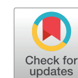
Colloids and Surfaces B: Biointerfaces 162 (2018) 246–255

Contents lists available at ScienceDirect

Colloids and Surfaces B: Biointerfaces

journal homepage: www.elsevier.com/locate/colsurfb

Architecture and physicochemical characterization of *Bacillus* biofilm as a potential enzyme immobilization factory



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A R T I C L E I N F O

Article history:
Received 23 August 2017
Received in revised form 9 November 2017
Accepted 22 November 2017
Available online 23 November 2017

Keywords:
Biofilm
Rheological properties
Lipase immobilization
Bacillus

A B S T R A C T

Biocatalysis for industrial application is based on the use of enzymes to perform complex transformations. However, these systems have some disadvantage related to the costs of the biocatalyst. In this work, an alternative strategy for producing green immobilized biocatalysts based on biofilm was developed.A study of the rheological behavior of the biofilm from *Bacillus* sp. Mcn4, as well as the determination of its composition, was carried out. The dynamic rheological measurements, viscosity (G") and elasticity (G') module, showed that the biofilm presents appreciable elastic components, which is a recognized property for enzymes immobilization. After the partial purification, the exopolysaccharidewas identified as a levan with a non-Newtonian behavior. Extracellular DNA with fragments between 10,000 and 1000 bp was detected also in the biofilm, and amyloid protein in the extracellular matrix using a fluorescence technique was identified. *Bacillus* sp. Mcn4 biofilms were developed on different surfaces, being the most stable those developed on hydrophilic supports. The biofilm showed lipase activity suggesting the presence of constitutive lipases entrapped into the biofilm. Indeed, two enzymes with lipase activity were identified in native PAGE. These were used as biocatalysts, whose reuse showed a residual lipase activity after more than one cycle of catalysis. The components identified in the biofilm could be the main contributors of the rheological characteristic of this material, giving an exceptional environment to the lipase enzyme. Based on these findings, the current study proposes green and natural biopolymers matrix as support for the enzyme immobilization for industrial applications.

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1. Introduction

In the last few years, an increased interest has been focused on the biocatalysis as a chemical synthesis alternative because it represents an ecological and low-cost alternative. One of the advantages of the biocatalysis, from an economic perspective, is the possibility of reusing the enzyme. Then the enzymatic immobilization presents a great potential because it permits to obtains re-usable biocatalysts in successive batches or continuous process [1]. Numerous materials have been reported as possible scaffold for enzyme immobilization [1]. Recently, some authors have studied the biofilm surface as new biotechnological material for enzyme immobilization [2,3]. This approach is attractive since it is scalable, represents a strategy for site-specific enzyme immobilization, and has the potential to stabilize enzymes under denaturing environmental conditions. The interest in biofilm enzyme immobilization is based on the conjugation strategies that employ a spontaneous process with biosynthetic materials, thus providing a simple way for enzyme immobilization [3].

Bacterial biofilms are interesting from a functional and structural point of view. Biofilms are spatially organized communities of microorganisms in an extracellular polymeric substance (EPS) [4] mainly composed of exopolysaccharides, proteins and extracellular DNA (eDNA) [5]. Exopolysaccharides play an important role in establishing and maintaining biofilm structure, and also mediate cell-to-cell and cell-to-surface interactions [6–8]. The formation of biofilms has been studied for some time now because of the advantages they offer, i.e. in wastewater treatment, microbial fuel

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https://doi.org/10.1016/j.colsurfb.2017.11.057
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cells for energy production, and also as a source of enzymes for fermentation, and textile or paper industries [9,10].

Bacillus species are often isolated from biofilms having harmful or beneficial effects in both industrial and natural environments [11]. Over the last decade, this Gram-positive genus has emerged as an important model for studying the molecular mechanisms of biofilm formation [5,12,13]. In addition, *B. subtilis* and *B. licheniformis* are very amenable to genetic manipulation. In the case of *B. subtilis* and *B. licheniformis* biofilms, relevant initial steps in the study of the structure and dynamics of the nonionic polysaccharide levan and the interaction among eDNA and amyloid-like proteins within the matrix have been performed [11,14].

It is known that *B. subtilis* does not form biofilm when the TasA protein is absent even in the presence of levan [6]. This result indicates that levan alone cannot support the formation of *Bacillus* biofilm, and that the presence of the other components, i. e. eDNA and proteins, modifies the rheological properties of the biopolymer [14]. In that sense, there is an increasing interest in the study of other components of the biofilm matrix, with the aim of controlling biofilm formation [15]. Special attention has received the amyloid protein, which play determinant roles in the structural integrity of biofilms [5,16]. Indeed, it was evidenced that eDNA-amyloid complexes play a key role in the modulation of the mechanical resistance of *B. licheniformis* biofilms [11]. At the same time, other proteins with enzymatic activities are also present in the matrix. For example, the main extracellular enzymes produced by *Pseudomonas aeruginosa* in the biofilm are hydrolases, including lipases. The simultaneous production and interaction of the polysaccharide alginate and the lipase LipA was reported in *P. aeruginosa* suggesting a role for this interaction in the enzyme immobilization and accumulation within biofilms [17].

Lipases (EC 3.1.1.3) are biotechnologically relevant enzymes. In addition to their natural function (hydrolysis of triglycerides), lipases are also able to catalyze synthesis (i.e. esterification, alcoholysis, and acidolysis) reactions in presence of organic solvents.

The versatility of lipases makes them one of the most important enzymes for commercial use, such as food processing, fats hydrolysis, oleochemical and detergent industry and pharmaceutical processing [18]. However, lipases are denatured and inactivated under several condition including improper pH and temperature. Besides, it is difficult the reusability of free lipases, which hinders the more expansive commercial use of these enzymes. An alternative to solve the reuse and stability is the immobilization of the enzyme. There are different immobilization systems using different supports for lipases immobilization, such as synthetic organic polymers, hydrogels and inorganic supports [19]. It has been found that the immobilized lipases showed higher activity over free ones.

The industrial use of immobilized lipases requires different characteristics of the biocatalyst depending on the particular application. Consequently, a continued research within the immobilization technology is necessary to provide solutions for each industrial application.

Based on the above mentioned, the aim of this work was to identify and characterize the natural components of *Bacillus* sp. Mcn4 biofilm and to evaluate the application of biofilm as a natural enzyme immobilization platform.

2. Materials and methods

2.1. Microorganisms and lipase activity on agar plates

A collection of 70 isolates of *Bacillus* sp. previously obtained from different oil fields and natural environments were screened for biofilm formation and lipase activity. For more details see Supplementary Information (S1-S2A).

2.2. Enzyme and biofilm production

In order to provide a large surface for biofilm development, bacterial growth was carried out at 30 °C in 500 mL flasks which were shaken (250 rpm) or kept under static conditions. They contained 200 mL of YP broth (yeast extract 0.5% and peptone from meat 1.0%), inoculated at a cell density of 1.56×10^6 CFU/mL with pre-cultures at mid-exponential growth phase of the selected *Bacillus* isolates. After 48 h of incubation, the supernatant obtained was utilized as an enzyme source, and the biofilms were manually collected for further evaluations.

Lipase activity was measured spectrophotometrically at 405 nm utilizing p-nitrophenyl acetate (p-NPA) or palmitate (p-NPP) as substrates. See Supplementary Information (S1-S2B).

2.3. Exopolysaccharide characterization

The exopolysaccharide was extracted and partially purified from the mature biofilm following the technique of Bales et al., [20] with modification (S1).

Exopolysaccharide samples were then lyophilized and resuspended in sterile bidistilled water up to a concentration of 1 g/L (w/V). Acid hydrolysis of exopolysaccharides was carried out in autoclave for 10 min at 121 °C in a solution of 2 M HCl. Reducing sugars released were quantified using the dinitrosalicylic acid method [21].

To identify the functional groups present in the exopolysaccharide a Fourier Transform Infrared (FTIR) analysis was carried out. The assay was performed using a potassium bromide tablets at room temperature, and 7 atmospheres of pressure in Perkin Elmer 1600 FTIR equipment (Institute of Physical Chemistry, Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucumán, Argentina).

2.4. Aggregation kinetics of biofilm amyloid protein

A thioflavin-T (ThT) experiment was conducted to analyze the formation of amyloid aggregate from biofilm proteins at 37 °C. Protein samples were obtained from the biofilm described above. They were first sonicated with a probe-type sonifier under controlled temperature. 5 μl of a dilution 1/10 of a homogeneous samples were mixed 95 μl of 20 mM HEPES pH 7.4, containing ThT 25 mM. The fluorescence emission was registered with an ISS PC1 spectrofluorimeter (Champaign, IL, USA) with excitation/emission wavelengths of 450 nm and 482 nm respectively [22].

2.5. Extracellular DNA characterization

For eDNA extraction, 0.20 g of biofilm was previously treated with formaldehyde to fix the cells and prevent cell lysis. The biofilm was resuspended in 1.5 mL of a buffer containing 50 mM TRIS and 20 mM EDTA, and disrupted in an ultrasonic water bath for 5 min. Cells were removed after centrifugation (16,000 × g, 5 min) and eDNA was precipitated with phenol-chloroform-isoamylic alcohol (49:49:1) following standard procedures. Random amplification of the eDNA was performed independently with primers XD9 (5′-GAAGTCGTCC-3′) [23], OPI-02mod (5′-GCTCGGAGGAGAGG-3′) [24], and M13b (5′-GAGGGTGGCGGTTCT-3′) [25]. See Supplementary Information (S1).

2.6. Evaluation of protein fraction from biofilm matrix

Biofilm proteins were extracted from 1.5 g of biofilm, and resuspended 10 mM phosphate buffer (pH 7.0). Formaldehyde was added to biofilm samples to fix the cells. After disruption in an ultrasonic water bath for 5 min, samples were centrifuged for 5 min

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