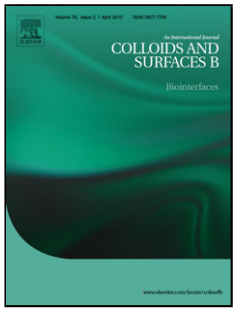

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Architecture and physicochemical characterization of *Bacillus* biofilm as a potential enzyme immobilization factory

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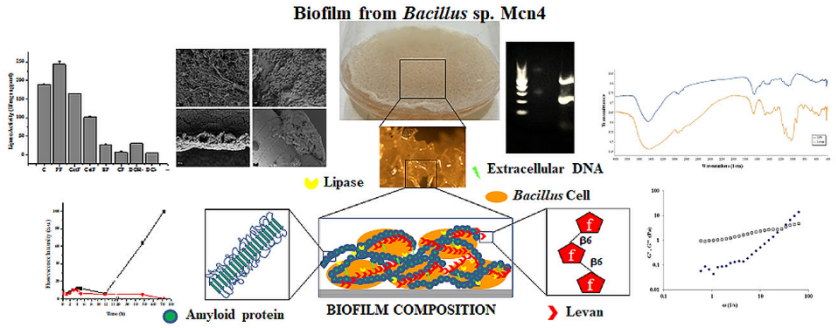
Graphical abstract



Highlights

- The biofilm presented a shear thinning behavior and appreciable elastic components.
- Extracellular DNA was quantified and fragments of different size were detected
- Amyloid protein production appears with the culture time.
- Entrapped lipase was detected in the biofilm.
- Biofilm with lipase activity was used as a biocatalyst.

Abstract

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.

Keywords: Biofilm; Rheological properties; Lipase immobilization; *Bacillus*

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 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 x 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 2M 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 20mM HEPES pH 7.4, containing ThT 25mM. 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 minutes. 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 minutes, samples were centrifuged for 5 minutes at 16,000×g. Supernatants were recovered, one volume of cold ethanol was added and samples were incubated for 8 h at -20 °C to allow protein precipitation and then centrifuged at 16,000×g. Pellets were resuspended in 10 mM phosphate buffer (pH 7.0). Total protein concentrations were determined with Lowry method [26] using fraction V of bovine serum albumin [27] as standard and separated by 10 % native-PAGE. See Supplementary Information (S1).

2.7. Rheological measurements

Rheological experiments were performed using an air-bearing Physica MCR 301 Anton Paar Rheometer controlled with Anton Paar Physica Rheo Plus software (Austria). All measurements were carried out using 25 mm parallel plate geometry (PP25 / measurements system) and a P-PTD200 accessory system (Peltier-controlled temperature). Exopolysaccharide aqueous solution samples (0.1 % w/V), were prepared in sterile double distilled water. See Supplementary Information (S1).

2.8. Influence of supporting material for biofilm develop

Biofilm development with lipase activity was carried out on supports with different physicochemical characteristics, ionic charge, hydrophobicity and porosity. The supports used for biofilm immobilization were polyurethane foam, cotton fibers, cellophane film, brick particles, ceramic particles, glass beads, Dowex Hydroxyl (OH^-), Dowex Chloride (Cl^-), Dowex 50W Proton (H^+), Amberlite IR 120 Sodium (Na+) and Amberlite CR 1320 Calcium (Ca^{2+}). 1 g of each support, except for cellophane (0.5 g), and cotton fibers or polyurethane foam (0.15 g), was placed in 100 mL glass vials. 10 mL of YP liquid medium was used to imbibe the supports, which were inoculated with 100 µL of a pre-culture in YP medium, corresponding to a cell concentration of 1.56 x 10^6 CFU/mL of *Bacillus* sp. Mcn4.

2.9. Scanning Electron Microscopy (SEM)

Microscopic characterization of the biofilm was performed using a ZEISS SUPRA 55 VP scanning electron microscope from CIME (CONICET - UNT,

Tucumán, Argentina). The specimens with biofilms were fixed in Karnosvsky buffer (2.5% glutaraldehyde, 1.5% paraformaldehyde) pH 7.2, for 30 min. Then, the samples were dehydrated in a graded ethanol series (30%, 50%, 70%, and 90%) and acetone (100%) dried with liquid CO_2 and finally, coated with gold by sputtering. Samples without biofilms were used as controls.

2.10. Residual lipase activity of the new biocatalysts

Biocatalyst residual activity was evaluated against repeated cycles of catalysis. Supports with higher lipolytic activity were selected for this purpose. Catalysis cycles were carried out for 1 h at 37 °C using p-NPP as a substrate. Biocatalysts were thoroughly washed after each enzymatic determination with abundant 10 mM phosphate buffer. After that, were centrifuged at 16,000×g for 5 min, and supernatants were removed.

Results and Discussion

3.1. Identification of natural components of *Bacillus* sp. Mcn4 biofilm

A previous work showed that strain Mcn4 has a close phylogenetic affiliation with *Bacillus licheniformis* [28]. This strain was found to form robust pellicles (floating biofilms) and solid surface-associated biofilms in standing culture (S2B). In this sense, the *Bacillus* genus has largely been evaluated as a biofilm producer [16, 29].

The extracellular matrix not only differs from species to species, but also within the same species depending on nutrient conditions and environmental stresses. Polysaccharides, proteins, and nucleic acids are important components of the matrix that help to maintain the structural integrity of biofilms, mediating both cell-to-cell and cell-to-surface interactions [7, 30]. This is shown in rheological studies of biofilms and EPS matrix studies, which, along with this study, have demonstrated this phenomenon [31-33]. The EPS matrix is considered as key to understand the biofilms mode of life. In the present work, a robust protocol was applied in order to extract the exopolysaccharide from *Bacillus* sp. Mcn4 biofilm. The yields ranged from 10-15 mg/L of partially purified exopolysaccharide from biofilm obtained from 1 L of culture. These results are consistent with those obtained by Bales *et al*. [20] for exopolysaccharide from different microorganisms. The functional groups in the exopolysaccharide were identified

by FT-IR spectroscopy. Fig. 1A shows an exopolysaccharide spectrum, which is characterized by a dominant band at 3430 cm^{-1}, assigned to the hydroxyl stretching vibration of the polysaccharide. The band centered at 2935 cm^{-1} could be attributed to the C-H stretching vibration corresponding to the methyl and methylene groups [34]. The absence of peaks among 1700-1775 cm^{-1}suggests that neither glucuronic acid nor diacyl esters are present in the exopolysaccharide of *Bacillus* Mcn4. The strong absorption observed at 1645 cm^{-1} could correspond to the amide stretching >C = O (amide band I) suggesting the presence of the C=O group [35]. The region between 1000 and 1200 cm^{-1} is considered the fingerprint region for carbohydrates [36, 37]. This region is dominated by the ring vibration overlapping the stretching vibration of the (C-OH) side group and by the glycosidic band vibration (C-O-C) consistent with what observed by other authors [38, 39]. As the exopolysaccharide structure was suspected to be that of a levan, its FTIR spectrum was compared with a commercial levan (Fig. 1A). Both were similar and the few differences observed were possibly due to the higher level of proteins associated to the exopolysaccharide from *Bacillus* sp. Mcn4.

Even though polysaccharide production is a common property in bacteria, its composition vary significantly. The levan structure is not uniform and is determined by the microorganism and the culture condition [10].

Polysaccharides have been reported to play a significant role in amyloid formation of a wide range of proteins/peptides associated with native biological functions [40, 41]. The amyloid structure has been linked to the pathogenesis of various human diseases. Although, there are a growing number of reports indicating that they are not only the result of abnormal folding, but are also purposely produced under certain non-pathological conditions to provide beneficial properties to certain organisms [42]. In our work, the ability to produce amyloid aggregates as part of the metabolism of *Bacillus* sp. Mcn4was evaluated by the classical ThT assay, which specifically binds to cross α-sheets structures that are hallmark in amyloid structure [43, 44]. Amyloid aggregation was not significantly detected in the control sample at 37 ºC, suggesting that the protein remained in its native state (Fig. 1B). However, when *Bacillus* sp. Mcn4 was incubated at 37ºC without agitation, it was possible to observe a sigmoidal polymerization curve with a lag phase followed by exponential growth of amyloid fibers, which continued increasing after 48 h (Fig. 1B). The detectable

ThT fluorescence emission has been reported by other authors. For example, the amyloid structure was observed in the biofilm of *B. licheniformis*, *Pseudomonas aeruginosa*, and *Weissella confuse*, among others [11, 43].

The most frequent question in the study of the role of functional amyloids in the building of an extracellular bacterial matrix is which monomeric proteins assist or trigger the assembly into fibers, a reaction thermodynamically unfavorable [45]. Recent studies have suggested that extracellular DNA tends to form a composite by incorporating itself into proteins or amyloidogenic peptides, and accelerating the polymerization of bacterial amyloid [46, 47]. Thus, eDNA and amyloids could form a compact ultrastructure providing stability to the matrix [11].

It has been shown that eDNA promotes cell-cell aggregation and cell-substrate adhesion [33]. This last characteristic, together with the capacity of binding to other polymers (i. e, proteins and exopolysaccharides) explains the importance of eDNA in the architecture of the biofilm providing a scaffold for the structure of the matrix.

eDNA associated with *Bacillus* sp. Mcn4 biofilm was qualitatively and quantitatively characterized. The quantification of the eDNA concentration showed a value of 2.72 mg/g biofilm. Under the utilized conditions, the amplification of the eDNA by PCR with the unspecific primer OPI-02mod gave a negative result, while the XD9 and M13b primers produced two bands of 10,000 and 1500 bp, and 2500 and 1000 bp, respectively (Fig. 1C). These results show for the first time that these primers could be used for the characterization of eDNA present in biofilms obtained under different conditions and, in consequence, with different biochemical properties.

As mentioned above, the biofilm matrix is a structure that provides benefits for the entire cellular community forming it, protecting them from several environmental stresses [48].

Bacillus genus is well known for its ability to produce extracellular enzymes, including lipases [49]. Biofilm formation and lipase production by *Bacillus* sp. Mcn4 were evaluated in a liquid medium under static and shaken growth conditions. When *Bacillus* sp. Mcn4 grew under static conditions, biofilm developed in the air-liquid interface while no biofilm formation could be observed after shaking (S2B). Additionally, under static conditions, the bacteria excreted extracellular polymers likely promoting biofilm formation [50].

Lipase activity was determined in the supernatant of agitated cultures, the supernatant of the biofilm, and the biofilm matrix. *Bacillus* sp. Mcn4 showed higher lipase activities in the biofilm matrix in comparison to supernatant from agitated cultures (Table 1). Noteworthy, lipase activity was not detected in the supernatant when the production was made under static condition.

The presence of several hydrolytic enzymes, including lipases, has already been described within bacterial biofilm, particularly in *Pseudomonas* strains [17]. Up to date the relationship of lipase activities with *Bacillus* biofilms has not been evaluated.

The protein concentration in the biofilm of Mcn4 strain was determined and the profile was evaluated in native polyacrylamide gel **(**Fig. 1D). Total protein concentration was 0.373 mg/g biofilm with a specific lipase activity of 508.45 U/mg proteins for C2 and 26.27 U/mg proteins for C16 (Table 1).

The zymogram showed two bands of lipase activity in the biofilm of about 135 and 70 KDa, respectively. In contrast, the lipase activity produced under shaken cultures only showed the band with highest molecular weight (Fig. 1E). Proteins were more abundant in the biofilm than in the supernatant (Fig. 1E). This difference between extracellular and biofilm protein concentration was also observed by Morikawa *et al.* [51] in the *Bacillus subtilis* B-1 strain. The enzymes could be retained within the biofilm due to its interactions with biofilm components such as exopolysaccharides. In this regard, the interaction of a lipase with alginate exopolysaccharide present in the biofilm of *Pseudomonas aeruginosa* was demonstrated by Tielen *et al.* [17]. Although it deserves a further research, the reasons for these different enzymatic profiles are beyond the scope of this work.

3.2. Rheological characterization of exopolysaccharide and Biofilm

The main mechanical properties of the biofilm structure are governed by the extracellular matrix components [52].

Material properties of biofilms have been studied using different microbial species [53, 54], growth methods as well as techniques like bulk scale rheometry [55, 56], small-scale bulk rheometry using passive microrheology [31-33, 40]. Different models have been proposed to explain the rheological behavior of

Bacillus strains [14, 57, 58]. Here, our current interest lies in the rheological characterization of biofilms produced by *Bacillus* sp. Mcn4.

Biofilms react in a time-dependent manner with a combination of distinct elastic and viscous components to external stress [8, 31, 32, 59, 60].

The rheological behavior of the isolated exopolysaccharide from *Bacillus* sp. Mcn4 was characterized under rotating measurement conditions by recording viscosity (η) and shear stress (τ) curves as functions of shear rate ($\dot{\gamma}$). For exopolysaccharide aqueous solutions, the shear stress as function of shear rate is characteristic of a shear thinning fluid (Fig. 2A). Furthermore, the dependence of viscosity with shear rate shows a decreasing viscosity with the increasing shear rate (Fig. 2B). The behavior of τ and η with the temperature in all the measured shear rate range was as expected, i.e. the lowest values for higher temperatures.

The exopolysaccharides solution produced by the microorganism shows a non-newtonian, shear thinning behavior, as shown in Fig. 2 A and B. The rheological parameters obtained from the fitting of shear stress experimental values, are given in Table 2. It can be seen that $n<1$ for both temperatures, showing that the exopolysaccharide solution is, indeed, non-newtonian. The flow index is observed to increase with temperature indicating that the shear thinning character is higher for solutions at lower temperatures as can be seen from Fig. 2 A and B, especially for low shear rates. At higher shear rate values both curves become smoother, tending to the second Newtonian plateau. The value of the consistency K (as a viscosity related constant) is lower at higher temperature, which is reasonable since, in general, the viscosity decrease with temperature increase.

The viscosity behavior of the exopolysaccharide solution observed in Fig. 2B is qualitatively comparable with the results obtained by other authors in the study of different microorganisms like *Rhizobium* sp. [61], *Xanthomonas* [62], *Bacillus subtilis* subs. *subtilis* NCIB 3610 [14, 63], *Paenibacillus* sp. [64].

In the case of biofilms, a non-Newtonian, shear thinning behavior is observed in Fig. 2C and D indicating a yield stress in the case of shear stress behavior as a function of shear rate (Fig. 2D). Therefore, the shear stress was fitted using the Bingham model as follows:

$$\tau = \tau_0 + \eta_P \dot{\gamma}$$

Where τ (Pa) is the shear stress, τ_0 (Pa) is the yield stress, η_P (mPa.s) is called plastic viscosity and $\dot{\gamma}$ (1/s) is the shear rate.

The linear fit gave τ_0 = 4.30 Pa and for η_P= 0.45 mPa.s with R^2= 0.997.

An important shear thinning behavior may be observed for viscosity as a function of shear rate (Fig. 2C). The viscosity curve was very similar to the viscosity curve obtained for Benigar *et al*. 2016 for the native biofilm nBF1, at least within the zone where the shear rate ranges are coincident.

Dynamic rheological measurements were also realized. The studies carried out under dynamic conditions (oscillatory flow) are typical tools for the characterization of viscoelastic properties of materials. Viscoelasticity is a material property of complex fluids, whereby the material exhibits both viscous and elastic behaviours. Viscosity is related to energy dissipation during flow mainly due to sliding and deformation of the material (atomic or molecular flow) which causes the ideally viscous materials to be irreversibly deformed over time to relieve stress. Conversely, elasticity is related to the energy stored during the flow due to orientation and deformation of the material (atomic or molecular stretching), which causes the ideally elastic material to deform instantly to relieve stress and return to their original state after the perturbing force is removed [61].

The dependence of the viscous and elastic modulus, G' and G'', with the angular frequency can be observed in Fig. 2E. It is evident that the biofilm exhibit viscoelastic properties. The experimental data can be qualitatively described by the relations for a Maxwell material.

For the biofilm, G' and G'' modulus increased with increasing frequency, with higher frequency dependence of G'.

Up to an angular frequency of approximately 27 1/s, the biofilm presents the typical behavior of concentrated solutions which indicates that the viscous flow predominate over the elastic response (G " > G ') at low frequencies [65-67]. Biofilm components's entanglement occurs at low frequencies, while it breaks during the oscillation period which allows the viscous flow to prevail over the elastic character. However, the two moduli curves crossover at a particular angular frequency value which is characteristic for the material structure and describes the average relaxation time $\tau = 1/\omega$. We

obtained a characteristic value of the order of 35 ms for a crossing frequency of 27.89 1/s. Also, a characteristic modulus G can be defined as the modulus G´= G" at the crossover [68], in this case, 2.84 Pa. The elastic character exceeds the viscous flow for increasing frequencies. In this situation the sample acts as a lattice and the result is G'> G". The loss tangent (tgδ = G" /G') indicates the relative contribution of the elastic and viscous components to the rheological properties of the material. It can be seen that tgδ shows values that are greater than unity at low frequencies, while they decrease below unity when frequency increases. The decrease in tgδ with the increase in frequency indicates that the system was in the pre-gel regime [65].

In the linear viscoelastic regime, viscoelastic functions such as the loss and the storage modulus remain constant under the applied stress. Fig. 2F shows measured values of G' and G'' versus the applied stress (with frequency fixed at 10 Hertz). A region of linear viscoelastic behavior is observed at shear stress values between 1 and 4.35 Pa, although the fall, showing non-linearity, is very smooth up to the maximum value contemplated of the stress. The viscoelastic studies suggest that this biofilm should be classified with appreciable elastic components.

3.3. Biofilm structure and adhesion on modified surfaces

The adhesive properties of eDNA and exopolysaccharide in mediating initial adhesion of microorganisms are highly versatile, but they are dependent on the physicochemical properties of the surface. Samples of biofilm were prepared for imaging with SEM (Fig. 3).

The SEM revealed biofilm architecture of heterogeneous agglomerates on its structure (Fig. 3A). The biofilm "aerial" structures appeared to be composed of bundles of interlaced cells that rose from the bacterial mat (Fig. 3A). Interestingly, bundle structures partially resisted the fixation and dehydration steps required for SEM preparation. The pattern observed did not permit the visualization of cells embedded in the biofilm (Fig. 3A). These observations disclosed the specific architecture of immersed *B. licheniformis* biofilm with the presence of bundle structures and confirmed that these structures are embedded in a highly hydrated extracellular matrix [69].

The solid surface-associated biofilms with different hydrophobicity properties or ionic charge were also evaluated. The best grown biofilm was observed on

polyurethane foam, cotton fibers and cellophane film. A large amount of exopolysaccharide covering the cell was observed on the three supports (Fig. 3 B, C, and D). The biopolymer could be distinguished as a network-like structure extending over and between bacterial cells. In addition, the mesh-like biopolymer structure could explain the stability of the large clusters of tightly spaced bacteria that permit the nutrient entrance inside the cluster [70].

The pattern of biofilm distribution on cellophane film was the most homogeneous (Fig. 3D). Interestingly, the bundle-like "aerial" structures observed in the interfacial biofilm were not present on this support and, as in the interfacial biofilm, bacterial cells were not detected (Fig. 3 A and D). The biofilm pattern on brick powder and ceramic particles was different. Many cell agglomerates were observed on these supports, probably due to a less amount of exopolysaccharide production (Fig. 3 E, F and G).

In this case, the adhesion of *Bacillus* sp.Mcn4 to the more hydrophilic support of cellophane film or cotton fibers (cellulose backbone) showed the hydrophilic nature of this strain.

The best biofilm development on charged particles was observed on negative charge supports, OH^- and Cl^- resin (Fig. 3H and I). SEM revealed the presence of numerous aggregates of bacterial cells enclosed in exopolysaccharide. No biofilm formation was observed (Fig. 3 G, J, K and L) on glass beads and positive charge supports (Na^+ and Ca^{2+}). The extracellular polymeric component of biofilm can influence bacterial adherence since ionic strength can affect the electrostatic properties of surfaces and the conformation of exopolysaccharide such as eDNA [71]. This extracellular polymer could influence the adherence of *Bacillus* sp. Mcn4 on the negative charge support.

3.4. *Bacillus* biofilm as scaffold for lipase immobilization

Biofilm matrix of *Bacillus* sp. Mcn4 represents a reservoir of lipase molecules. The constitutive lipase produced by *Bacillus* sp.Mcn4 was immobilized by the spontaneous entrapping bond formation.

The biofilm developed in an air-liquid interface of the growth medium and on the best supports previously tested were evaluated as immobilization systems (Fig. 4). The higher lipase activity was observed in the biofilm developed on polyurethane foam, cotton fibers and cellophane film (Fig. 4A). All of these

biocatalysts were able to hydrolyze p-NPP, but the highest activity was found in biofilm developed on polyurethane foam.

Considering biofilm composition, there are several points for enzyme immobilization. In fact, the simultaneous production of lipase and exopolisaccharide, this last one working as immobilization system, was described for *P. aeruginosa*. In this case a lipase was bound to an alginate and became dependent on the negatively charged polysaccharide, indicating ionic interactions between the molecules [17]. In our case, the *Bacillus* sp. Mcn4 lipase could be bound to the neutrally charged levan present in the biofilm. Levan is a biocompatible support with active groups that may be conjugated to biomolecules such as proteins.

Another component that could be involved in lipase immobilization is the amyloid protein present in the biofilm. In a previous work we reported the use of amyloid proteins of hen egg lysozyme induced by heparin for lipase immobilization [44]. In this case the amyloid protein itself in the Mcn4 biofilm work as a support for the immobilization of the constitutive lipase.

The best biocatalysts were re-used to evaluate the recycled in more than one catalysis cycle. The three biocatalysts conserved lipase activity at least in three catalysis cycles but the highest residual lipase activity was observed in polyurethane foam with 67% in the second cycle and 42% in the third cycle (Fig. 4B). The biological interaction between lipase and biofilm stabilized the enzyme that could be used for more than one biocatalysis cycle.

The best of this biofilm-based support for enzyme immobilization is that the conjugation proceeds were natural, without the need for any chemical treatment steps, and provided a simple way to immobilize enzymes. The material was produced biosynthetically, which is a green alternative [72].

3. Conclusions

In this work a first approach of using the biofilm of *Bacillus* sp. Mcn4 as a support for enzyme immobilization was proposed. We demonstrated that the biofilm extracellular matrix represents a great green support. Initially, we studied the components present in the *Bacillus* sp. Mcn4 biofilm. The exopolysaccharide was partially purified with similar characteristics to levan. In addition, eDNA with different sizes were detected and amyloid protein was observed in the extracellular

matrix, which shows an exponential increase along of incubation. The biofilm rheological behavior in terms of viscoelastic components gives it structural properties allowing its application as a support system for the immobilization. The presence of extracellular enzymes such as lipases was observed also in the biofilm. The physical and chemical properties influenced the development of more stable biofilms. Biocatalysts with immobilized lipase activity showed residual activity in more than a catalysis cycle. This biocatalyst could be useful in pharmaceutical and food industries, wastewater treatment or biofuel production.

Currently, more studies are being done for investigating all the potential of this green support for enzyme immobilization.

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Supporting Information

The complete methods are given in S1. Lipolytic activity of *Bacillus* spp. strains and Biofilm of *Bacillus* sp. Mcn4 developed in static condition are given in Figure S2. This Material is available free of charge via Journal Website.

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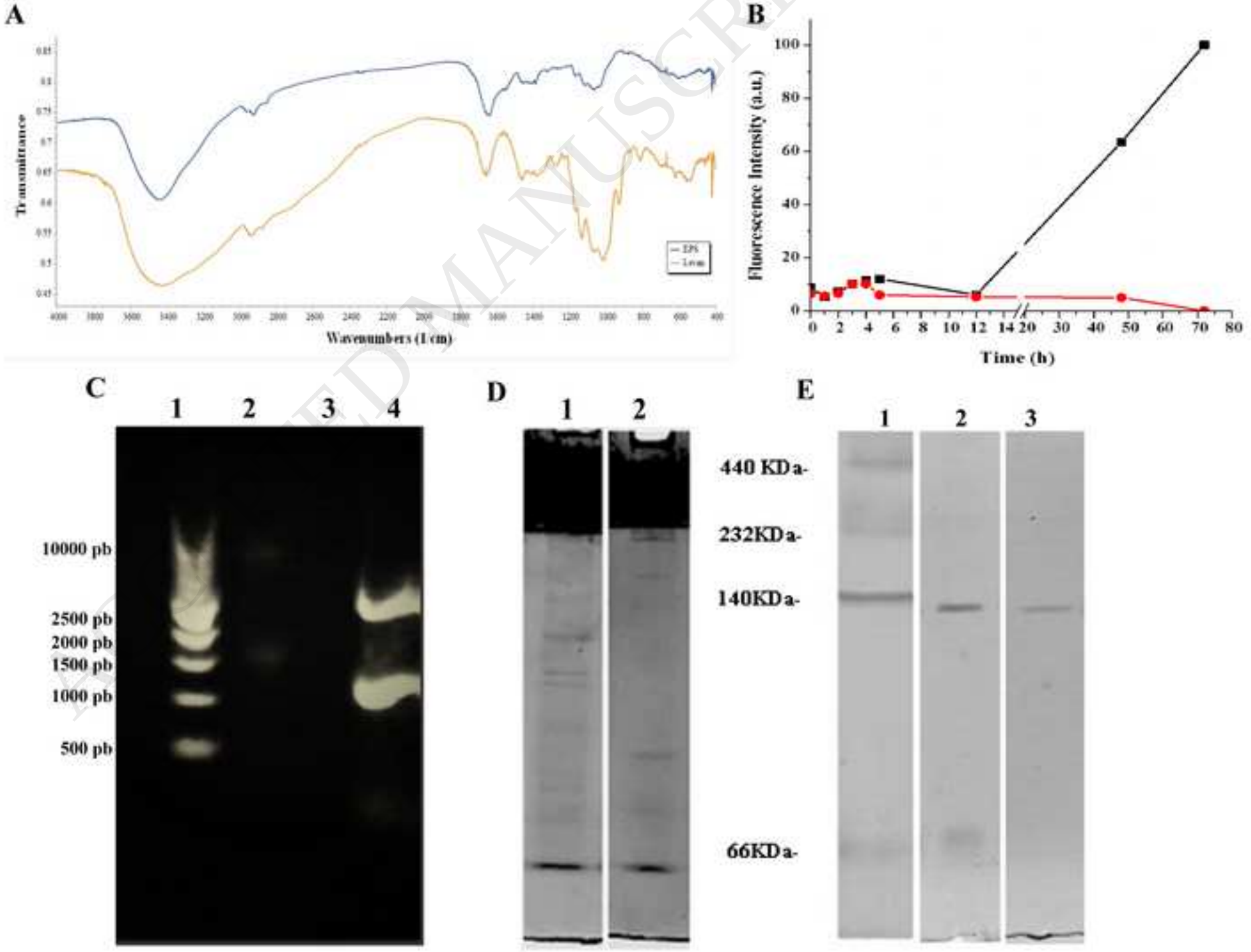
Figure 1.A-FT-IR spectra of: Blue line- exopolysaccharide from biofilm of *Bacillus* sp. Mcn4 and Red line- Levan. **B**- Amyloid aggregation kinetics measured by ThT fluorescence emission. Full Square: sample of biofilms. Full Circle: control protein of growing media. **C**- eDNA amplification **1**- Molecular weight standards, **2**-XD9 primer, **3**-OPI-02mod primer, **4**-M13b primer. **D**-Protein profile Native-PAGE analysis of: **1**- Biofilm; **2**-Supernatant. **E**-Zymogram showing lipase activity. **1**-Molecular weight standards, **2**-Activity biofilm under static condition, 3-Activity in supernatant under agitation.

Figure 2. A- Shear Stress (Pa) vs Shear Rate (1/s) plot of exopolysaccharide aqueous solution (0.1 % w/V) measured at 5 ºC (□) and 20 ºC(■). **B**- Viscosity (mPa.s) vs Shear Rate (1/s) plot of 0.1 wt % exopolysaccharide aqueous solution measured at 5 ºC (□) and 20 ºC(♦). **C**- Viscosity (mPa.s) curves vs Shear Rate (1/s) plot of biofilm measured at 37 °C. **D**- Shear Stress (Pa) vs Shear Rate (1/s) plot of biofilm. **E**- Double logarithmic plots of G´ and G" vs ω (Angular Frequency) of the biofilm measured at 37 °C. **F**-Double logarithmic plots of G´ and G" vs τ (Shear Stress) measured at 37 °C.

Figure 3. Biofilm of *Bacillus* sp. Mcn4 developed in an air-liquid interface and adhered on hydrophobic, hydrophilic and charge-modified surfaces. Each biofilm structure was observed by SEM. A-Biofilm development at on an air-liquid interface (10.00K X; 25.00K X); Biofilm development on: B-Polyurethane Foam (200X; 10.00K X; 25.00K X) C- Cotton Fibers (250X; 2.50K X; 10.00K X); D-Cellophane Film (250 X; 25.00K X; 50.00K X); E-Brick Powder (150K X; 2.50K X; 10.00K X); F-Ceramic Particles (150 X; 2.50K X; 10.00K X) ; G-Glass Beads (250X; 10.00K X; 25.00K X); H-Dowex OH^- Particles (250X; 10.00K X; 25.00K X); I-Dowex Cl^- Particles (200X; 2.50K X; 10.00K X); J-Amberlite Na^+ Particles (300X; 2.50K X; 10.00K X); K-Dowex H^+ Particles (700X; 2.50K X; 10.00K X); L-Amberlite Ca^{2+} Particles (700X; 10.00K X; 25.00K X).

Figure 4.**A-** Lipase activity was evaluated in the biofilm developed in an air-liquid interface of the growth medium and on Polyurethane Foam (PF), Cotton Fibers (CotF), Cellophane Film (CelF), Brick Powder (BP), Ceramic Particles (CP), Dowex OH^- (DOH^-), Dowex Cl^- (DCl^-) Particles. **B-** Recycling potential of PF (■), CotF (■) and CelF (■) in hydrolysis reaction.

Figure(s)


A
Transmittance
Wavenumbers (1/cm)
B
Fluorescence Intensity (a.u.)
Time (h)
C
1
2
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4
10000 pb
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2000 pb
1500 pb
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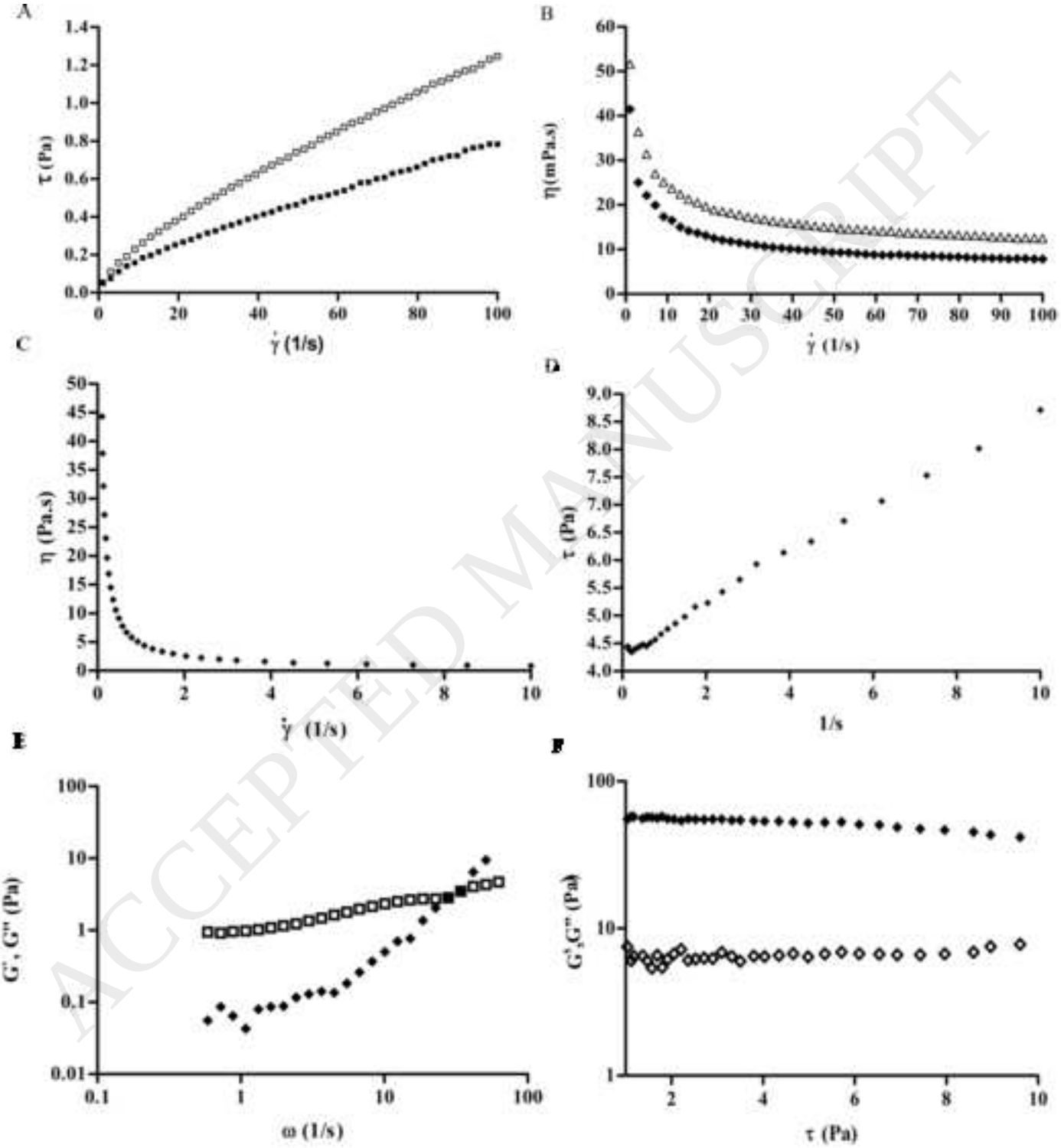
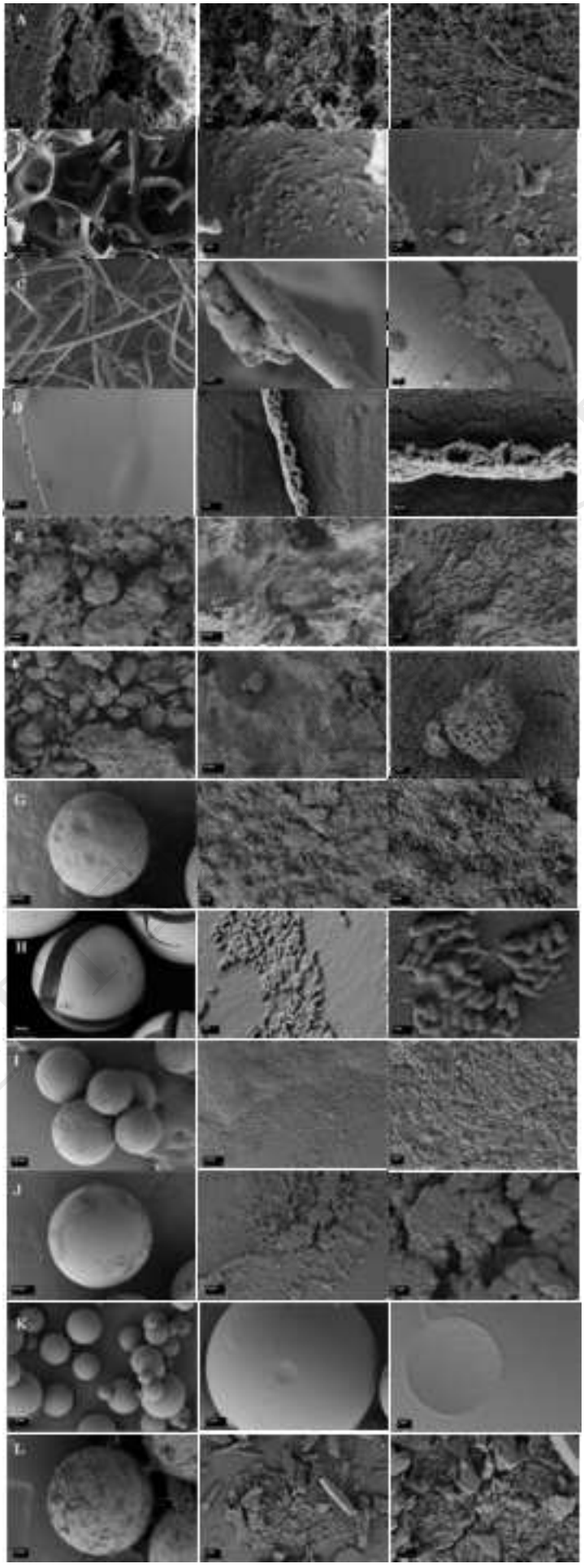
2
3
440 KDa-
232KDa-
140KDa-
66KDa-





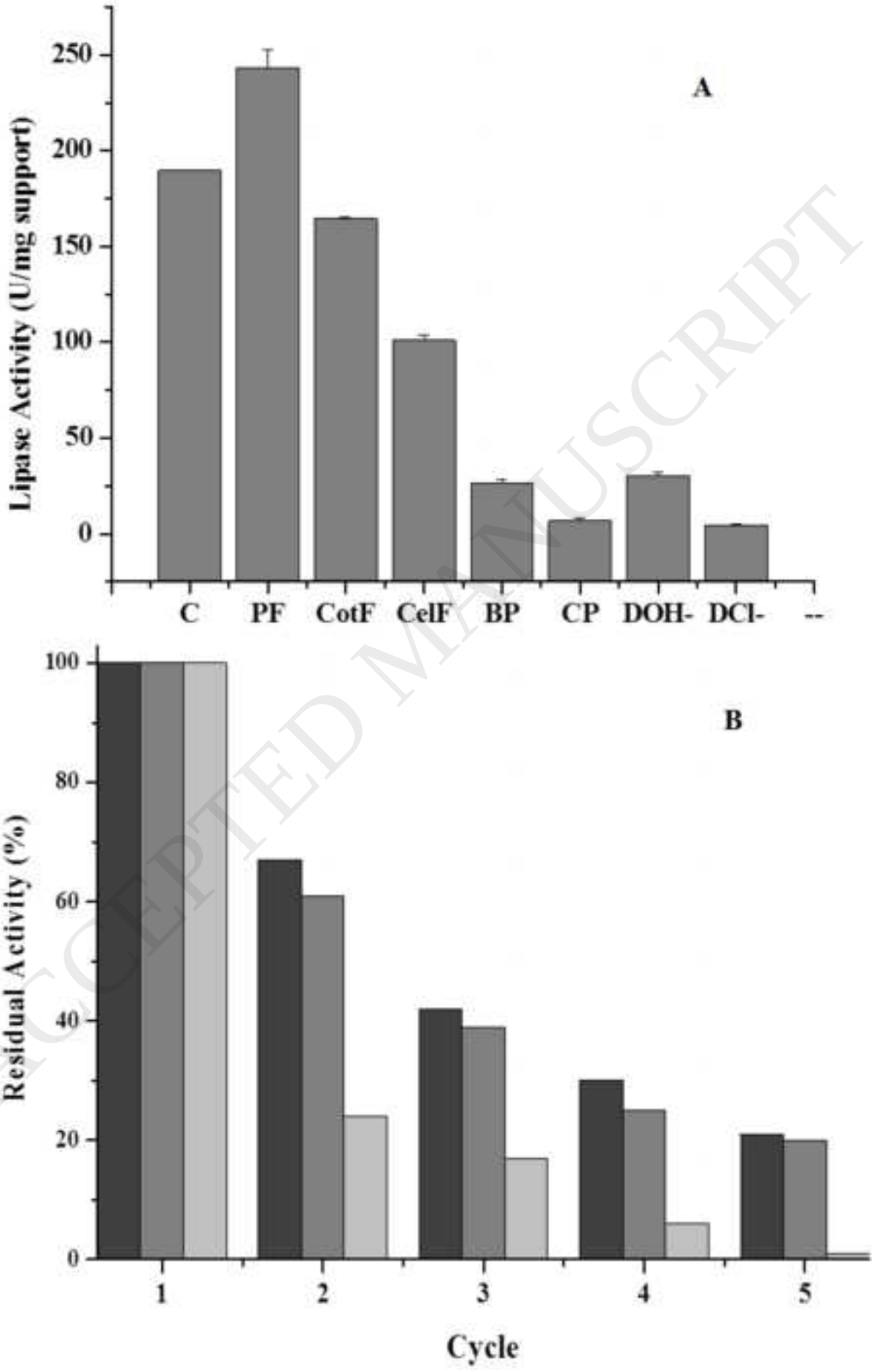


Table 1. Lipase activity from *Bacillus* sp Mcn4 was determined in both the supernatant and into of biofilm in static and agitation condition, using C2 (acetate) and C16 (palmitate) p-nitrophenyl derivates.

Lipase	**Static condition**				**Agitation condition**			
	Biofilm		**Supernatant[1]**		**Biofilm[2]**		**Supernatant**	
	p-NPA	**p-NPP**	**p-NPA**	**p-NPP**	**p-NPA**	**p-NPP**	**p-NPA**	**p-NPP**
Activity (U/mL)	19.34	2.130	-	-	-	-	12.90	0.890
Specific Activity (U/mg)	508.4	26.27	-	-	-	-	79.58	5.490

Each value presented here is an average of triplicates of three independent trials. The mean standard deviation for each value is < ± 5.0%

1-No lipase activity was detected; 2- No biofilm was formed

Table 2. Data on Temperature, Consistency Index and Flow Behavior Index for the fitting of shear stress vs shear rate for EPS aqueous solution, with Power Law Model.

	T (ºC)	**K (Pa.s^n)**	***n***	**R^2**
EPS aqueous solution	5	52.01	0.684	0.994
	20	30.49	0.715	0.991