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Review

Bioautography detection in thin-layer chromatography

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ABSTRACT

Bioautography is a microbial detection method hyphenated with planar chromatography techniques. It is based mainly on antimicrobial or antifungal properties of analyzed substances. The review discusses three versions of bioautography, i.e. contact, immersion and direct bioautography. The more concern is given to the last one. Many applications are quoted, not only for testing various groups of compounds, but also for investigating biochemical processes and factors influencing bacterial growth. Additionally, related methods, which can be included into direct bioautography, are discussed. The most promising among them seems to be TLC-bioluminescence screening.

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1. Introduction

Bioautography belongs to microbiological screening methods commonly used for the detection of antimicrobial activity (Fig. 1). The screening can be defined as the first procedure, which is applied to an analyzed sample, in order to establish the presence or absence of given analytes [1]. Basically speaking, it is a simple measurement providing a "yes/no" response [2]. Quite often, screening methods give higher sensitivity than any other methods. Moreover, they are simple, cheap, time-saving and do not require sophisticated equipment. Bioautography screening methods are based on the biological activities, e.g. antibacterial, antifungal, antitumour, and antiprotozoae of the tested substances [3]. This detection method can be successfully combined with layer liquid chromatography techniques, such as thin-layer chromatography (TLC), high-performance thin-layer chromatography (HPTLC), overpressured-layer chromatography (OPLC) and planar electrochromatography (PEC). In this review, the name TLC-bioautography is used mostly in its wide-ranging meaning concerning any planar technique linked to bioautography. In so-called direct bioautography, i.e. bioautography hyphenated directly with thin-layer chromatography (TLC-DB), both separation and microbial detection are performed on the same TLC plate. Generally, the method measures antibacterial properties of analyzed substances, i.e. changes in bacterial growth. However, other mechanisms of action can be considered, e.g. disturbing vital cell processes as it takes place when bioautography is performed using luminescent bacteria, in so-called TLC-bioluminescence method [4,5]. Both TLC-DB and TLC-bioluminescence enable searching for biological active substances in complicated mixtures and matrices, and can be included into effect-directed analysis (EDA), a new approach in environmental and hazard management based on biological response [6,7].

2. Microbiological screening methods

2.1. Diffusion methods

Diffusion methods are frequently used in testing antimicrobial susceptibility of pure substances, preferably polar than non-polar

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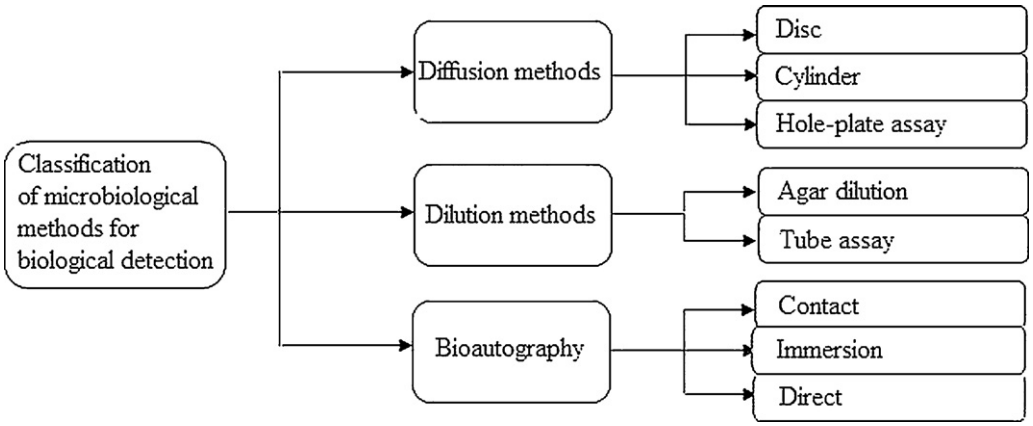

Fig. 1. The classification of microbiological methods for biological detection.

ones [8–10]. The disc method is the official one for quantitative detection of inhibitory substances in milk in the USA [11,12]. In this procedure, filter paper discs (about 6 mm diameter), containing the test compound, are placed on the agar surface previously inoculated with the test microorganisms (dipping a filter paper into a test compound solution should be avoided – it is advised to spot the substance on the disc surface). The antimicrobial agent diffuses into the agar and inhibits germination and growth of the tested microorganism. The Petri dishes are incubated and the zones of inhibition growth are measured. The similar procedure is carried out in E-test, where stripes are used instead of discs [13,14]. In the cylinder method, stainless steel or porcelain cylinders of uniform size (usually 8 mm × 6 mm × 10 mm) are placed on the inoculated agar surface of a Petri dish, and filled with samples and standards. After incubation, the cylinders are removed and the inhibition zones are measured. The cylinder method is the official one for quantitative detection of β-lactam residues [12,15,16]. In the hole-plate assay, a few millimeter diameter holes are cut in the inoculated agar surface and filled with the samples. The tested compound solution diffuses into agar medium causing growth inhibition of the microorganisms. The Petri dishes are left at room temperature, prior to incubation. Then, the zones of growth inhibition are measured [17]. The minimum inhibitory concentration (MIC) is determined visually, as the lowest test compound concentration, which causes recognizable zones of inhibition growth. However, diffusion methods are less suitable to determine the MIC values than dilution ones, because it is impossible to measure the amount of the test compound diffused into the agar medium (Fig. 2).

2.2. Dilution methods

The main advantage of dilution methods is possibility to estimate the concentration of the test compound in the agar medium or in the broth suspension; for this reason, they are commonly used for determination of MIC values [18]. The application range includes complex extracts, pure substances, and both polar and non polar samples. In the agar dilution procedure, various concentrations of the tested compound are mixed with a nutrient agar. The agar plates are inoculated and then incubated. The lowest concentration of the antimicrobial substance, at which no microorganism growth is detected, gives the MIC value. In the tube assay, various concentrations of the tested compound are mixed with bacterial suspension in series of tubes – the lowest concentration causing inhibition in microorganism growth corresponds to the MIC value. In the broth micro-dilution assay, the microorganisms are grown in the plate wells, to which various concentrations of the tested compound are added. The growth of the microorganisms is indicated by the presence of turbidity in the wells [19].

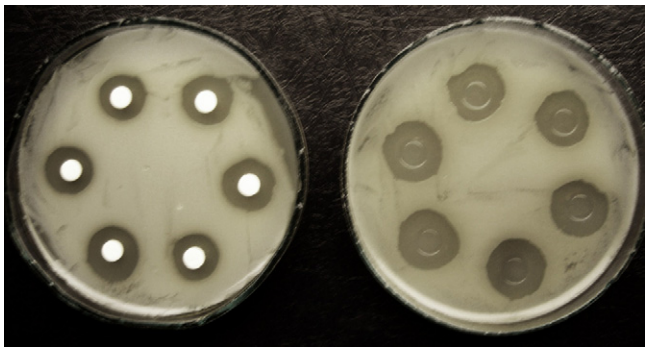

Fig. 2. Diffusion bioassays for flumequine standard solutions: agar disc (on the left) and agar cylinder (on the right) method. Test bacteria: *Bacillus subtilis*.

2.3. Bioautography

The procedure in bioautographic methods is similar to the one used in agar diffusion methods. The difference is that the tested compounds diffuse to inoculated agar medium from the chromatographic layer, which is adsorbent or paper [20,21]. In the contact bioautography, the TLC plate or paper chromatograms are placed on the inoculated agar surface for some minutes or hours to allow diffusion. Next, the plate is removed and the agar layer is incubated. The zones of inhibition growth appear in the places, where the antimicrobial compounds were in contact with the agar layer. In the immersion (agar-overlay) bioautography, the plate is first immersed in or cover with agar medium, which after solidification is seeded with the tested microorganisms and then incubated [22–24]. In order to enable better diffusion of the tested compound into the agar surface, the plates can stay at low temperature for a few hours before incubation. This method is a combination of contact and direct bioautography, because the antimicrobial compounds are transferred from the chromatogram to the agar medium, as in a contact method, but the agar layer remains onto the chromatogram surface during the incubation and visualization, as in direct bioautography.

Among the all bioautographic methods, the most widely applied is direct bioautography [3,25,26]. The principle of this method is that a developed TLC plate is dipped in a suspension of microorganisms growing in a proper broth and then incubated in a humid atmosphere. A silica surface of the TLC plate covered with the broth medium becomes a source of nutrients and enables growth of the microorganisms directly on it. However, in the places where antimicrobial agents were spotted, the inhibition zones of the microorganism growth are formed. Visualization of these zones is usually carried out using dehydrogenase activity-detecting