ORIGINAL RESEARCH

Development of Bioactive Solid Support for Immobilized *Lactococcus lactis* Biofilms in Bioreactors for the Production of Nisin

Luis J. Bastarrachea[1] · **David W. Britt[2]** · **Ali Demirci[3]**

Abstract

It is essential to increase microbial population during the fermentation processes. Polypropylene was modified through reactive blending for it to have anhydride groups able to covalently bind with the polycation chitosan and for it to harbor *Lactococcus lactis* biofilms to produce the antimicrobial peptide nisin. Biofilm development was conducted for 5 cycles of fermentation in rich and minimal media. After 5 batch cycles of fermentation for biofilm growth in rich media, the chitosan-modified polypropylene contained 6.4 ± 0.4 log(CFU/cm^2), whereas the polypropylene support without chitosan contained 5.9 ± 0.4 log(CFU/cm^2). Infrared spectroscopy and electron and atomic force microscopy analyses confirmed higher nutrient and biomass deposition on the chitosan-modified polypropylene. After 24 h of fermentation in rich media, the *L. lactis* biofilms grown over the chitosan-modified polypropylene support produced a maximum nisin concentration of 523.5 ± 256.7 IU/mL, while cells in suspension produced 240.6 ± 5.1 IU/mL at the same period. *L. lactis* biofilms grown in minimal media over the chitosan-modified polypropylene support produced a maximum nisin concentration after 24 h of incubation in rich medium of 8.5 ± 3.9 IU/mL. Through reactive blending, it is possible to prepare supports that can harbor biofilms able to significantly increase the production of metabolites during fermentations.

Keywords Cell immobilization · Surface modification · Biofilms · Fermentation technology · Lactic acid bacteria · Antimicrobials · Nisin

Introduction

Microorganisms can be used in the industry through fermentation processes to produce a wide variety of metabolites of interest, such as alcohols, polymers, organic acids, enzymes, or antimicrobial agents. This is done by growing microorganisms at the necessary fermentation conditions (temperature, gas composition, pH, and nutrients) for them to produce a compound of interest through their inherent metabolic mechanisms (Ercan et al., 2015a). It is essential to increase microbial population during the fermentation for enhanced fermentation process. Therefore, cell immobilization can improve the efficiency of fermentation processes. With this approach, cells are allowed to bind to the surface of a suitable solid support, which can result in a higher productivity as compared to fermentation processes in which only suspended cells are used (Verbelen et al., 2006). An additional advantage of fermentation systems that employ immobilization is the repeated use of the support during many repeated fermentation batches without re-inoculation, as well as a reduced need for downstream purification of the compound of interest. Cells can attach to surfaces through electrostatic and hydrophobic interactions, or be immobilized through bioconjugation techniques (Hermanson, 2008; Oliveira, 1997). They can also be entrapped within the matrices of porous materials (Verbelen et al., 2006) Attachment of cells relies on the chemistry and topography of the solid support surface (surface charge, roughness, porosity, hydrophilicity, or hydrophobicity) (Klein & Ziehr, 1990), all properties that can be tuned efficiently through bioconjugation and polymer modification techniques (Bastarrachea et al., 2015; Goddard & Hotchkiss, 2007). While cell immobilization

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has been widely studied, it has not been extensively used for industrial applications, yet, due to the poor stability of the supporting materials (made frequently of protein and polysaccharide gels) (Champagne et al., 2010; Genari et al., 2003; Krasaekoopt et al., 2003; Lamboley et al., 2003; Lemay et al., 2002) and loss of viability of the immobilized cells (Champagne et al., 2007; Lamboley et al., 2003; Morin et al., 1992). Thus, there remains a need for the development of stable, efficient, and reusable solid supports that can be used in bioreactors at an industrial scale.

Cell immobilization can be done through two main types of mechanisms: active and passive immobilization (Ercan et al., 2015a; Ercan & Demirci, 2015). In active immobilization, microbial cells are entrapped within the matrix of a porous material (such as gels of polysaccharides or proteins), or attached through covalent bond formation between the chemical functional groups present in the cells and the functional groups present in the solid support (bioconjugation). In passive immobilization, cells deposit on the surface of the solid support and attach to its surface through electrostatic interactions, followed by biofilm formation of the microorganism of interest. Active immobilization can be ineffective due to the fragility and lack of stability of the types of supports used in cell entrapment and due to the toxicity of some coupling agents used to promote covalent bond formation between the cells and the solid supports, which may adversely affect cell growth and viability. For this reason, passive immobilization through the formation of biofilm has shown to be a more promising method to enhance fermentation processes (Ercan et al., 2015a). The formation of a microbial biofilm on a solid support requires a series of steps and conditions. First, a limited number of cells deposit on the surface of the solid support through electrostatic, hydrophobic, or Van der Waals interactions. This translates into reversible and weak interactions between the solid support and the cells, which eventually become irreversible interactions, promoted by bacterial adhesive appendages (fimbriae) and the production of extracellular polymeric substances (EPS) that can create bridges between the cells and the solid support. The irreversible attachment is enforced by multiple covalent, ionic, dipole–dipole, and hydrophobic interactions between the cells and the solid support, mediated by fimbriae and EPS (Ercan et al., 2015a; Missirlis & Katsikogianni, 2007). Once the biofilm has developed an irreversible attachment, the cells contained in it are able to produce metabolites of interest. If the necessary conditions of temperature, pH, and nutrient availability are maintained, cells may continue to reproduce, which may eventually lead to detachment of portions of the biofilm, which can be replaced by the multiplication of remaining cells in the biofilm (Ercan et al., 2015a). The formation

of biofilms is also promoted as a form of adaptation and survival by bacteria against harsh conditions of extreme pH, high salt concentration, or lack of nutrient availability (Kubota et al., 2008).

An ideal solid support for biofilm development must promote cell adhesion, be mechanically strong (able to resist physical manipulation and shear), and be a low-cost and widely available material. Additional key factors are the surface area for cell attachment and architecture of the support to optimize fluid dynamics during metabolite production (Ercan et al., 2015b). Different types of microorganisms may require a specific range of surface properties to be able to attach to the surface of a particular material. Several species of bacteria show affinity for hydrophobic surfaces (Ercan et al., 2015a; Pereira et al., 2000), while others such as some species of *Lactobacilli* develop biofilms on hydrophilic surfaces (Ercan et al., 2015a; Ho et al., 1997). However, the poor physical stability, narrowly defined surface chemistry, and limited surface area of biofilm-harboring supports (very often made of fragile materials such as protein and polysaccharide gels) has limited translation of immobilized biofilms in industrial applications to improve agriculturally relevant bioprocessing and engineering systems (Ercan et al., 2015b).

One possible alternative to improve the applicability of immobilized cells systems is the chemical modification of commonly used polymeric plastics through reactive blending or reactive extrusion. Polymer blending is a common practice in the plastic industry, with diverse applications in transportation, electronics, appliances, and packaging (Backer & Hu, 2001). Polymer blends can be miscible or immiscible, with the majority of blends being immiscible. To make polymers miscible and compatible for blending and to form covalent bonds with other polymers, they can be modified through the introduction of reactive groups within their side chains, block copolymers, or coupling agents. An additional advantage of these techniques when they are employed to modify the surface of materials is the preservation of the functional physical and mechanical properties of the original bulk material (Bastarrachea, 2019; Bastarrachea & Goddard, 2016; Bastarrachea et al., 2021; Gagon et al., 2020a, b). One attractive alternative to modify commonly used polymers to promote biofilm formation is chitosan. Chitosan has been extensively explored as a food preservative due to its effect on the stability of quality attributes such as pH and total volatile basic nitrogen (TVB-N), as well as its inhibition toward microbial growth in certain circumstances (Feng et al., 2016). However, due to the polycationic nature of this biopolymer, it has also been widely studied as an aide for biofilm formation, and its effectiveness has been confirmed for a variety of applications with different types of cells (mammalian cells and

bacteria) (Machluf, 2005; Nedović et al., 2005; Willaert & Baron, 1996). The positive charge and hydrophilicity make chitosan ideal for lactic acid bacteria surface immobilization for bioproduction because (as explained earlier) lactic acid bacteria possess a hydrophilic surface and therefore have affinity toward surfaces with the same surface character.

There are numerous products that can be produced by microbial fermentations including nisin, which is an antimicrobial peptide produced by several strains of *Lactococcus lactis*. It has Generally Recognized as Safe (GRAS) status given by the US Food and Drug Administration (Food & Drug Administration, 1988), and can be applied to several types of foods, such as meat and dairy products, as well as alcoholic beverages, to inactivate or inhibit mostly Gram-positive bacteria (Delves-Broughton, 2005; Qi et al., 2011). In addition, it possesses thermal stability, low toxicity to humans, and is unlikely to cause antibiotic resistance (Qi et al., 2011). It has also been investigated for cancer treatment and for infections (Kamarajan et al., 2015). Its antimicrobial mechanism is believed to involve disruption of cell wall synthesis and cell membrane disruption (Peschel & Sahl, 2006). Moreover, recent studies have discovered that the antimicrobial effect of nisin can be enhanced in combination with natural extracts, such as grape seed extracts, which has been attributed to the combined effect of cell membrane disruption by nisin and the alteration of metabolic pathways (Zhao et al., 2020a, b).

Therefore, this study has been undertaken to develop a modified plastic support made with polypropylene and chitosan via reactive blending and evaluate its performance to harbor *Lactococcus lactis* biofilms for the production of nisin.

Materials and Methods

Materials

D-Glucose, Tween® 20, agar (lot 191843), acetic acid (95%), acetone, absolute ethanol, glutaraldehyde (25%), hydrochloric acid (1.0 N), Na_2HPO_4, and D-lactose monohydrate were from Fisher Scientific (Pittsburgh, PA). KH_2PO_4 and NaCl were from VWR (Life Science, Philadelphia, PA). $MgSO_4 \cdot 7H_2O$ and yeast extract (YE) were from Alfa Aesar (Thermo Fisher Scientific, Waltham, MA). Isotactic polypropylene (PP) and methyl vinyl ether/maleic anhydride copolymer (MVE) were from Scientific Polymer Products (Ontario, NY). Glass beads (500–750 μm) were from Acros Organics (Fair Lawn, NJ). Peptone water (PW) and Nutrient Broth (NB) were from Oxoid (Thermo Fisher Scientific, Waltham, MA). M17 broth was from Difco, Becton Dickinson (Sparks, MD). Low molecular weight chitosan

(50–190 kDa) was from Sigma-Aldrich (St. Louis, MO). Anhydrous calcium sulfate was from Drierite (W. A. Hammond DRIERITE Co. LTD, Xenia, OH). Polybond 7200, a maleic-anhydride grafted PP (PP-*g*-MA), was kindly provided by Dr. John Yun from SI group, Inc. (Niskayuna, NY). Nisin® P (ultrapure Nisin Z) was supplied by Handary S. A. (Brussels, Belgium).

Biofilm Solid Support Preparation

The preparation of the PP support for the formation of *L. lactis* biofilms was based on a previous work of Gagon et al. (2020a) and Bastarrachea et al. (2021). A 1:1 (mass basis) mixture of PP and PP-*g*-MA pellets was fed into a bench-top Laboratory Mixing Extruder (LME) with a 1/8 in orifice strand die (Dynisco, Franklin, MA) at 180 °C and 30 rpm. The strands of the resulting mixture (referred to as PP-MA) were fed into a chopper to obtain pellets that where hot-pressed at 200 °C and 60 MPa, to obtain films with a thickness of 0.3 ± 0.03 mm. These PP-MA were cut into 2.0×2.0 cm coupons and cleaned in an ultrasonic water bath by immersing them first in acetone and then in deionized (DI) water (2 cycles of 10 min for each solvent at 40 kHz). After cleaning, coupons were allowed to dry overnight under anhydrous calcium sulfate (relative humidity of < 30%). Once dried, the coupons were spin coated at 2000 rpm for 1 min on both sides first with 0.3 mL of MVE in acetone (1 mg/mL) and then with 0.3 mL of chitosan in 1% acetic acid in DI water (15 mg/mL), using an anti-corrosion spin coater (VTC-200P-110, MTI Corporation, Richmond, CA). After spin coating, coupons were first allowed to dry in anhydrous calcium sulfate for 30 min and then heat-cured for 1 h at 185 °C. These coupons were referred to as PP-MVE-CHI. Figure 1 shows a depiction of the preparation of PP-MVE-CHI and subsequent deposition of nutrients and cells.

Lactococcus lactis Biofilm Formation on Solid Support

Lactococcus lactis (ATCC 11454) was obtained from the culture collection of the Department of Nutrition, Dietetics and Food Sciences at Utah State University (Logan, UT). A loopful of frozen stock in 25% glycerol (–80 °C) was inoculated onto M17 agar with 1% (m/v) D-lactose monohydrate. The M17 agar plates were incubated for 24 h at 32 °C, and a single colony was inoculated into 9 mL of M17 broth with 1% (m/v) D-lactose monohydrate. The inoculated M17 broth was incubated for 24 h at 32 °C, and a loopful was inoculated onto new M17 agar with 1% D-lactose monohydrate. These M17 agar plates were incubated again for 24 h at the same temperature, and the resulting plates with colonies were stored at 4 °C for not more than 3 weeks.

Fig. 1 Depiction of PP-MVE-CHI preparation and the subsequent deposition of nutrients from growth medium and cells. Prepared in Adobe Illustrate
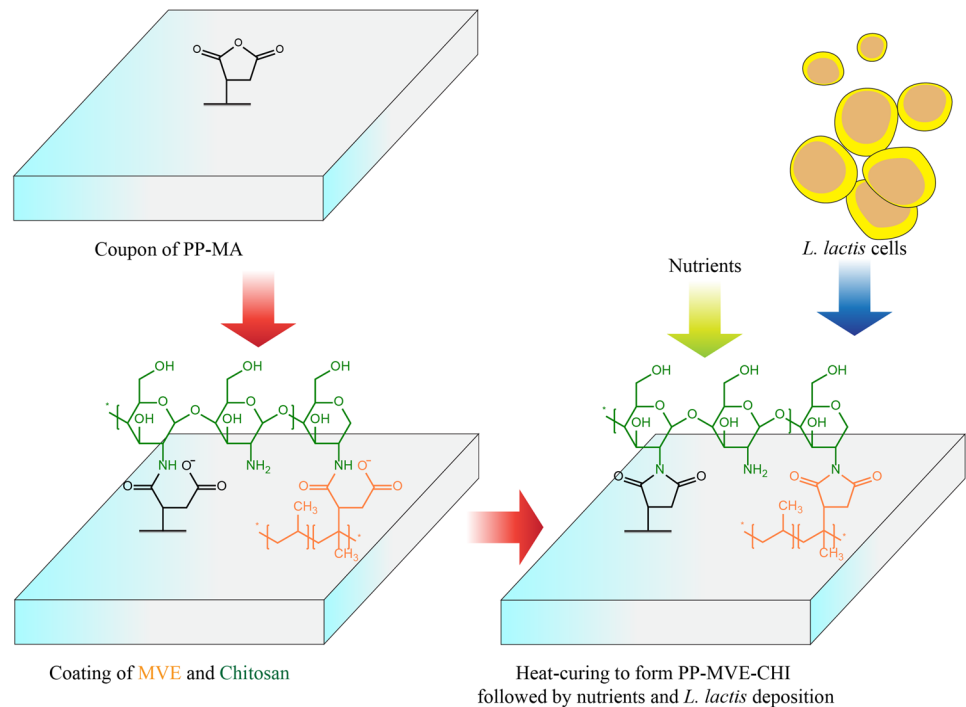
To prepare suspensions for biofilm formation, a single *L. lactis* colony from M17 agar was first inoculated into 9 mL of M17 broth and incubated at 32 °C for 24 h. Then, a 10% (v/v) dilution of this broth was prepared with sterile nisin fermentation-rich medium including D-glucose (80 g/L), peptone (10 g/L), YE (10 g/L), KH_2PO_4 (10 g/L), NaCl (2 g/L), and $MgSO_4 \cdot 7H_2O$ (0.2 g/L) and incubated for 24 h at 32 °C (for simplicity, this medium will be referred to as just rich medium). In parallel, as-prepared PP-MA and PP-MVE-CHI coupons were individually immersed in 20 mL of rich medium for initial nutrient deposition on their surface also for 24 h at 32 °C. After 24 h of incubation, the inoculated rich media were centrifuged at $1949 \times g$ for 10 min and the supernatant was replaced twice with 20 mL of new sterile rich medium after each centrifugation. A 1% dilution of this suspension was prepared with new sterile rich medium, to have an initial cell population of ~ 7 log(CFU/mL), and the PP-MA and PP-MVE-CHI coupons that had been subjected to 24 h of incubation in sterile rich medium were individually transferred into 20 mL of this ~ 7 log(CFU/mL) *L. lactis* suspension for initial cell attachment, which was followed by 48 h of incubation at 32 °C. After these 48 h of incubation for initial cell attachment, the PP-MA and PP-MVE-CHI coupons were subjected to 5 cycles of repeated batch fermentations for biofilm development (48 h per cycle) in which the growth medium was replaced at the end of each batch with sterile medium (20 mL per coupon). Two different fermentation media were used in these cycles: rich medium (with the ingredients listed above) and minimal medium (with the same ingredients listed above with the

exception of peptone and YE). The use of minimal medium was considered to explore the possibility of biofilm development with less ingredients. This process was replicated in at least 3 independent replicates.

Determination of Cell Density on Solid Support

To determine the number of cells attached on the PP-MA and PP-MVE-CHI coupons after the 5 cycles of repeated batch fermentations in either rich or minimal media, a published protocol of Ho et al. (1997) was followed. Briefly, the coupons were first transferred to 20 mL of sterile peptone water (PW, 0.1% (w/v) peptone) and vortexed vigorously to remove loose bacteria for 5 s. Then, the coupons were aseptically transferred to 10 mL of sterile PW with 5 g of glass beads to be vigorously vortexed in three 30-s cycles to remove the attached cells. Serial 10% dilutions in sterile PW were prepared from the vortexed suspension, and 100 μL of each dilution was inoculated onto M17 agar, which was followed by incubation at 32 °C for 48 h, and colony enumeration. The results were expressed as $\log(CFU/cm^2)$, taking into account the total surface area of individual coupons (8 cm^2). The support (PP-MA or PP-MVE-CHI) with the highest cell density was chosen to test the production of nisin.

ATR-FTIR

The surface chemistry of PP-MA and PP-MVE-CHI coupons was analyzed as described in our previous works (Bastarrachea, 2019; Bastarrachea et al., 2021; Gagon

et al., 2020a, b). Briefly, the coupons were analyzed with an IRTracer-100 infrared spectrometer (Shimadzu Corporation, Kyoto, Japan) with a diamond ATR crystal (Quest Single Reflection ATR Accessory, Specac Limited, Orpington, UK). Multiple spots on samples from independent replicates were analyzed through Happ-Genzel apodization (4 cm^{-1}, 32 scans per spot). Spectra were interpreted with the software KnowItAll (Biorad Laboratories, Philadelphia, PA). PP-MA and PP-MVE-CHI coupons were analyzed as prepared and after the 5 cycles of repeated batch fermentations. For the latter case, coupons were first individually vortexed vigorously for 5 s in 20 mL of sterile DI water to remove loose cells and then allowed to dry under anhydrous calcium sulfate until the excess humidity had evaporated, to be then analyzed through ATR-FTIR as explained above.

Contact Angle Goniometry

A droplet of DI water (1 µL) was applied on the surface of as-prepared PP-MA and PP-MVE-CHI coupons, and the static contact angle (θ_s) was measured with a VCA Optima digital contact angle instrument (AST Products, Billerica, MA). At least 3 measurements were performed from independently prepared samples of either PP-MA or PP-MVE-CHI.

SEM

After the 5 cycles of repeated batch fermentations in either rich or minimal media explained before, the type of supports (PP-MA or PP-MVE-CHI) with higher cell density were subjected to SEM analysis as follows. The coupons were first individually immersed for 1 h at room temperature in 20 mL of 2% glutaraldehyde in sterile Sorensen's buffer (133 mM Na$_2$HPO$_4$ and 133 mM KH$_2$PO$_4$ in DI water, pH 7.0). Then, the coupons were rinsed 3 times in 20 mL of sterile Sorensen's buffer (10 min per rinse) to remove loose bacteria. This was followed by gentle rinses (immersions) in aqueous ethanol solutions as follows: 2 rinses in 20 mL of 70% (v/v) absolute ethanol in DI water (5 min per rinse), 1 rinse in 20 mL of 90% (v/v) absolute ethanol in DI water (5 min), 1 rinse in 20 mL of 95% (v/v) absolute ethanol in DI water (5 min), and 3 rinses in 20 mL of absolute ethanol (10 min per rinse). Coupons were thereafter dried through critical point drying (CPD) and sputter-coated with a 10-nm layer of gold and palladium using a rotary sputter coater system EMS150R ES (Electron Microscopy Sciences, Hatfield, PA). SEM analysis was conducted with a FEI Quanta FEG 650 (Field Electron and Ion Company, Hillsboro, OR) under low vacuum at an accelerating voltage of 10 kV.

AFM

The sputter-coated PP-MA and PP-MVE-CHI samples were imaged with AFM to provide surface roughness, and topographical analyses not attainable with SEM images of the coupons were obtained with a Nanoscope III Bioscope (Digital Instruments, Inc., Tonawanda, NY) under taping mode using Budget Sensors-Tap 300 aluminum-coated cantilevers with a tip radius of curvature < 10 nm, 125 µm length, 30 µm width, 4 µm thickness and a 40 N/m force constant. Images were collected at 256 × 256 pixel resolution and 1 Hz over a range of scan sizes and angles. Sample surface roughness was analyzed on raw images using the instrument software.

Nisin Analysis

After completion of the 5 cycles of repeated batch fermentations in rich and minimal media and the cell density determination, the type of support (PP-MA or PP-MVE-CHI) with higher cell density was subjected to a single cycle of fermentation to challenge the ability of its *L. lactis* biofilms to produce nisin. After the 5 repeated batch fermentations, multiple coupons were transferred individually to 20 mL of sterile rich media and incubated for up to 48 h at 32 °C. Fermentation samples were collected over time for nisin analysis, which was conducted through agar diffusion assay as explained below (Pongtharangkul & Demirci, 2004, 2006a).

Samples were randomly selected over specific times, and the coupons were separated from the fermentation broth, and its pH was adjusted to 3.0 with sterile HCl (1.0 N) to inhibit further bacterial growth. Then, Tween® 20 was added to the sample to a final concentration of 0.1% (v/v) to prevent nisin from attaching to the tube's inner walls. The volume of medium was then heated to 90 °C for 5 min in a water bath to inactivate proteolytic enzymes, which was followed by 15 min of centrifugation at 3800 × g and 4 °C. The supernatant was separated and stored at –20 °C until analysis. As control, 20 mL volumes of rich medium with suspended *L. lactis* cells (initial inoculum of ~ 7 log(CFU/mL)) were used. For agar diffusion assay, the nisin-sensitive bacterium *Micrococcus luteus* (ATCC 4698) was employed (because of its sensitivity to nisin as reported in the cited references in the previous paragraph). A loopful of frozen *M. luteus* stock in 25% glycerol (from the Department of Nutrition, Dietetics and Food Sciences at Utah State University) was inoculated onto NB agar and incubated for 48 h at 32 °C. Then, a single colony was inoculated into 9 mL of NB and inoculated at 32 °C for 48 h. A loopful of this broth was inoculated onto new NB agar and incubated for 48 h at 32 °C, and the resulting plates were stored at 4 °C for not more than 3 weeks. For the agar diffusion assay, a single colony of *M. luteus* was inoculated into 9 mL of NB and incubated for 24 h at

32 °C. Then, a 5% (v/v) dilution of this broth was prepared with sterile and melted (40–50 °C) NB with 0.75% (w/v) agar and 1% (v/v) Tween® 20, to have a final *M. luteus* load of ~ 8 log(CFU/mL). Agar plates were prepared by pouring 25 mL of this medium into individual sterile Petri dishes (100 × 15 mm). The agar plates were allowed to solidify at room temperature for 3 h. Then, 7.0-mm diameter wells were made on the agar using a sterile stainless-steel cork-borer. A nisin standard curve was prepared by diluting Nisin® P in sterile diluted HCl (0.02 N), with multiple concentrations in the range of 0–500 International Units per mL (IU/mL), with 1 g of pure nisin being equal to 40×10^6 IU (Davies & Delves-Broughton, 2000). The fermentation samples frozen at −20 °C were allowed to thaw, and 10 and/or 2.5% (v/v) dilutions were made in sterile HCl solution (0.02 N). Then, 100 μL of diluted samples and nisin standards were applied individually into the wells made on the diffusion assay agar. The agar plates were stored at 4 °C for 24 h to allow the diffusion of nisin from the wells, and then incubated at 32 °C for 48 h for the survived cells to grow and the zones of inhibition to develop. Finally, diameters of inhibition zone were measured with a Fisherbrand™ Traceable™ Digital Caliper (Fisher Scientific, Pittsburg, PA), and nisin concentrations were calculated based on the nisin standard curve constructed by plotting the diameters of inhibition zones in mm versus the logarithm of the corresponding nisin concentration in IU/mL. At least 3 replicates were performed for this evaluation.

Statistical Analysis

When appropriate, significance between treatments was determined through analysis of variance (ANOVA) and Tukey's pairwise comparisons using Minitab® (Minitab Inc.,

State College, PA). Linear or nonlinear regression analyses were done using SigmaPlot® (Systat Software, San Jose, CA). Significance was set at $\alpha = 0.05$.

Results and Discussion

### Biofilm Solid Support Cell Density and Surface Characterization

When rich media was used in the 5 cycles of repeated batch fermentations for biofilm development, *L. lactis* cell density of the PP-MVE-CHI coupons (6.4 ± 0.4 log(CFU/cm²)) was on average 284% higher than the cell density from PP-MA (5.9 ± 0.4 log(CFU/cm²)). When minimal media was used, the cells were not culturable, even after allowing the inoculated M17 plates to incubate for more than 48 h at 32 °C. Although the measured cell density between the PP-MA and PP-MVE-CHI subjected to the repeated batch fermentations in rich media was not significant ($P > 0.05$), PP-MVE-CHI was selected for the microscopy evaluations and nisin fermentation evaluations as presented below, given its apparent ability to bind more nutrients and cells.

The values of the static contact angle (θ_s) for as-prepared PP-MA and PP-MVE-CHI were $105.0 \pm 3.0°$ and $88.0 \pm 1.0°$, respectively. This implies that PP-MA is hydrophobic and PP-MVE-CHI is slightly hydrophilic ($\theta_s < 90°$) (Goddard & Hotchkiss, 2007).

Figure 2 shows the ATR-FTIR spectra of the as-prepared PP-MA and PP-MVE-CHI coupons, and the PP-MA and PP-MVE-CHI coupons after the 5 cycles of repeated batch fermentations in rich medium (RM) and minimal medium (MM). The as-prepared PP-MA coupons show the characteristic carbonyl C=O (1780 cm⁻¹) and carboxylic acids

Fig. 2 ATR-FTIR spectra of PP-MA (left) and PP-MVE-CHI (right) after 5 cycles of repeated batch fermentations in either minimal medium (MM) or rich medium (RM). Prepared in SigmaPlot
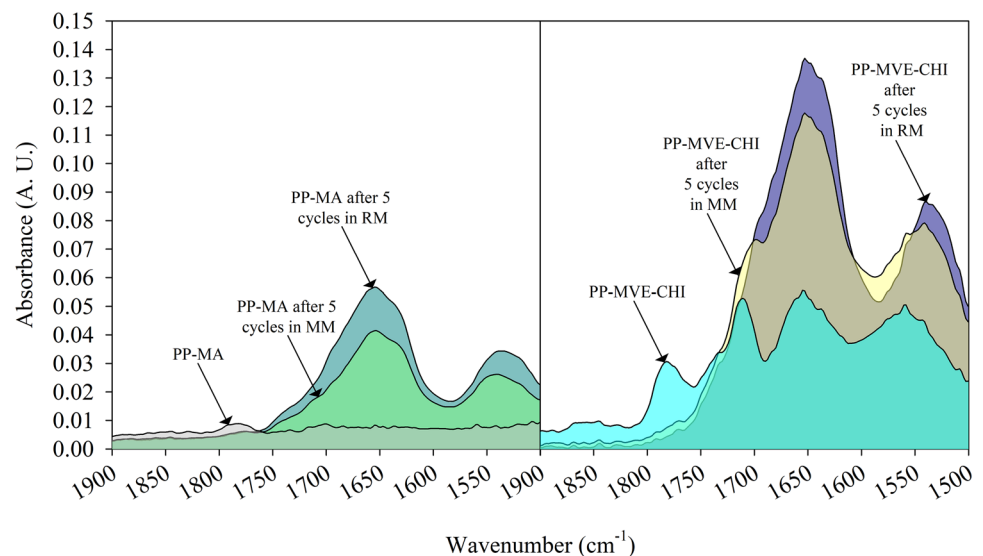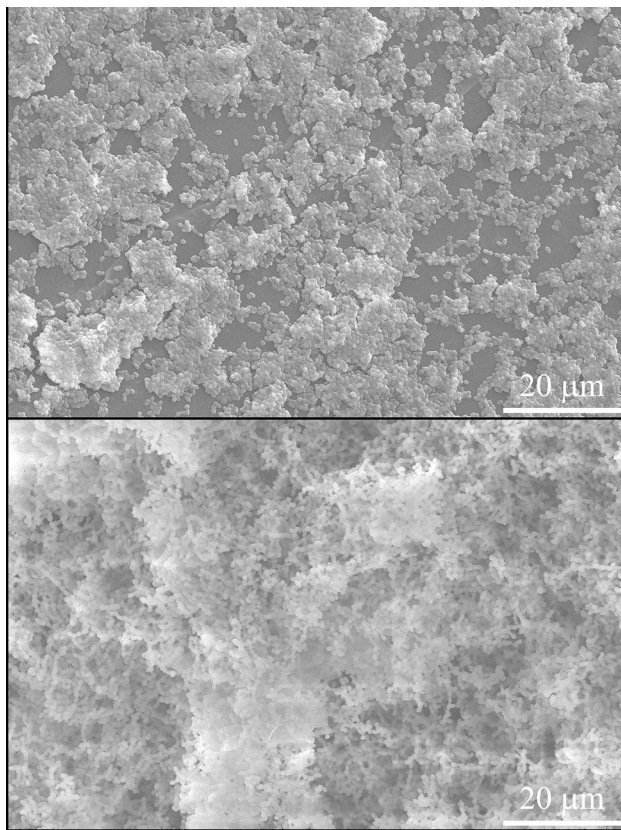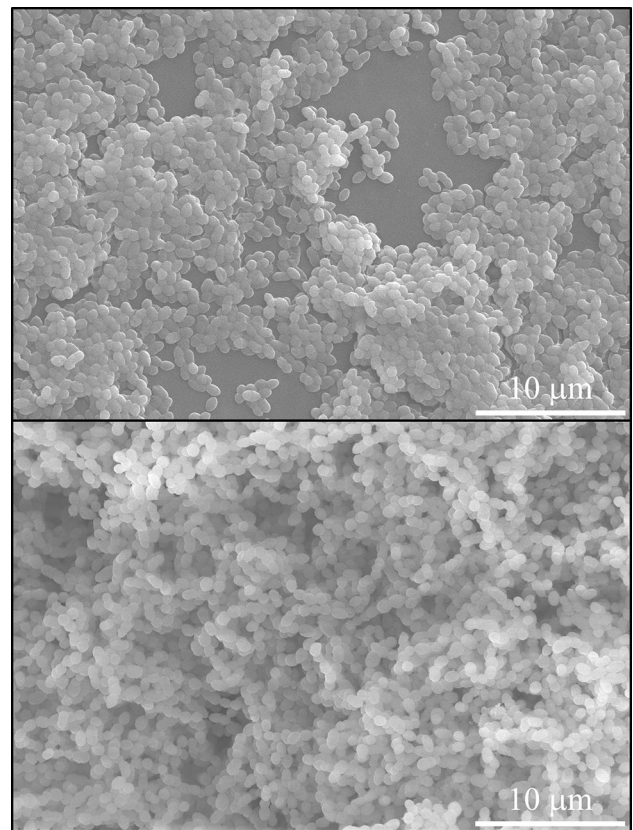
Fig. 3 2000×SEM image of *L. lactis* biofilms on PP-MVE-CHI grown in minimal (above) or rich (below) media after 5 cycles of repeated batch fermentations
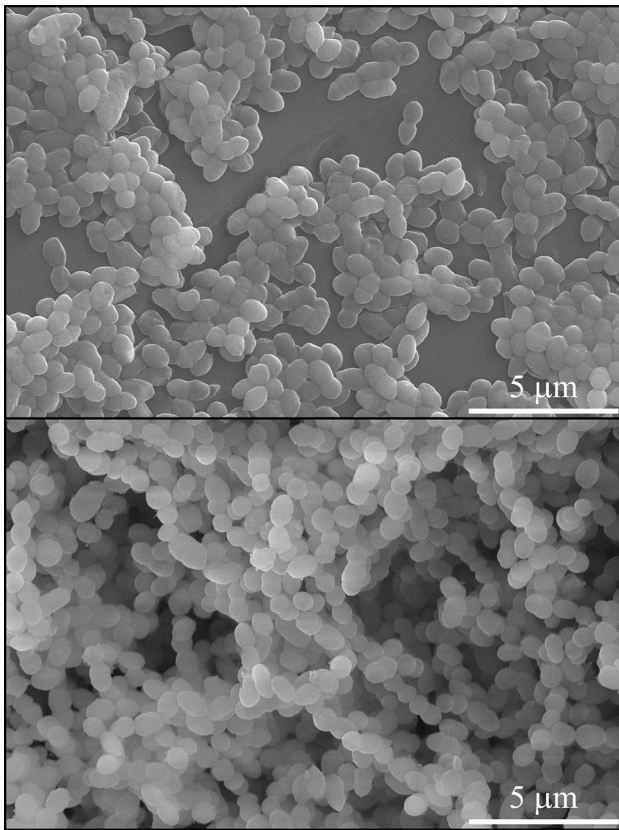

Fig. 4 5000×SEM image of *L. lactis* biofilms on PP-MVE-CHI grown in minimal (above) or rich (below) media after 5 cycles of repeated batch fermentations

–COO– (1720 cm^{-1}) vibration bands from the maleic anhydride groups. The as-prepared PP-MVE-CHI coupons show the expected carbonyl from the imide bond vibration at 1755–1680 cm^{-1}, which confirms crosslinking between MVE and chitosan. After the 5 cycles of repeated batch fermentations in either rich or minimal media, the formation and/or concentration increase of amides can be corroborated from their C = O and N–H vibration bands at 1755–1680 cm^{-1} and 1570–1515 cm^{-1}, respectively (Gagon et al., 2020a; Smith, 1998). These bands exhibit higher absorbance from samples exposed to rich media, which suggests higher concentration of biomass and nutrients, and the presence of those functional groups is most prominent on PP-MVE-CHI. As explained before, due to its apparent ability to harbor more cells and nutrients, PP-MVE-CHI was selected for the microscopy evaluations and nisin fermentation evaluations as presented below, given its apparent ability to bind more nutrients and cells.

The higher cell density exhibited by PP-MVE-CHI as compared to the control PP-MA can be interpreted as a direct result of the higher hydrophilicity (lower value of θ_s) it has. It has been reported that lactic acid bacteria have affinity for hydrophilic surfaces (Ercan et al., 2015a; Ho et al.,

1997). Similarly, hydrophilic surfaces are prone to fouling of organic matter (Barish & Goddard, 2013), which in the case of PP-MVE-CHI supports the apparent higher deposition of *L. lactis* cells and nutrients as compared to PP-MA. The high concentration of biomass and organic matter was also corroborated by the higher absorbances of the organic functional groups (amides) that can be attributed to proteins (Fig. 2) and that are also characteristic of lactic acid bacteria (Santos et al., 2015). We hypothesize that the higher nutrient deposition on the surface of PP-MVE-CHI as compared to the unmodified support promotes a higher production of nisin (as discussed later), possibly as a result of an effectively higher concentration of nutrients in the vicinity of the biofilms.

## Scanning Electron Microscopy and Atomic Force Microscopy

Figures 3, 4, 5, and 6 show *L. lactis* biofilms formed on PP-MVE-CHI after the 5 cycles of repeated batch fermentations in either minimal or rich media. For the former case, the biofilms exhibited a smaller thickness. In contrast, when rich medium was used, a thick layer of biofilm developed that
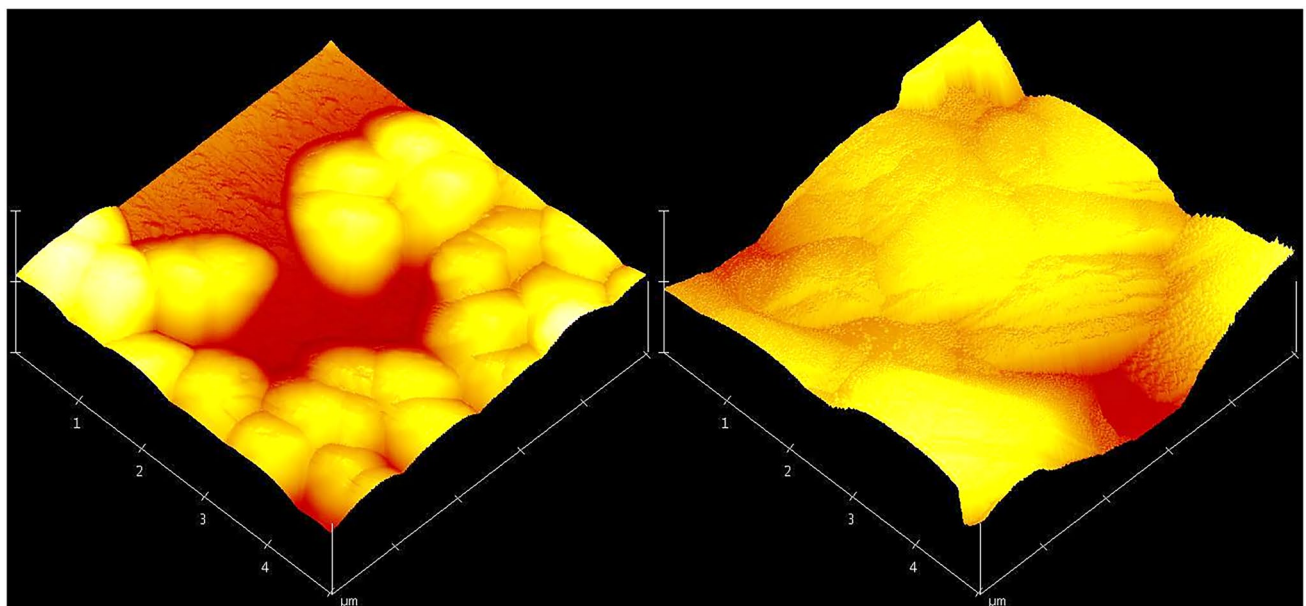
Fig. 5 10,000×SEM image of *L. lactis* biofilms on PP-MVE-CHI grown in minimal (above) or rich (below) media after 5 cycles of repeated batch fermentations

Fig. 6 20,000×SEM image of *L. lactis* biofilms on PP-MVE-CHI grown in minimal (above) or rich (below) media after 5 cycles of repeated batch fermentations

covered most of the support's surface. This was corroborated by AFM analysis (Fig. 7), which confirms that when minimal medium was employed, the biofilms were composed of
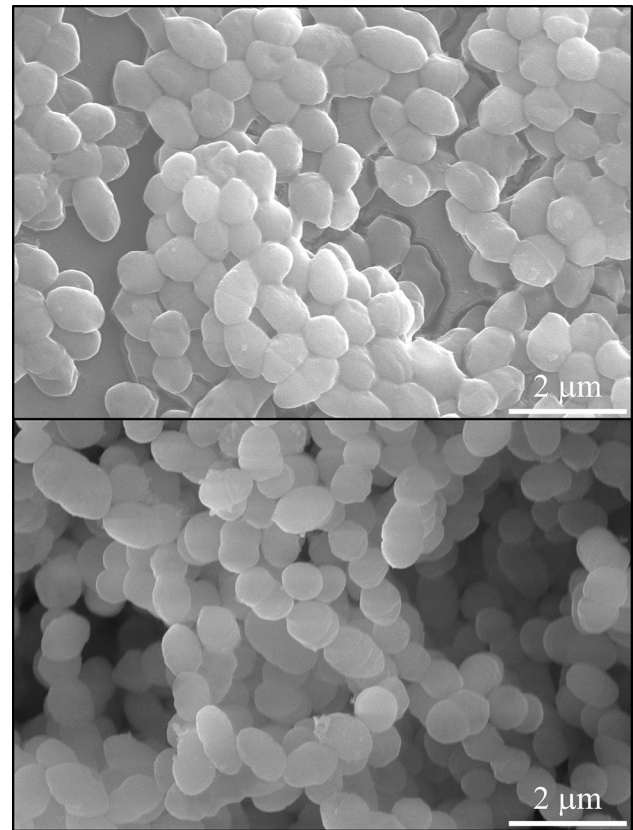
a layer of bacteria with the thickness of a few cells, while thick multicellular layers were found when rich medium was used. This apparent difference in cell size or morphology

Fig. 7 AFM images of *L. lactis* biofilms on PP-MVE-CHI grown in minimal (left) or rich (right) media after 5 cycles of repeated batch fermentations
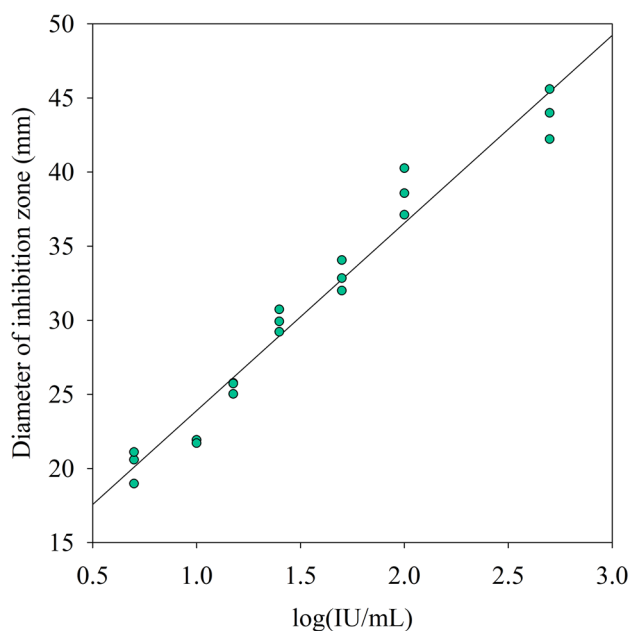
Table 1 Nisin production (IU/mL) by control *L. lactis* suspended cells and biofilms on PP-MVE-CHI supports. Treatments that share the same lowercase letter are not significantly different ($P > 0.05$)

Time (h)	Control (suspended *L. lactis* cells)	*L. lactis* biofilms on PP-MVE-CHI after 5 cycles in minimal medium	*L. lactis* biofilms on PP-MVE-CHI after 5 cycles in rich medium
0	0.0 ± 0.0a	0.0 ± 0.0a	0.0 ± 0.0a
2	0.0 ± 0.0a	0.0 ± 0.0a	9.8 ± 1.5a
4	0.0 ± 0.0a	0.0 ± 0.0a	20.3 ± 10.9a
6	5.9 ± 5.2a	0.0 ± 0.0a	100.1 ± 47.5ab
8	30.5 ± 17.0a	0.0 ± 0.0a	148.7 ± 38.0abc
24	240.6 ± 5.1bc	8.5 ± 3.9a	523.5 ± 256.7e
48	445.9 ± 51.4de	6.8 ± 0.8a	295.2 ± 177.1cd

may be attributed to unfavorable conditions, specifically for the case of the biofilms grown in minimal media. It has been observed in previous research works that antimicrobial agents or a harsh environment can promote visible changes in cell morphology (Chen et al., 2019, 2020, 2022).

Even though *L. lactis* biofilms formed under repeated batch fermentations in minimal media were not culturable, their presence was confirmed through SEM and AFM on the surface of PP-MVE-CHI (Figs. 3–6 and 7). Some non-spore-former bacteria are able to induce a stage known as viable but non-culturable (VBNC), in which the cells reduce drastically their metabolic rates due to harsh conditions of nutrient depravation and unideal environment for reproduction (such as unideal pH, temperature, and gas composition), but can stay alive (Liu et al., 2018). This implies that given the right conditions of growth, VBNC cells are eventually able to resuscitate and reproduce.

Nisin Fermentation and Analysis

Table 1 shows the nisin production over time by the control *L. lactis* suspended cells and the PP-MVE-CHI coupons with *L. lactis* biofilms formed in either minimal or rich media after the 5 repeated batch fermentations. After 24 h of incubation, the biofilms from PP-MVE-CHI grown in rich media were able to produce a significantly higher ($P < 0.05$) concentration of nisin (523.5 ± 256.7 IU/mL),

Fig. 8 Typical nisin agar diffusion assay standard curve. Prepared in SigmaPlot
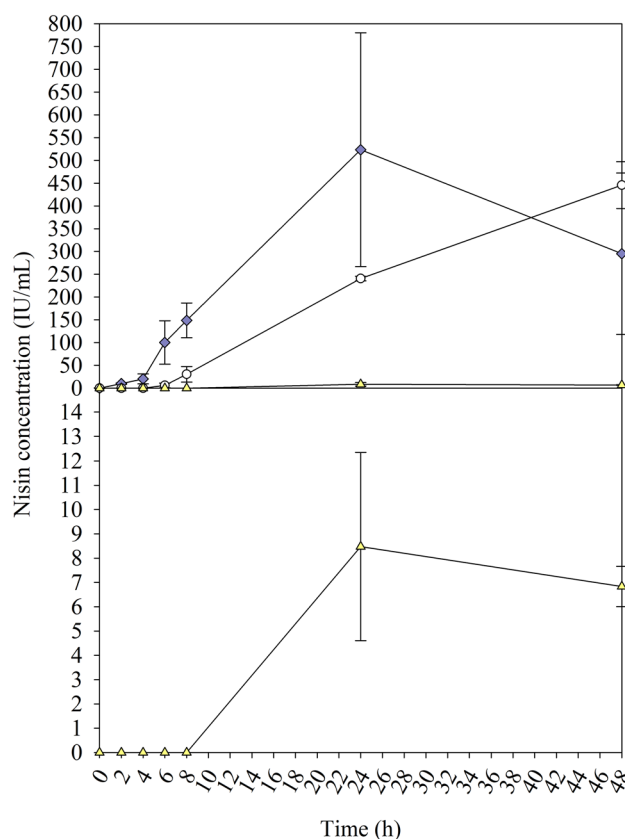
Fig. 9 Nisin production by *L. lactis* biofilm on PP-MVE-CHI, formed in either rich media (blue-filled diamond) or minimal media (yellow-filled triangle), and the suspended cells controls (empty circle). Prepared in SigmaPlot

an amount > 100% higher than the control suspended cells at the same time of incubation (240.6 ± 5.1 IU/mL). However, after 48 h, the concentration of nisin in the fermentation broth in contact with the biofilms from PP-MVE-CHI grown in rich medium had declined to 295.2 ± 177.1 IU/mL, which was lower than what was given by the suspended cells (445.9 ± 51.4 IU/mL) at that time of incubation, although both treatments were not significantly different ($P > 0.05$). The *L. lactis* biofilms from PP-MVE-CHI grown in minimal media were able to produce detectable levels of nisin only after 24 h of incubation, and the nisin concentrations produced at 24 and 48 h (8.5 ± 3.9 and 6.8 ± 0.8 IU/mL, respectively) were not significantly different ($P > 0.05$) from the nisin concentrations produced by the control suspended cells until 8 h of incubation. The agar diffusion assay produced reliable standard curves with high correlation ($R^2 \approx 1.0$) as shown in Fig. 8. Figure 9 shows the nisin production over time from the different treatments.

The reduced metabolic rate by the VBNC cells of *L. lactis* biofilms on PP-MVE-CHI (grown with minimal media) was confirmed in this study by the drastically lower amount of nisin they were able to produce as compared to the control suspended cells and *L. lactis* biofilms on PP-MVE-CHI (grown with rich media). The decline in nisin concentration after 48 h by *L. lactis* biofilms on PP-MVE-CHI grown with rich media may be a result of proteolytic activity by the cells (Pongtharangkul & Demirci, 2006b). Despite this latter phenomenon, the highest production of nisin was given by this latter treatment after 24 h of incubation, which suggests the potential of this concept of cell immobilization to increase substantially the production of metabolites in industrial fermentations.

Conclusions

In this study, it was demonstrated that through polymer modification, it is possible to produce supports able to harbor biofilms with high cell density and productivity. For the case of lactic acid bacteria (specifically *L. lactis*), a more favorable biofilm formation can be obtained with hydrophilic surfaces. This type of modified material is also able to bind more nutrients than the unmodified polypropylene. In this study, the chitosan-modified polypropylene was able to harbor ~284% more cells than the chitosan-free polypropylene, and those biofilms were able to produce ~100% more nisin than suspended cells in rich medium. The use of minimal media for biofilm growth can produce viable but not culturable biofilms, which results in limited metabolite production in a short incubation time. Future work to further improve this concept may include tuning or increasing the hydrophilicity of the modified solid supports to promote an even higher cell density that can in turn translate into higher productivity, increasing the surface area of exposed biofilm to the fermentation media,

as well as the optimization of the fermentation conditions to obtain the highest yields possible.

Data Availability All data generated are included in this article.

Declarations

Conflict of Interest The authors declare no competing interests.

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