L. cremoris* cells expressing the cellulase gene *cel9A* from different promoters (PepN, B6, P8, LDH, or PnisA induced with 10 ng/mL or 25 ng/mL nisin). C: control sample (concentrated conditioned medium of *L. cremoris* NZ9000 containing empty pNZ8148 plasmid). M: protein weight marker. The experiment was conducted in three biological replicates for each sample and representative gel is shown. (c) Comparison of the amounts of secreted Cel9A cellulase expressed from different promoters. The raw intensities of SDS-PAGE bands corresponding to the Cel9A expressed from constitutive promoters or PnisA promoter induced with 10 ng/mL of nisin were relativized to the Cel9A expressed from PnisA promoter induced with 25 ng/mL of nisin. Data are presented as mean ± standard deviation (SD) from three biological replicates. (d) The activity of Cel9A cellulase on carboxymethyl cellulose (CMC). Cellulase Cel9A was expressed under constitutive promoters (left; B6, PepN, P8 and LDH) and under the inducible promoter PnisA (right) induced with 10 ng/mL (Pnis10) or 25 ng/mL (Pnis25) nisin. Biological replicates are numbered from 1 to 3.

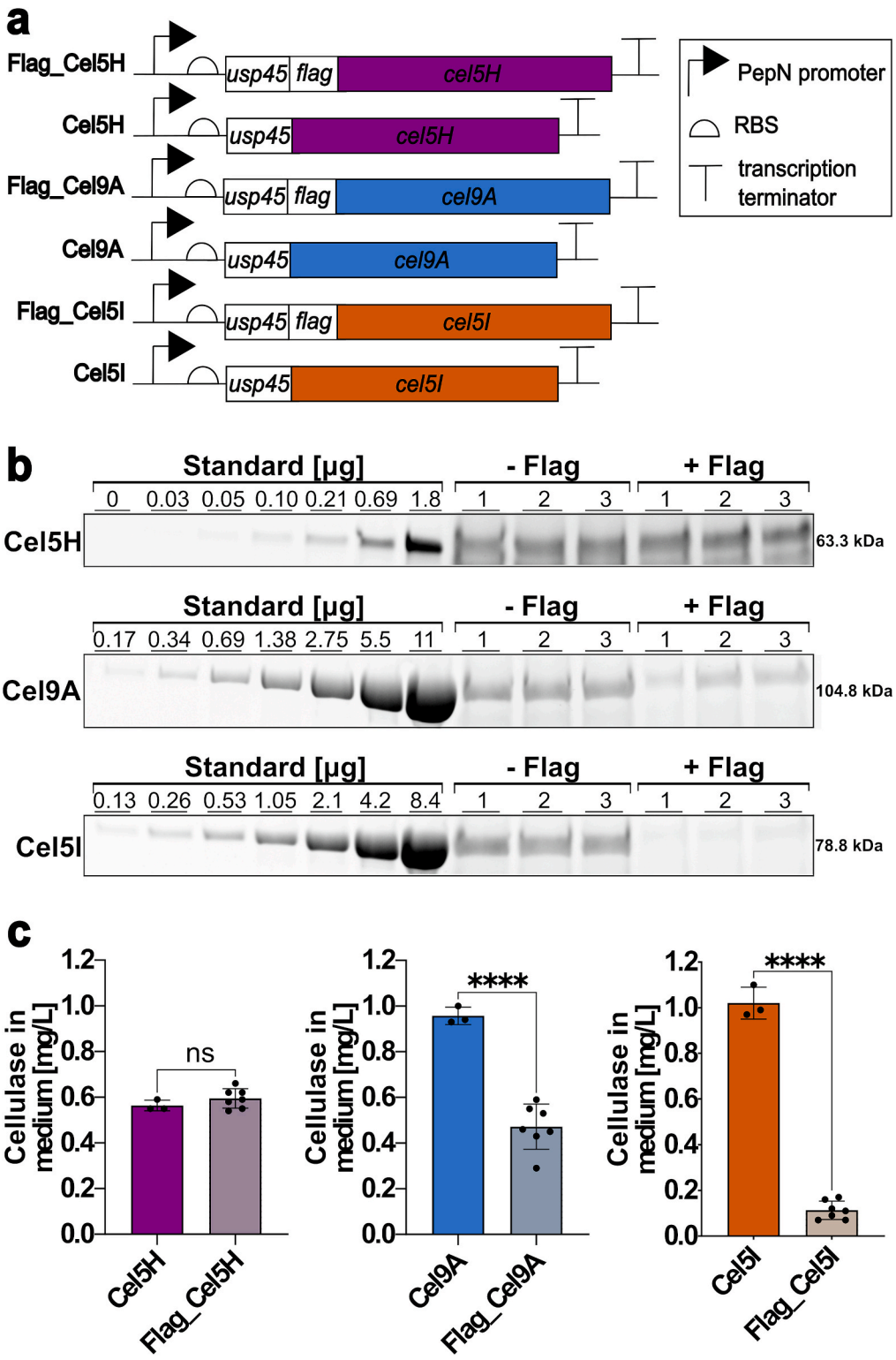

Fig. 2. Secretion of cellulases Cel9A, Cel5I and Cel5H from *L. cremoris* NZ9000 cells. (a) Expression constructs designed for the secretion of cellulases Cel9A, Cel5I and Cel5H with or without Flag tag. *usp45*: signal peptide for protein secretion. Flag: protein tag. (b) SDS-PAGE gels of concentrated conditioned media of *L. cremoris* cells secreting cellulases Cel5I, Cel5H or Cel9A with (+ Flag) or without (− Flag) the N-terminal Flag tag. Each sample marked with a number (1, 2, 3) represents an individual biological replicate. The standards are purified recombinant cellulases (Cel5H, Cel5I, Cel9A) of known concentration obtained from *Escherichia coli* (Franche et al., 2016; Vita et al., 2019). Purified Cel5H (unpublished) was contributed by HP Fierobe. (c) Estimated concentrations of secreted cellulases with or without Flag tag in the conditioned media after overnight expression in *L. cremoris* under the constitutive promoter PepN. Data are presented as mean ± standard deviation (SD) of three biological replicates. Statistical analysis was performed using unpaired *t*-test. **** $P < 0.0001$, ns $P > 0.05$.

secreted cellulases Cel9A and Cel5I, as they were produced in 2-fold and 9-fold higher amounts without the tag, respectively. Removal of the protein tag had no significant impact on the secretion of Cel5H cellulase. The *L. cremoris* cells secreted a higher amount of (tag free) Cel9A and Cel5I compared to Cel5H, with average yields ~ 1 mg/L (Cel9A and Cel5I) and 0.6 mg/L (Cel5H) (Fig. 2c). Different secretion efficiency among the recombinant cellulases may result from differences in their inherent folding properties, structural stability, and compatibility with the *L. cremoris* secretion pathway.

3.3. Surface display of cellulases on L. cremoris cells

In addition to *L. cremoris* cells that secrete recombinant cellulases, the *L. cremoris* with cellulases attached to their cell surface were also

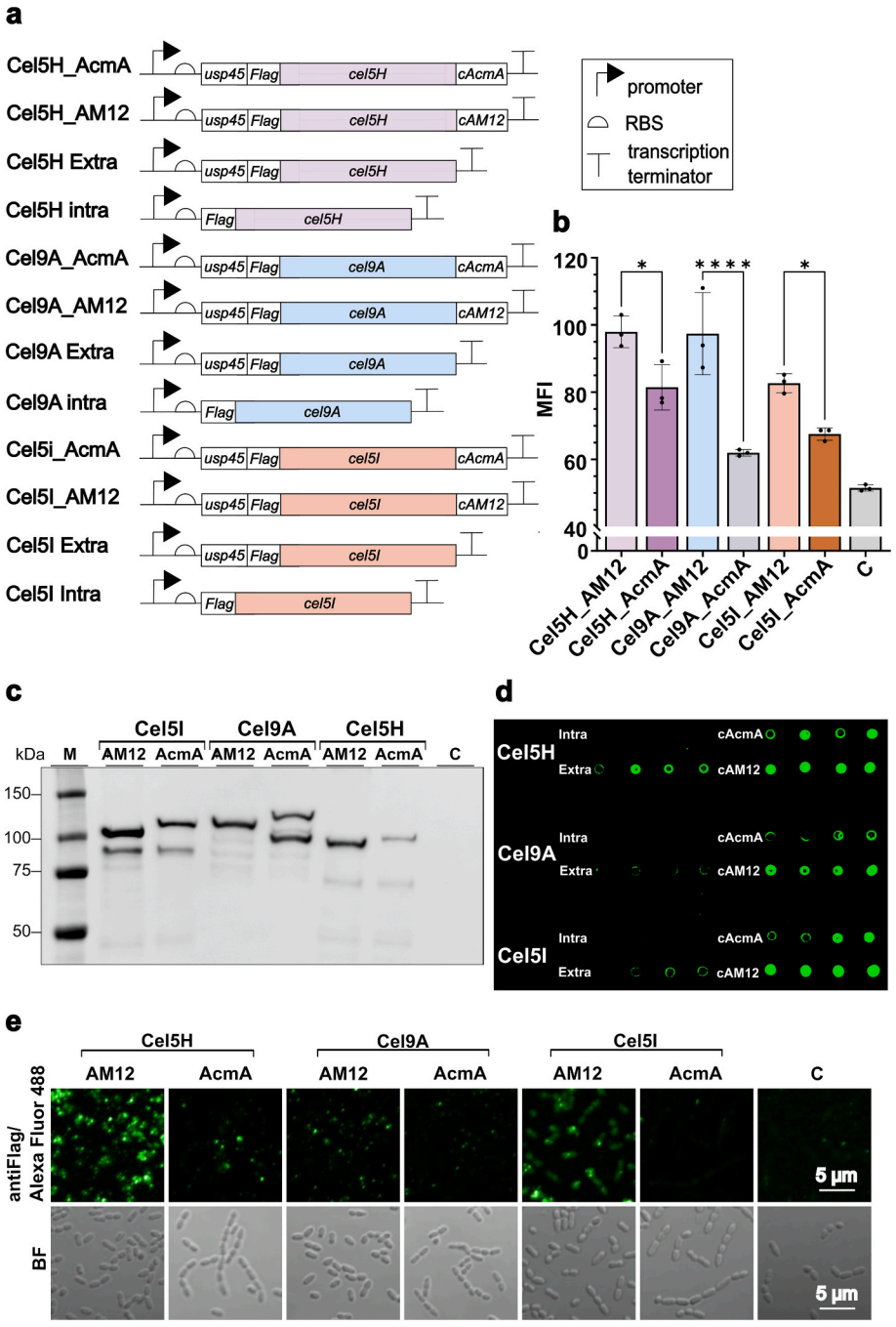

Fig. 3. Surface display of cellulases on *L. cremoris* cells. (a) Expression constructs for the surface display of the cellulases Cel9A, Cel5I and Cel5H on *L. cremoris* cells. usp45: signal peptide for protein secretion. Flag: protein tag. cAcmA and cAM12: noncovalent protein anchor domains. (b) Flow cytometry of *L. cremoris* cells displaying the cellulases Cel5H, Cel9A or Cel5I on their surface with two different anchors (cAcmA and cAM12). MFI: mean fluorescence intensity. Values are expressed as mean ± SD based on three biological replicates. Statistical analysis was performed using one-way ANOVA. **** $P < 0.0001$, * $P < 0.05$. (c) Western blot of the digested cell wall fractions of *L. cremoris* cells with expressed cellulases Cel5H, Cel9A, Cel5I displayed on cell surface with anchors cAM12 or cAcmA. M: protein weight marker. (d) Dot blot of *L. cremoris* cells producing Cel5H, Cel9A or Cel5 cellulase intracellularly (Intra), secreting cellulase (Extra) or displaying cellulase on their surface with anchors cAcmA or cAM12. (e) Representative confocal immunofluorescence microscopy images of *L. cremoris* cells displaying Cel5H, Cel9A or Cel5I on their surface with anchors cAcmA or cAM12. BF: bright-field. Bar scale: 5 µm. In panels b, c and e, C (control) corresponds to *L. cremoris* cells NZ9000 transformed with empty plasmid pNZ8148.

engineered. Two different noncovalent anchors for surface display of cellulases were tested: the anchor domain of the endogenous *L. cremoris* autolysin N–acetylglucosamidase AcmA (Ravnikar et al., 2010) and the anchor domain of the endolysin from lactococcal bacteriophage AM12 (Plavec et. al., 2019) (Fig. 3a). The surface display was evaluated with four different methods. Western blot analysis of digested cell wall fractions of *L. cremoris* cells confirmed the presence of bands of molecular weights corresponding to that of cellulases fused with anchor domain (Fig. 3c). In addition, bands of lower molecular weights were also observed, which might indicate partial degradation of cellulases with endogenous proteases. To further confirm that cellulases fused with anchor were displayed on cell surface, dot blot analysis was also performed with intact *L. cremoris* cells using the same primary and secondary antibodies as in western blot analysis (Fig. 3d). The control samples used in dot blot assay were *L. cremoris* cells that produced cellulases intracellularly (lacking anchor and secretion signal) or secreted cellulases (lacking anchor). Since no signal was observed in control samples containing intracellular cellulases, we can confirm that the antibodies that were used do not detect cellulases inside the cell and also do not interact non-specifically with the *L. cremoris* cell surface. The secreted cellulases Cel9A and Cel5I without anchor domain were detected at minimal level on the cell surface, while a slightly higher level for secreted Cel5H cellulase was observed. This could be due to nonspecific interactions between Cel5H and *L. cremoris* cell surface, or incomplete secretion. All three cellulases that contained either cAcmA domain or cAM12 domain produced intensive dot blot signal on the cell surface of *L. cremoris* cells. Successful surface display of cellulases by both anchor domains was also confirmed with flow cytometry (Fig. 3b) and confocal fluorescence microscopy (Fig. 3e) of *L. cremoris* cells. All four methods showed that cAM12 anchor enables more efficient surface display of cellulases in *L. cremoris*. Therefore, this anchor was selected for further work.

3.4. Functionality of secreted and surface-displayed cellulases

We evaluated the cellulolytic activity of the produced recombinant cellulases (without the Flag tag) using amorphous phosphoric acid-swollen cellulose (PASC) and microcrystalline Avicel cellulose as substrates (Fig. 4). To assay the activity of secreted cellulases, dialyzed medium of overnight cultures of *L. cremoris* NZ9000 cells secreting the heterologous cellulases were used. For assessing the activity of surface-displayed cellulases we used the *L. cremoris* NZ3900 strain for surface display instead of the NZ9000 strain, as the cellobiose metabolism in the NZ3900 strain is under catabolic repression. Therefore, the NZ3900 strain is unable to import and metabolize cellobiose in the presence of glucose (Linares et al., 2010), which lowered the consumption of cellobiose by the cells during the activity assay. Notably, the surface display of cellulases on NZ3900 and NZ9000 strains is comparable (Supplementary Fig. S2).

We measured the amount of reducing sugars released from amorphous or microcrystalline cellulose after 24 h of incubation. The highest activity on amorphous cellulose was observed with the secreted Cel5H cellulase, producing over 35-fold more reducing sugars compared to the negative control (dialyzed medium of cells not producing cellulases). Notably, all cellulases showed higher activity when secreted compared to when displayed on the cell surface (Fig. 4a). As expected, the activity of all cellulases on amorphous cellulose was markedly higher than that on microcrystalline cellulose. The highest activity on microcrystalline cellulose was found for the dialyzed medium containing secreted Cel5I cellulase, producing around 3-fold more soluble reducing sugars

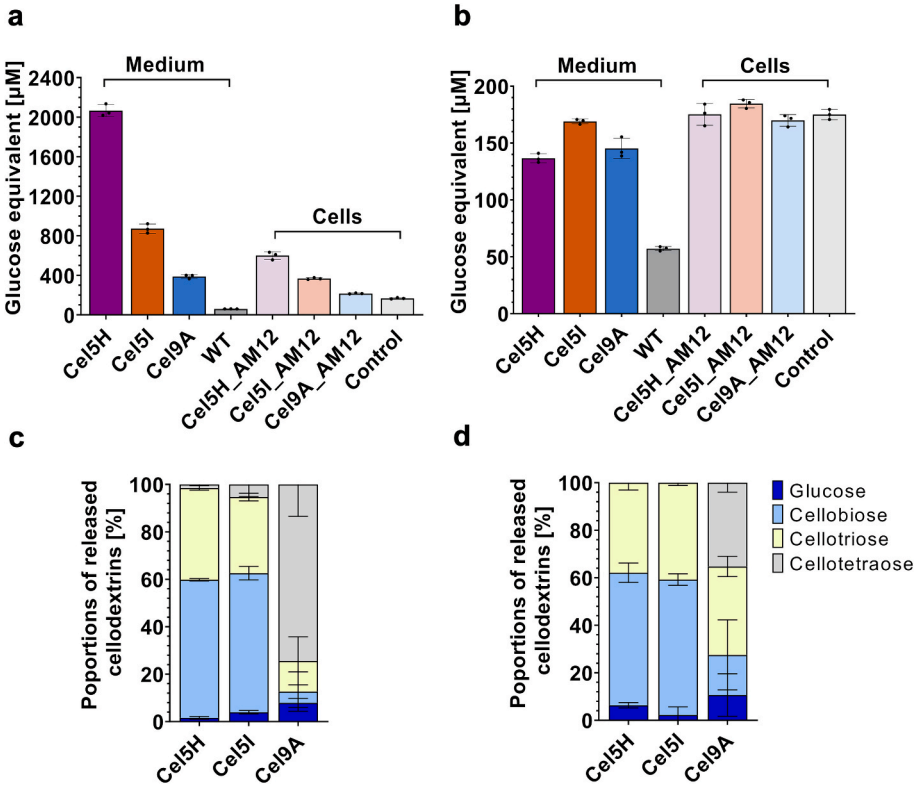

Fig. 4. Cellulolytic activity of cellulases on insoluble cellulose determined by the amount and proportions of soluble reducing sugars released by the cellulases Cel5H, Cel5I and Cel9A from PASC (a, c) or Avicel (b, d). Upper panels (a, b) show the amount of released soluble reducing sugars by secreted (Medium) or surface-displayed cellulases (Cells). Control corresponds to the conditioned medium of *L. cremoris* NZ9000 cells (a) or *L. cremoris* NZ3900 cells (b), both transformed with empty plasmid pNZ8148. Lower panels show proportions of the cellodextrins released by the secreted cellulases (c, d), whereby the values obtained using the negative control (conditioned medium of *L. cremoris* NZ9000 transformed with empty plasmid) were subtracted. In all graphs, the values are expressed as mean ± SD based on three independent measurements.

compared to the negative control (Fig. 4b). Higher activity on microcrystalline cellulose was again observed for each secreted cellulase compared to the cells with the corresponding surface-displayed cellulases. The latter did not exhibit any notable activity on microcrystalline cellulose, as the amounts of reducing sugars produced were similar to that of control cells not displaying cellulases. The activity detected in these control samples was likely caused by the intracellular components that could be released from the cells during the assay and the residual components of the growth medium that could also reduce the ferricyanide, contributing to the background signal.

HPAEC-PAD was used to measure the proportions of released cellodextrins for each secreted cellulase. The most abundant product released

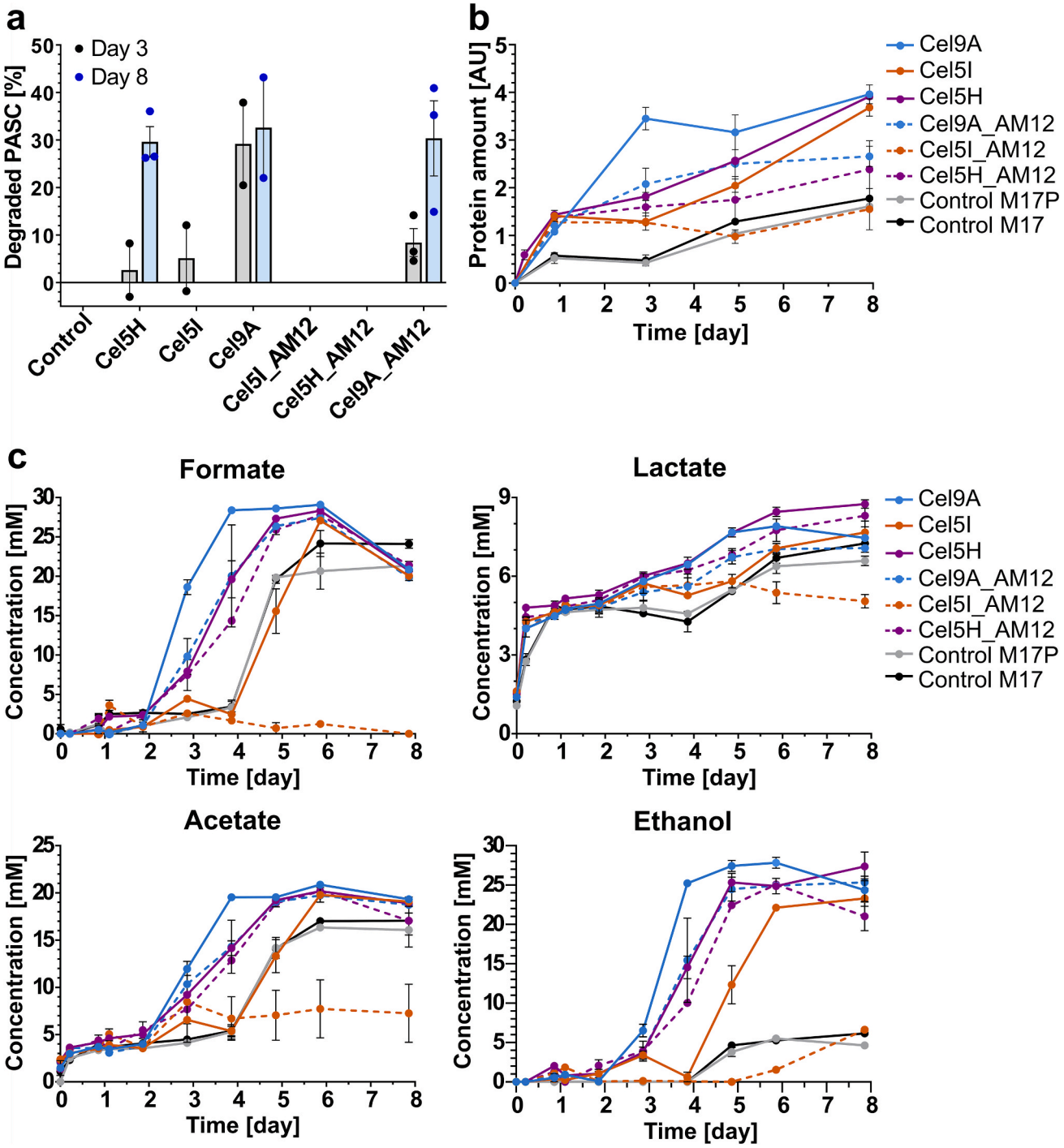

Fig. 5. Growth of genetically modified *L. cremoris* strains on PASC. (a) Percentage of degraded PASC for each *L. cremoris* strain. The amount of glucose released from the remaining PASC was determined by HPAEC-PAD before the start of cultivation (time 0) and after 3 and 8 days of cultivation. The glucose concentration values determined at time 0 corresponded to 100 % of the PASC present in the culture medium. Values are expressed as mean ± SD based on at least two biological replicates. (b) The total protein content in cell lysates (AU, arbitrary units) at different time points. (c) Formate, lactate, acetate and ethanol production during growth of *L. cremoris* strains on PASC. Values are expressed as mean ± SD based on three biological replicates. The experiment was conducted with *L. cremoris* cells either secreting cellulases Cel9A, Cel5I and Cel5H (solid lines) or displaying these cellulases on cell surface via cAM12 anchor (dashed lines). Controls: *L. cremoris* NZ9000 transformed with empty plasmid, grown in M17 medium (Control M17) or M17 medium with PASC (Control M17P). Values are expressed as mean ± SD based on three biological replicates.

by the cellulases Cel5I and Cel5H either from PASC (Fig. 4c) or Avicel (Fig. 4d) was cellobiose, followed by cellotriose. In contrast, cellulase Cel9A produced a significantly higher proportion of cellotetraose than cellotriose and cellobiose from PASC (Fig. 4c). On Avicel, the proportions of cellotriose and cellobiose released by Cel9A increased (Fig. 4d). For all three cellulases, glucose was among the least abundant products produced from both substrates (Fig. 4c, 4d).

Since cellulases contain at least one carbohydrate-binding module (CBM) in addition to the catalytic domain, we further confirmed the functionality of the heterologously expressed secreted cellulases in regards to their ability to bind to different cellulose substrates (PASC, Avicel, Sigmacell, filter paper) (Supplementary Fig. S3a). In addition, all *L. cremoris* strains displaying cellulases on their surface showed significantly higher binding to filter paper than the control strain without the displayed cellulases (Supplementary Fig. S3b).

3.5. Growth of genetically modified L. cremoris strains on PASC

Since the activity of cellulases on crystalline cellulose was very low (Fig. 4d), the growth of *L. cremoris* was only tested on amorphous cellulose. The growth was tested for *L. cremoris* NZ9000 strains that secrete cellulases and *L. cremoris* NZ9000 strains with surface-displayed cellulases via cAM12 anchor. Only the strains expressing the cellulases without the Flag tag were used. The growth was characterized by measuring the amount of residual PASC (Fig. 5a), the amounts of total cell proteins (Fig. 5b, S4) and release of fermentation products (Fig. 5c, S8) during the 8-day cultivation. Based on the total amount of protein biomass, produced metabolic acids and ethanol, *L. cremoris* NZ9000 secreting cellulase Cel9A exhibited the fastest growth and metabolite production (Fig. 5b, S4, 5c, S8). This strain degraded ~ 30 % PASC already by the third day of cultivation; however, no significant further degradation was observed by the end of the cultivation period (Fig. 5a). A similar amount of degraded PASC was also observed for the strain secreting the cellulase Cel5H and for the strain with surface-displayed cellulase Cel9A on the last day of growth (Fig. 5a). No significant PASC degradation was observed for any of the other strains during the cultivation period. Notably, all strains that secreted cellulases grew better and produced more ethanol and metabolic acids than the strains displaying the same cellulases on their cell surface. Some growth and metabolic acid production were also observed for the control strain, which does not produce heterologous cellulases (Fig. 5b, S4, 5c, S8). This growth was attributed to the presence of glucose and other components in the rich M17 medium. Ethanol was the major metabolite that contributed to differences between the control strain and strains producing cellulases (Fig. 5c, S8). Among all the strains that produce cellulases, the strain displaying the cellulase Cel5I showed the lowest growth performance, comparable to that of the control strain, whereby it produced even less ethanol and metabolic acids. With regards to the fermentation end-products during the growth on PASC, the cells producing the cellulases released significantly higher concentrations of ethanol (~25 mM) compared to the control strain (~5 mM). Also, the levels of formate and acetate increased faster throughout cultivation with the cells producing the cellulases; however, the final amounts produced were similar in all strains, including the control cells not producing cellulases. Of all measured fermentation products, lactic acid was the least abundant, reaching concentrations from 5 mM to 8 mM in the medium (Fig. 5c, S8).

4. Discussion

To grow on cellulose as the sole carbon source, bacteria must produce extracellular enzymes that degrade this polysaccharide, transporters that import cellulose degradation products, and suitable intracellular enzymes that metabolize the imported cellodextrins. While *L. cremoris* NZ9000 has intrinsic ability to transport and metabolize the cellulose degradation products, it lacks the genes encoding functional cellulases. In this study, successful constitutive expression of selected heterologous cellulases Cel9A, Cel5H and Cel5I in *L. cremoris* NZ9000 cells was achieved. The majority of studies employed inducible expression of cellulases in LAB, as this might avoid the possible issues with high metabolic burden caused by the constitutively expressing protein (Tarraran and Mazzoli, 2018). However, constitutive expression of cellulases is preferred over inducible expression, as cellulases are essential for growth on cellulose. Also, the use of inducible promoters in LAB has several disadvantages. Induction is most commonly triggered with bacteriocins (Sørvig et al., 2003; Mierau and Kleerebezem, 2005), which impact the growth and are not suitable for use in bioprocesses involving cocultures with other microorganisms. Induction of cellulase expression during longer culturing times (several days) and with slower growth would demand extensive optimizations to achieve optimal expression of cellulases throughout the whole growth period, because of the bactericidal nature of inducer, its adhesion and degradation. Apart from this, inducers would also incur additional costs which makes their use financially unsustainable at the industrial scale (Tarraran and Mazzoli, 2018).

In naturally occurring cellulolytic bacteria cellulases are either secreted or noncovalently anchored to the cell surface. To date, only Moraïs et al. (2014) have attempted to bind the individual cellulases to the surface of LAB, which was achieved using the sortase signal motif LPxTG that enables covalent binding. However, in the naturally occurring cellulolytic bacteria, the independent cell surface cellulases that are not complexed in cellulosomes are bound to the cell surface noncovalently, generally via their surface layer homology (SLH) domains (Munir and Levin, 2016). To better mimic that natural binding mode of cellulases in our *L. cremoris* strain, we employed the noncovalent anchors cAcmA and cAM12 for surface display of cellulases. Despite previously shown to be comparable (Plavec et al., 2019), cAM12 anchor provided a better surface display of all three heterologous cellulases in this study. Nevertheless, the three cellulases differed in their capacity to bind onto the cell surface, with Cel5I being the most abundant in the cell wall fraction (Fig. 3c) and Cel5H being the most exposed on the cell surface and readily detectable with specific antibodies (Fig. 3b, 3d, 3e).

Media containing secreted heterologous cellulases from *L. cremoris* were shown to have higher activity on both crystalline and amorphous cellulose than *L. cremoris* with cellulases displayed on cell surface. This could be explained by the spatially limited amount of surface-displayed cellulases, and more limited contact of surface-displayed cellulases with their substrate, as already reported by Moraïs et al. (2014). Secreted Cel5H showed the highest activity on amorphous cellulose, although its concentration in the medium was the lowest. This indicates high intrinsic activity of this cellulase, which is in line with Cel5H being one of the most active endoglucanases with high amorphous cellulose-degrading ability in its native organism *Saccharophagus degradans* (Watson et. al., 2009). As expected, the activities of all cellulases were lower on crystalline cellulose, as the crystalline regions of cellulose are less tractable to enzyme activity (Chen, 2014). The observed compositions of cellodextrins that were released from cellulose with each cellulase (Fig. 4c, 4b) were mostly comparable to previous studies (Franche et al., 2016; Vita et. al., 2019; Watson et. al., 2009). Cel9A released lower proportion of cellobiose and glucose from crystalline cellulose than in previous study (Vita et. al., 2019), possibly due to its lower concentration. Cellotetraose, the main soluble cellodextrin produced by Cel9A, could be degraded to cellobiose and glucose with extended incubation, as previously reported (Vita et al., 2019). Importantly, all three cellulases were also shown to bind to different cellulosic materials, which confirms the functionality of their carbohydrate–binding module (CBM) (Supplementary Fig. S3a, S3b). Overall, both cellulose-binding ability and cellulose-degrading activity observed in this study show that all three heterologously expressed cellulases in *L. cremoris* NZ9000 function correctly.

A particular challenge for lactic acid bacteria that produce heterologous cellulases is growth on cellulosic substrate. To date, only a limited

number of studies have attempted to cultivate engineered lactic acid bacteria on cellulose. Two studies showed that *L. lactis* and *Lactiplantibacillus plantarum* expressing heterologous cellulases grew and produced lactic acid in M17 medium supplemented with cellodextrins, which are already the degradation products of cellulose (Okano et al., 2010; Gandini et al., 2017). Liu et al. (2017) reported successful production of lactic acid and degradation of wheat straw and filter paper with engineered *L. cremoris*; however, this occurred only in the presence of M17 medium supplemented with additional glucose. In the study by Stern et al. (2018), the engineered *L. plantarum* failed to grow on the wheat straw as a sole carbon source supplemented with chemically defined medium. In our growth experiment, amorphous cellulose (PASC) was used as a major carbon source, supplemented with M17 medium with no additional glucose.

Engineered *L. cremoris* strains that secreted cellulases Cel5H and Cel9A degraded PASC during the cultivation, resulting in faster growth of these strains compared to the *L. cremoris* cells not producing cellulases. Even though the cellulase activity assays indicated higher intrinsic activity of Cel5H over Cel9A (Fig. 4c), the *L. cremoris* strain secreting Cel9A grew faster and produced larger total amount of metabolic acids and ethanol than the strain secreting Cel5H (Supplementary Fig. S8). This discrepancy between the lower Cel9A activity observed in the *in vitro* assays and the higher Cel9A activity observed during the cultivation on PASC might arose due to several reasons. First, Cel9A binds stronger to cellulose than Cel5H (Supplementary Fig. S3a), which possibly leads to higher stability of Cel9A under culture conditions. This is supported by previous report of increase in cellulase pH-stability in the presence of insoluble substrate (Røjel et al., 2020). Furthermore, the Cel9A might be more susceptible to product inhibition than Cel5I and Cel5H in the *in vitro* activity assay (Fig. 4a) where cellobiose accumulates. This effect would be eliminated during the cultivation of bacteria on PASC, where cellulose degradation products are taken up by the cells. In fact, all recombinant *L. cremoris* strains take up and metabolize cellobiose comparably to glucose (Supplementary Fig. S7), and during growth on PASC, no cellobiose remained in the culture medium at any time point (results not shown). Although the 24 h *in vitro* activity assays indicated that the main degradation product of Cel9A is cellotetraose, this sugar could be further degraded to cellobiose and glucose with extended incubation as shown previously (Vita et al., 2019). Additionally, we also cannot rule out the possibility that *L. cremoris* NZ9000 utilizes more complex sugars like cellotriose and cellotetraose, since Gandini et al. (2017) have shown that phylogenetically closely related lactococcal species *Lactococcus lactis* IL1403 can metabolize cellooligosaccharides up to cellotetraose. Transporting longer cellodextrins into the cells could be energetically more economical if intracellular phosphorolytic cleavage of cellodextrins would be catalyzed by cellodextrin phosphorylase that yields glucose-1-phosphate and uses inorganic phosphate anion, rather than ATP, as phosphate donor (Chen, 2015). The *L. cremoris* strain secreting the Cel5I cellulase showed poorer growth on PASC as well as on glucose, compared to strains expressing Cel5H and Cel9A. This indicates higher metabolic burden Cel5I cellulase imposes on cells, as this strain also grew slower compared to the control strain without cellulases (Fig. 5, S6). This was also evident from low segregational stability of the Cel5I-expressing plasmid. In contrast, the strains secreting the Cel5H and Cel9A retained their corresponding plasmids for at least 10 days even without the selective pressure (Supplementary Fig. S5). This difference in segregational stability of plasmids can be also attributed to the inserted sequence as shown previously (Kiewiet et al., 1993; Dabert et al. 1992). The low segregational stability of Cel5I plasmid could be related to the production of large amounts of high molecular weight plasmid multimers that reduce growth rates (Kiewiet et al., 1993; Kusano et al. 1989), which might also explain the slower growth of strain secreting Cel5I. Considering that the use of antibiotics would be undesirable for actual application of the developed cellulose-degrading lactic acid bacteria, high plasmid stability demonstrated for Cel5H and Cel9A is of particular importance. Cel9A was also the most active PASC degrader when comparing the strains with surface-displayed cellulases (Fig. 5a). This underlines the superior activity of Cel9A under culture conditions compared to the other two cellulases.

During the growth of *L. cremoris* NZ9000 on PASC, large increase in overall production of metabolites (metabolic acids, ethanol) was observed when cellulases, particularly Cel9A, were expressed (Supplementary Fig. S8). This increase is comparable to the cumulative yield of metabolites produced by *L. cremoris* NZ9000 strain grown on glucose or cellobiose (Supplementary Fig. S9c). Since *L. cremoris* is homofermentative, its major fermentation product is lactate (Neves et al., 2005), as was also observed in our case when grown on glucose (Supplementary Fig. S9a) However, during growth on PASC, lactate was not the major metabolite produced by *L. cremoris* expressing the cellulases. Instead, higher levels of formate, acetate and ethanol were observed (Fig. 5c). Accordingly, we observed a similar profile of metabolites during growth of *L. cremoris* NZ9000 on cellobiose (Supplementary Fig. S9b), a degradation product of PASC. Therefore, the substrate catabolized by cells impacts the profile of the metabolites produced. This mixed-type fermentation is common in lactic acid bacteria and is pronounced under low levels of glucose and in the presence of oxygen, as was the case in our cultivation experiments. Under these conditions, the $NADH/NAD^+$ ratio in cells is reduced, which lowers both the activity and transcription of lactate dehydrogenase (Neves et al., 2005). Higher levels of lactate might be achieved under anaerobic cultivation conditions (Okano et al., 2010) or further metabolic engineering to prevent the formation of other fermentation end products. This should be taken into the account when designing a bioprocess focused on lactate production, and will be tested in our future study.

Ability of bacteria to grow on PASC confirms that the developed recombinant strains can express functional cellulases and metabolize cellulose-derived sugars released from cellulose. This capability suggests these strains have the potential to utilize the cellulose from other plant materials, such as plant biowastes. However, plant material has more complex composition, containing lignin and hemicellulose in addition to cellulose. Therefore, at this stage of the development, pretreatment (physical, chemical, physicochemical) of plant biowaste to remove lignin and hemicellulose and to make cellulose more easily accessible, would have to be included (Zhao et al., 2022). Although further studies are still required to fully unlock the carbon source from plant biowaste by using lactic acid bacteria, this study provides an important step forward in reaching this ultimate goal.

5. Conclusion

In this study, it was shown for the first time that genetically modified lactic acid bacteria *L. cremoris* can grow on insoluble cellulose substrate as a major carbon source, without the supplementation with glucose. This was achieved by heterologous production of single cellulases originating from different native cellulolytic bacteria in *L. cremoris* strain that can utilize cellulose degradation products. The cellulases were designed either for secretion or for display on the cell surface, whereby the cells that secreted cellulase Cel9A were the most effective in cellulose degradation during their cultivation on amorphous cellulosic substrate and produced the highest levels of metabolic acids and ethanol. This approach might contribute to the development of bioprocesses for transformation of cellulosic materials into valuable organic compounds using lactic acid bacteria. Future studies investigating coexpression of multiple cellulase-encoding genes with strong mutual synergy might provide further improvements in cellulose biotransformation by these microorganisms.

CRediT authorship contribution statement

Petra Štravs: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Hélène David:** Writing – review & editing, Investigation. **Henri-Pierre**

Fierobe: Writing – review & editing, Resources, Methodology, Investigation. **Stéphanie Perret:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Aleš Berlec:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2025.133177.

Data availability

Data will be made available on request.

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Glossary

CBM: carbohydrate-binding module
CMC: carboxymethyl cellulose
HPAEC-PAD: high-pressure anion exchange chromatography coupled with pulsed amperometric detection
PASC: phosphoric acid swollen cellulose