Fast HPTLC-direct bioautography using *Bacillus subtilis* for screening of antimicrobial components in plant extracts

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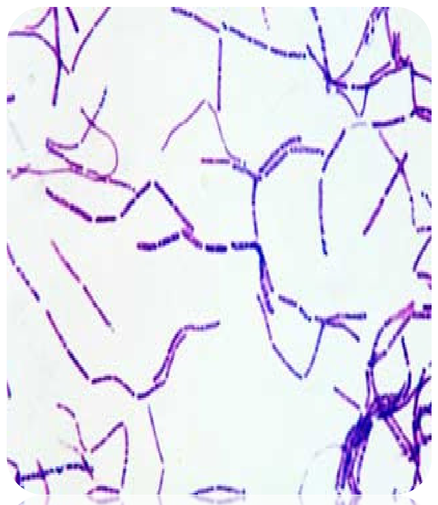
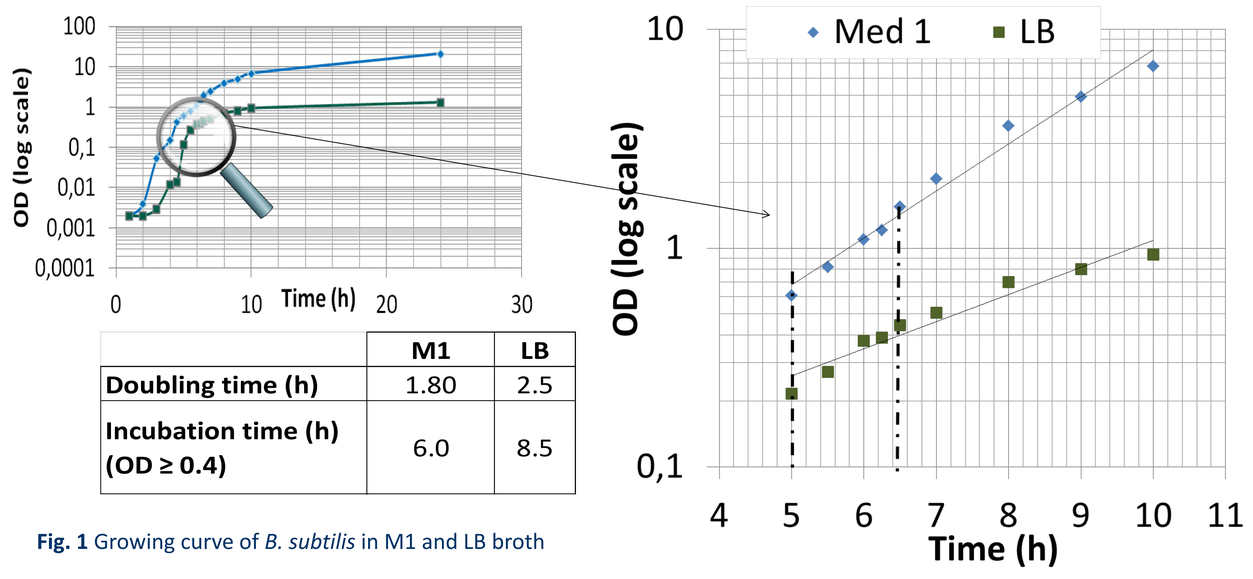


Introduction

Hyphenation of HPTLC with direct bioautography (HPTLC-DB) using *Bacillus subtilis* can play a key role for non-targeted identification of antibacterial substances in botanicals. Two limits are encountered: first, the method could be strongly affected by all factors influencing the growth rate of the bacteria since *B. subtilis* is incubated on the developed plates for a long time; secondly, the steps of this method are very time consuming, *i.e.* incubation of bacteria in broth and on plate as well as visualization [1-3]. The aim of the current study is to present an optimized, rapid and straightforward technique for HPTLC-DB using *B. subtilis* using a newly created synthetic medium (M1). To achieve this goal, growth rate of the bacteria and plate incubation times, as dependent variables, were compared with the simple Luria Broth (LB) medium. In order to prepare the bacterial suspension for immersion, the broth was inoculated with a spore suspension of *B. subtilis* in a flask, incubated at 37 °C for up to 6 h. The cell number was monitored by spectrometric measurement of the optical density (OD_{600}). In this method, an OD≥ 0.4 medium was prepared for immersion. These parameters were studied using an extract of *Ocimum basilicum L.*

Results and discussion

For optimization of the bioautography, microbiological aspects have to be studied with regard to chromatography. To select the best nutrient medium for a fast HPTLC-*B. subtilis* assay, LB was compared with the newly created M1. The M1 included peptone from soya bean, mineral salts ($MgSO_4 \times 7H_2O$, NaCl, NaH_2PO_4, K_2HPO_4 and $NH_4H_2PO_4$), glucose and yeast extract. The optimal viscosity of the broth was obtained by the addition of glycerol. While LB comprises sodium chloride, tryptone and yeast extract. The semi logarithmic plot of OD_{600} *versus* t showed that although the log phase time of both broth media was almost alike, the doubling time of *B.s.* in M1 (1.8 h) was 30 % shorter than in LB (2.5 h, Fig. 1). The broth incubation time to obtain the OD ≥ 0.4 took 6 h for M1 and 8.5 h for LB.



	M1	LB
Doubling time (h)	1.80	2.5
Incubation time (h) (OD ≥ 0.4)	6.0	8.5

Fig. 1 Growing curve of *B. subtilis* in M1 and LB broth

First, enzyme MTT reaction times between 10 and 120 min were investigated (Fig. 2, A-D), showing 60 min as an optimal choice. Secondly, plate incubation times were studied between 1 and 5 h (Fig. 2, E-H). The bioautogram was optimal for 2 h incubation (Fig. 2 H). For prolonged incubation times (5 h, Fig. 2 G), obviously a starving out was noticed for the newly created M1. Finally, HPTLC plates were also immersed in and compared with LB medium. The M1 bioautogram (Fig. 2, H) showed the best contrast between background and antimicrobial zone, if compared with that of LB (Fig. 2, I). The broth medium was adsorbed by silica gel particles and acted as a connector between adsorption material and bacteria accumulated on the very surface. It was also a source of food and energy for the bacteria. So the amount of nutrient and viscosity of the culture broth were very important. If the broth was not adequate in viscosity, the culture broth can cause increased diffusion of the adsorbed compounds on the plate or might inhomogeneously cover the layer.

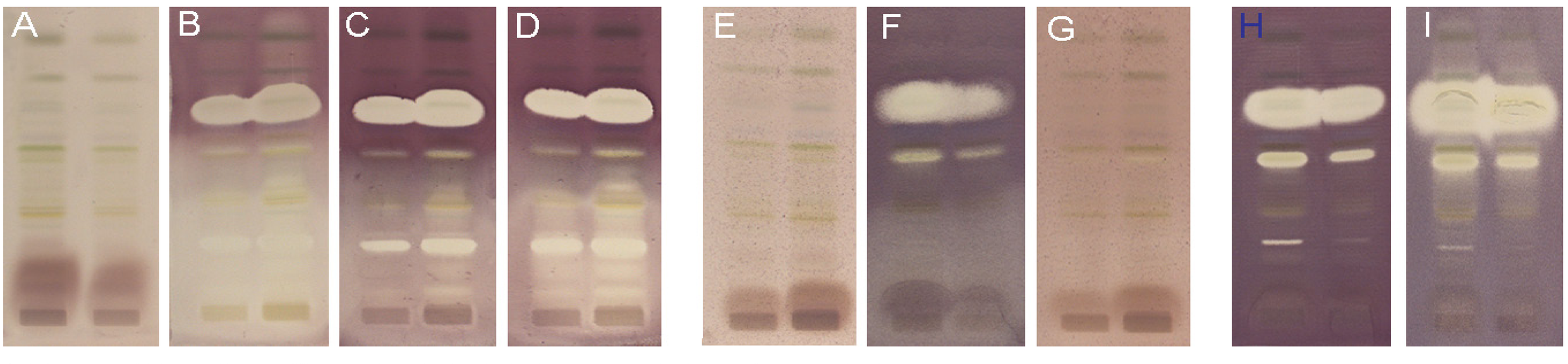


Fig. 2 Bioautograms of *Ocimum basilicum L.* at different plate incubation times (A-D) and enzyme MTT reaction times (E-H) for M1 (Table 1), and comparison with LB medium (I); different heating methods were compared as well (moistened plastic box at 37 °C (A) *versus* TLC Plate Heater at 50 °C)

	Incubation time (h)	Enzyme MTT reaction time (min)	in moistened plastic box 37°C	on TLC Plate Heater 50 °C	M1	LB
A	2	10	+		+	
B	2	45	+		+	
C	2	60	+		+	
D	2	120	+		+	
E	1	10		+	+	
F	3	10		+	+	
G	5	10		+	+	
H	2	10		+	+	
I	2	10		+		+

Table 1 Compilation of the different incubation times (A-D), enzyme MTT reaction times (E-H), both for M1, and then compared with LB medium (I), as well as different heating methods (moistened plastic box at 37 °C (A) *versus* TLC Plate Heater at 50 °C)

Conclusion

It was evident that the medium influenced the outcome of the bioautography. The results showed that after the enzyme MTT reaction step, the background color on the plates, and thus the contrast to the antibacterial zones, were much stronger with M1 than with LB. It was concluded that the procedure using M1 with 6 h broth incubation time combined with 2 h plate incubation time took the shortest time and generated the sharpest inhibition zones. The newly created, nutrient-rich M1 medium, which contained additionally glucose and an increased amount of salts, reduced doubling time and plate/broth incubation times of the bacteria.

References

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