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Characteristics of Biofilms Formed by *Bacillus subtilis* subsp. *spizizenii* Growing in a Simple Culture Medium with two Different Carbon Sources

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INTRODUCTION & AIM

Biofilms represent a key survival strategy, providing bacteria with effective protection against abiotic and biotic stress. In the laboratory, *Bacillus subtilis* subsp. *spizizenii* forms a biofilm at the liquid-air interface, whose matrix is primarily composed of polysaccharides, proteins, and nucleic acids. The aims of this study are: 1) to evaluate how the chemical composition of the matrix and the stability of the biofilm are modified when the bacteria grow using either glucose or glycerol as carbon sources. 2) to study the effect of temperature on biofilm production and planktonic growth.

METHOD

For the production of bacteria in a planktonic state, *Bacillus subtilis* subsp. *spizizenii* was cultivated in Minimal Salts Medium (MSM), containing 1 g/L K_2HPO_4, 0.3 g/L KH_2PO_4, 0.5 g/L NH_4Cl, 0.1 g/L NH_4NO_3, 0.1 g/L Na_2SO_4, 0.01 g/L $MgSO_4.7H_2O$, 1 mg/L $MnSO_4.4H_2O$, 1mg/L $FeSO_4.7H_2O$, 0.5 g/l $CaCl_2$, 0.01 g/L EDTA, 1L deionized water pH=7±0.4 with 55mM L-glutamic acid and, 1% glucose or 1% glycerol as carbon source. The bacteria was incubated at 150 rpm at different temperatures. Aliquots of 5 ml were taken at 0, 24, 48, 72 and, 96 h to measure the optical density at a wavelength of 610 nm. For the production of biofilm, the culture was maintained under static conditions for 96 h at different temperatures.

Stability of the biofilm developed at 20 and 4ºC: After 96 hours, when the biofilm had already formed, one set of Erlenmeyer flasks was kept at 20ºC, and another set was kept at 4ºC. Every 5 days, the biofilm was extracted and weighed from three Erlenmeyer flasks. The experiment lasted for 80 days.

Determination of exopolysaccharides present in the biofilm matrix:
The analysis was performed using high-resolution anion-exchange chromatography with a pulsed amperometric detector (HPAEC-PAD) on a Dionex BioLC-3000 system. A Carbopac P-20 column with a P-20 pre-column (Dionex) was used. For the determination of neutral sugars, the following solvents were used: Solvent A: 200 mM NaOH, Solvent B: water, Isocratic program: 8% A and 92% B, Flow rate: 0.5 ml/min, final concentration: 18 mM NaOH.

RESULTS & DISCUSSION

Biofilm production and microbial growth in a medium with Glucose or Glycerol

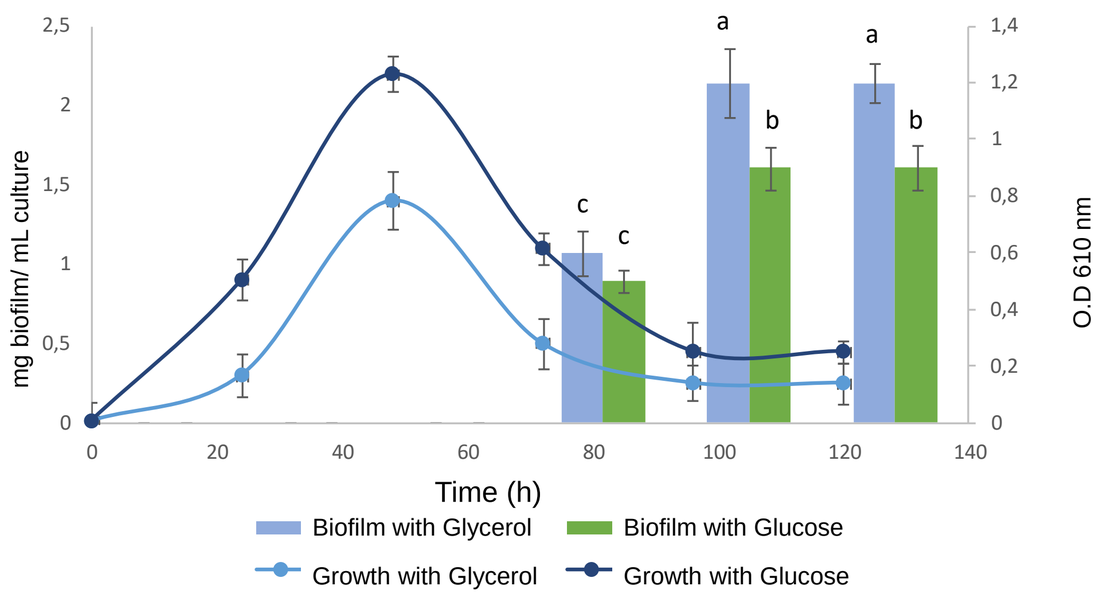

Fig. 1.Growth and biofilm formation by *B. subtilis* subsp. *spizizenii* grown in MMS with 1% glycerol or 1% glucose under agitation and static conditions at 30°C

Biofilm stability by *B. subtilis* at different temperatures

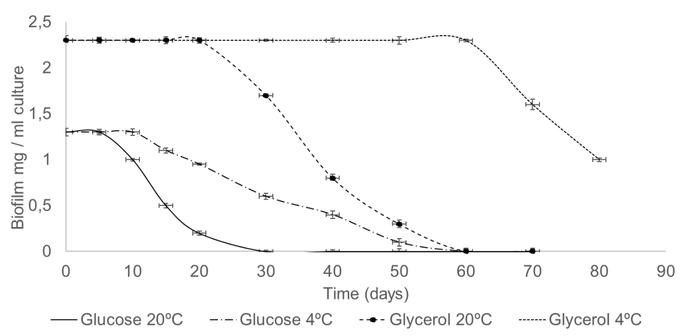

Fig.2. Biofilm stability by *B. subtilis* subsp. *spizizenii* grown in MMS with 1% glycerol or 1% glucose under static conditions and maintained at 20ºC and 4ºC

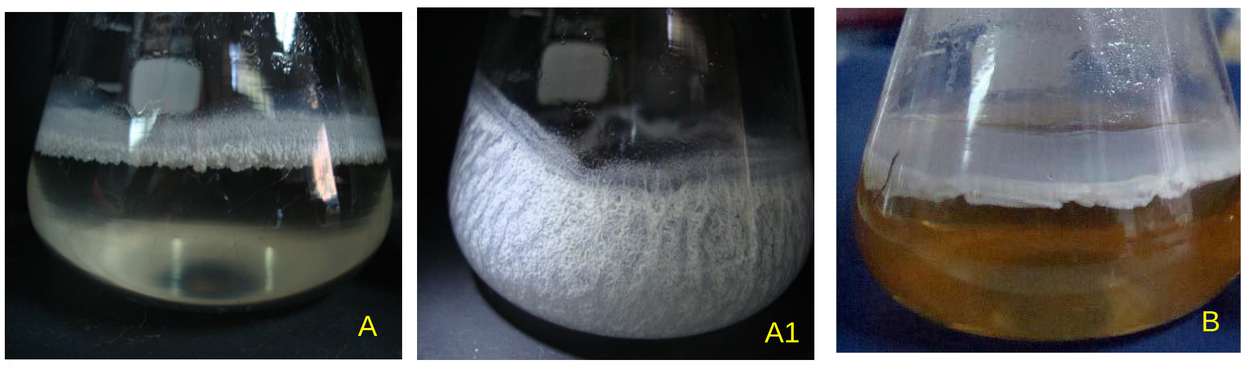

Photo.1. Biofilm formed by *B. subtilis* subsp. *spizizenii* grown in MMS, 55 mM L-glutamic acid, 1% glycerol (A) or glucosa (B) B) Biofilm adhered to the walls of the Erlenmeyer flask (A1)

Growth of *Bacillus* in its planktonic form at different temperatures

Fig.3. Growth of *B. subtilis* subsp. *spizizenii* in MMS with 1% glycerol and glutamic acid 55mM at different temperatures

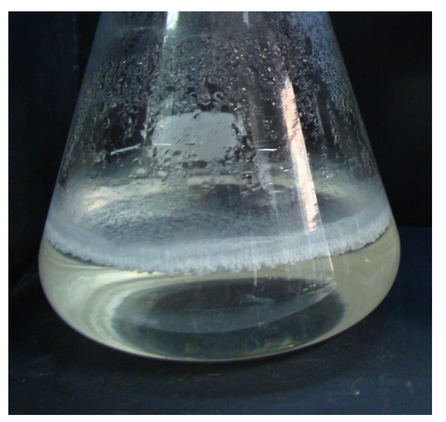

Photo. 2. Detail of the clear culture medium due to cell migration towards the biofilm

Growth of *Bacillus* under its biofilm structure at different temperatures

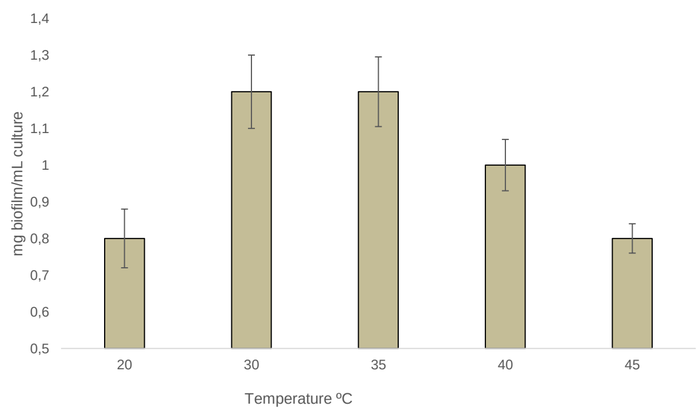

Fig.4. Biofilm yield of *B. subtilis* subsp. *spizizenii* in MMS with 1% glycerol and glutamic acid 55mM at different temperatures

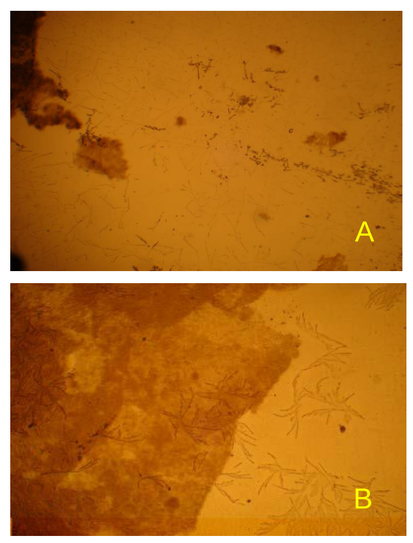

Photo.3:Detail of exopolysaccharides released from the matrix of the biofilm A) with glucose 1% B) with glycerol 1%. Magnification: 1000x

CONCLUSION

The biofilms obtained from different carbon sources show distinct differences.

- When the carbon source is glycerol, the biofilm structure is more robust and exhibits strong adhesion to glass.
- It is suggested that the substituents of the polysaccharide monomers may be crucial for their structural properties.
- The biofilm produced with glycerol was more stable, beginning to disintegrate after 20 days at 20°C and after 60 days at 4°C.
- The optimal temperature for the vegetative growth of *Bacillus* was not the same as for biofilm development, with 45°C being optimal for the vegetative and between 30-35°C for the biofilm.
- Moreover, in cases where the biofilm is formed at the liquid-air interface, planktonic cells in this culture migrate toward the biofilm, and as a result, the medium appears clear; this is likely due to nutrient depletion, which does not support planktonic growth.
- Due to the characteristics of the biofilm obtained from glycerol, it could be used as a bioinoculant for seeds.