A DEVELOPMENT OF A FAST CHROMATOGRAPHIC METHOD FOR INDOLEACETIC ACID DETERMINATION IN CELL CULTURES

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This study presents the development and validation of a novel high-performance liquid chromatography method employing fluorescence detection for the rapid determination of indoleacetic acid (IAA) in bacterial cell cultures. IAA, a pivotal plant growth hormone, influences various physiological processes and is often employed as a marker to screen the growth-promoting properties of rhizosphere and endophytic bacteria. Current methods for IAA determination face challenges such as lengthy and costly sample preparations, complex matrix interferences, and the need for expensive equipment. This method utilizes simple sample preparation and rapid analysis procedures, mitigating previous limitations. The method's performance was assessed using two bacterial strains, Bacillus subtilis 26D and Bacillus amyloliquefaciens IMV A4. The method was also validated according to ICH guidelines, confirming specificity, linearity, accuracy, and repeatability. The developed method demonstrated significant improvements in speed and environmental impact, utilizing minimal solvent volumes and offering quick turnaround times. This method is a valuable tool for the rapid screening of bacterial strains for IAA production, facilitating the optimization of growth conditions in agricultural biotechnology applications.

Keywords: IAA, HPLC-FLD, rapid screening, bacterial cultures, method development

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Introduction

Indoleacetic acid (IAA) is a plant growth hormone, also produced by some rhizosphere and endophytic bacteria, and is one of the modes of action of plant growth-promoting rhizobacteria. It accelerates root and xylem growth, promotes lateral root formation, and also improves plant stress tolerance to adverse environmental conditions, such as high soil salinity and drought (Khoso et al., 2024). IAA can be used as a marker in screening growth-promoting properties of various bacterial strains isolated from the rhizosphere of target plants, then strains that show highest yields may be isolated, grown in conditions optimized for IAA production, and subsequently used as biofertilizers. This step, however, is dependent on having access to a fast and reliable procedure for IAA determination. A number of methods were developed using a variety of techniques, such as the Ehrich reaction, combined with colorimetric measurement (Guo et al., 2010), high-performance liquid chromatography with diode-array detection (HPLC-DAD) (Ratnaningsih et al., 2023, Górka et al., 2017, Remane et al., 2015, Štefančič et al., 2007,), HPLC with mass-detection (HPLC-MS) (Hou et al., 2008), HPLC with fluorometric detection (HPLC-FLD) (Liu et al., 2022, Campanella et al., 2016, Mattivi et al., 1999), electrochemical sensing (Shao et al., 2023), high-performance thin-layer

chromatography (HPTLC) (Goswami et al., 2015), and capillary electrophoresis (Chen et al., 2018). IAA determination in bacterial cell cultures comes with a number of limitations, which influence the choice of analytical method and development of the procedure itself, with key limitations being matrix complexity and low analyte concentration. Various methods, therefore, possess a number of disadvantages, namely, interfering compounds in the case of light absorption-based methods, complex sample preparation techniques, such as solid-phase extraction or liquid-liquid extractions with a subsequent concentration of extracts, which introduce analyte losses and prolong analysis time, or requiring expensive equipment in case of HPLC-MS. HPLC-FLD is of particular interest in this case, as this mode of detection is more specific and sensitive compared to HPLC-DAD, while being more accessible than HPLC-MS. However, HPLC-FLD methods described in the literature are still using long and costly sample preparations, and the analysis itself is quite long. Therefore, a fast HPLC method with simple sample preparation, focused on IAA determination, needs to be developed.

Materials and Methods

Chemicals

Indoleacetic acid, 99 % purity, was purchased from Acros Organics. Sodium acetate, glacial acetic acid, and acetonitrile (gradient grade) were purchased from Sigma-Aldrich. All chemicals were purchased through authorized distributors in Ukraine. Water was prepared on-site, using the Millipore Milli-Q water purification system.

Samples

Two bacterial strains were chosen as model microorganisms for IAA production: *Bacillus subtilis* 26D and *Bacillus amyloliquefaciens* IMV A4. *B.subtillis* 26D is a known inducer of plant resistance to biotic stresses. It serves as a basis of biological preparations for plant protection and is quite often used in research to find promising strains for crop production as a reference strain. *B. subtilis* 26D is an endophytic strain (Oliynyk et al, 2017), isolated from internal tissues of cotton plants. Strain *Bacillus amyloliquefaciens* IMV A4 was isolated from pre-sterilized tomato seeds of the Karas variety. This is an endophytic strain characterized by antagonistic activity against phytopathogenic bacteria of the *Pseudomonas syringae* species.

Sample preparation

Bacterial strains were cultivated on LB media for 72 h at 27°C, 5 ml of media were transferred into a glass tube and frozen prior to the analysis. Frozen samples were thawed at room temperature and placed on an ultrasonic bath for 10 minutes. After that, 1.0 ml of media was diluted to 10.0 ml with the initial composition of mobile phase, centrifuged at 5000 RPM for 5 minutes, and filtered through a Nylon syringe filter with pore size 0.22 μm into a 1.5 ml HPLC vial, discarding first 2 ml of filtrate.

A reference solution was prepared by dissolving 10.0 mg of IAA in the initial composition of the mobile phase and diluting it to 100.0 ml with the same solvent. 1.0 ml of this solution was diluted to 10.0 ml with the same solvent to give an IAA concentration of 10 mg/L.

Equipment and chromatographic conditions

The fluorescence spectrum of the IAA solution was recorded on a Shimadzu RF-6000 spectrofluorimeter. Chromatographic system consisted of Agilent 1260 Infinity II HPLC with

fluorescence detector, and Zorbax Rapid Resolution SB-Aq column, 4.6 x 50 mm, with 3.5 µm particle size. A gradient elution method was employed, with 1g/L sodium acetate solution, adjusted to pH 3.8 with glacial acetic acid as solvent A, and acetonitrile as solvent B. The following time program was used: 16 % B at 0 min, increased to 40 % B at 5 min, again increased to 80 % B at 6.2 min, then returned to 16 % B at 6.6 min and held at 16 % B until 8 min. Mobile phase flow was 1.4 ml/min, and the column thermostat was kept at 25°C. Injection volume was 5 µl. The fluorescence detector was set at an excitation wavelength (EX) of 255 nm and an emission wavelength (EM) of 350 nm.

Method development

Preliminary experiments with IAA have shown that it does not completely dissolve in water at 100 mg/L concentration, even after sonication for 30 minutes with mild heating. Literature data suggested using dilute sodium hydroxide to aid in its dissolution (Goldbio, 2020). However, that would lead to the injection of a basic solution into the HPLC system, which is detrimental to the silica-based column. Therefore, a mixture of water and acetonitrile (80:20) was chosen as the solvent for IAA, in which it completely dissolved with light shaking at 100 mg/L concentration. A 3D fluorescence spectrum was then recorded (Figure 1), to establish the most sensitive and specific detection parameters, and it was determined that IAA shows two distinct fluorescence zones, at EX 255 nm / EM 350 nm, and EX 293 nm / EM 355 nm. The first set of conditions was then chosen as parameters for fluorescence detection.

As IAA is a weak acid, and the intended separation mode for its determination is reversed-phase HPLC, it is advantageous to have IAA present in the solution in its unionized form, which can be achieved by using an acidic buffer solution as a solvent instead of water. Acetate buffer was chosen because of greater acetate solubility in acetonitrile compared to phosphate, and because of its biodegradability and lesser environmental impact. The salt concentration was chosen to be 1 g/L for ease of preparation while still providing sufficient buffer capacity. Buffer pH was adjusted to 3.8, which is in line with the acetate buffer working range of 3.6-5.6, and would shift to higher values when mixed with acetonitrile during separation, while still being inside the optimal buffer capacity range.

Fluorometric detectors have an intrinsic limitation, namely, they are fragile and, therefore, cannot tolerate high pressure. Taking this into account, acetonitrile was chosen as an organic component of the mobile phase over methanol. This ensured that the detector cell would not be damaged by excess pressure, higher mobile phase velocities could be used, and a wide variety of mobile phase compositions could be employed in gradient elution. Another step to lower the pressure in the HPLC system is to use a short and wide column, which is why a column with dimensions 4.6 x 50 mm was used. In order to ensure adequate separation with such a short column, a column with a smaller particle size (3.5 µm instead of the usual 5 µm) was used. Finally, a column containing SB-Aq sorbent was chosen because of the high water content in the initial mobile phase, as these columns are designed to work in highly aqueous mobile phases, up to 100 % water.

Mobile phase composition was developed to ensure that IAA adequately resolves from unretained components at the start of chromatograms. Hence, the initial gradient step is a linear increase from highly aqueous mobile phase to greater acetonitrile content with more elution power. After IAA elution, acetonitrile content is increased again to 80 % to ensure that all strongly retained compounds also elute, leading to a return to the initial mobile phase composition and short re-

equilibration step. Sample preparation was also adjusted to use the initial mobile phase composition as a solvent to ensure that the viscous growth medium is sufficiently diluted not to cause problems with injections and not disturb chromatographic equilibrium by injecting the sample in a drastically different solvent. A sample dilution procedure was also developed to ensure that an optimal detector signal was obtained.

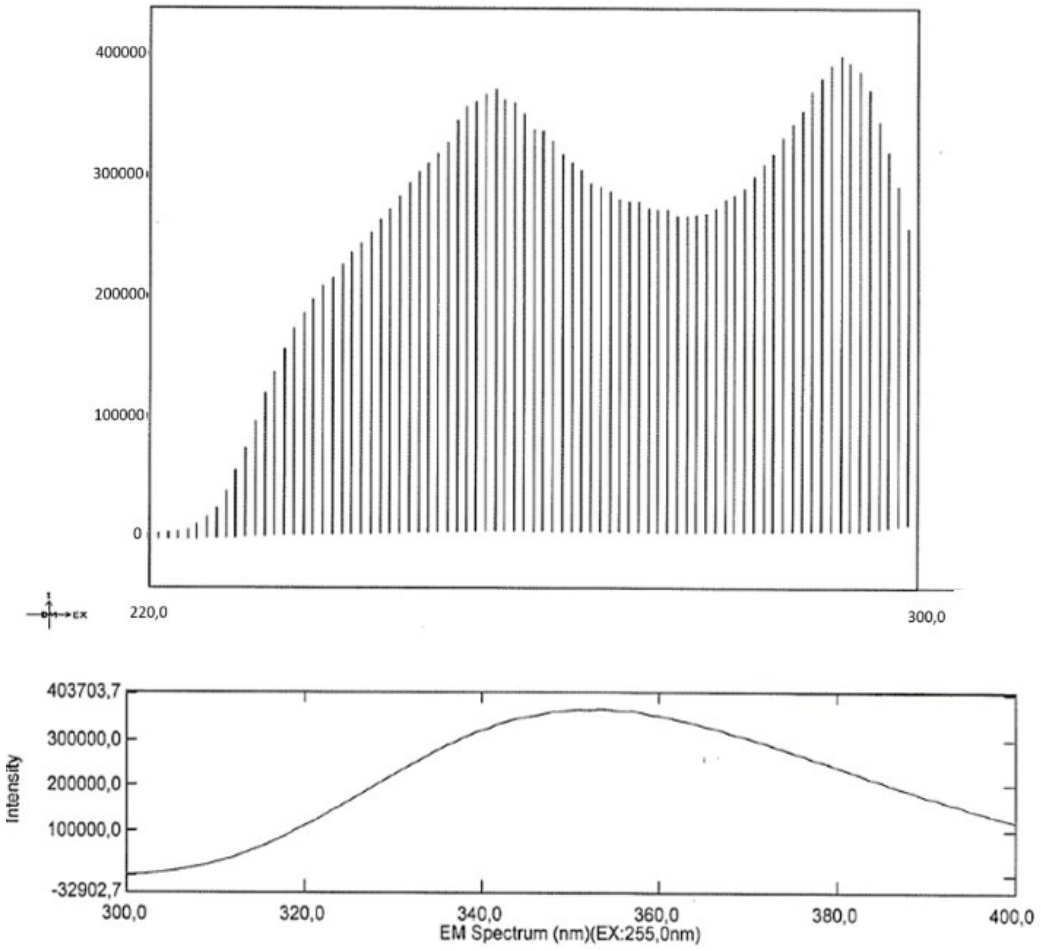

Figure 1. 3D Fluorescence spectrum of IAA. Top: Emission intensity as a function of excitation wavelength. Bottom: IAA emission spectrum at selected excitation wavelength (255.0 nm)

Method validation

The developed method was also validated in accordance with ICH guidelines (ICH, 2023), the following validation characteristics were studied: specificity, linearity, accuracy, repeatability, and quantitation limit. Method specificity was ensured by injection of blank solutions and sterile growth media, no peaks in the range of IAA peak were detected. Method linearity was studied in the range of 0.01 mg/L IAA to 15 mg/L IAA, with 10 mg/L being the target nominal concentration, with reference to which the IAA content is calculated. To study method linearity, a series of model solutions were prepared, with concentrations in the range of 0.1 % to 150 % of nominal. The method was found to be linear in this range (Figure 2), recovery of model solutions was 99.3 % in the range from 0.1mg/L to 15 mg/L IAA, and repeatability, measured as confidence interval of recovery values,

was 2.1 %. Recovery was also tested on spiked samples, where a test solution of 26D strain was spiked with 5 mg/L IAA during dilution, recovery was found to be 98.4 %. As the method used for IAA determination is a method that exhibits noise, a quantitation limit (LOQ) can, therefore, be confirmed using signal-noise ratio (S/N). LOQ was studied by injecting the solution with the lowest concentration in the studied range (0.01 mg/L) and S/N was found to be 34.1, which is more than the recommended value (10 for LOQ).

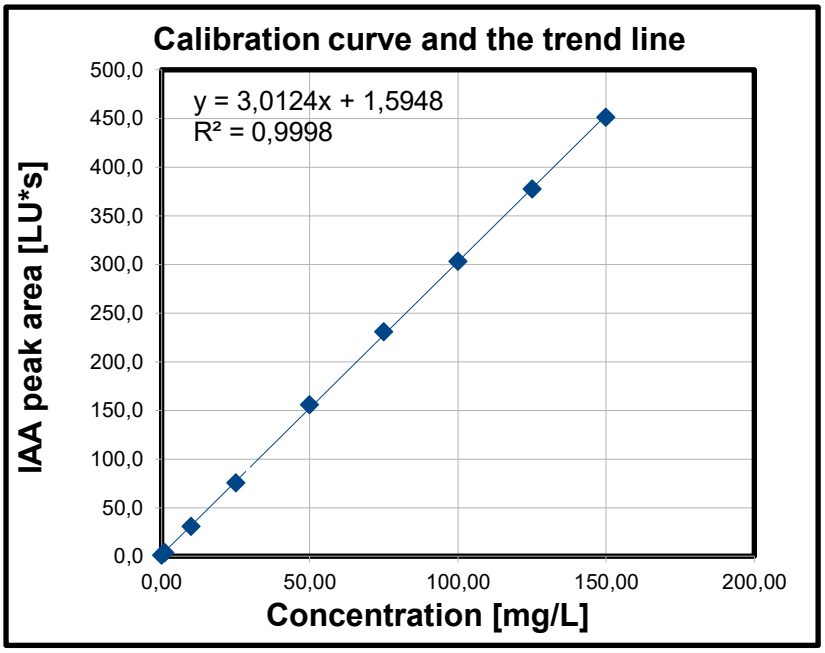

Figure 2. Linearity curve for IAA. Dots: Calibration curve. Line: Trend line. Insert: Trend line equation.

Results and Discussion

Samples of two bacterial strains were grown on culture media, as described above and tested for IAA production using a previously developed and validated method. All solutions were prepared in duplicate and injected twice. Chromatograms of test solutions show a characteristic picture of natural samples with minimal sample cleanup and preparation, with many partially or completely unresolved peak; however, IAA peak was well resolved from most other peaks and could be confidently identified and integrated, thus confirming that the developed method is fit for purpose. Chromatograms of the reference solution and one of the test solutions are displayed in Figure 3. and Figure 4., respectively.

B. subtillis 26D strain was found to produce about 1.4 mg/L IAA, while *B. amyloliquefaciens* IMV A4 produced about 9 mg/L IAA. Both values are well within the studied linear range of the method. The method was also found to be very fast and easy to implement, with a full analysis run taking at most a couple of hours and using only up to 500 ml of solvents for dilution and chromatographic analysis itself, minimizing its environmental impact.

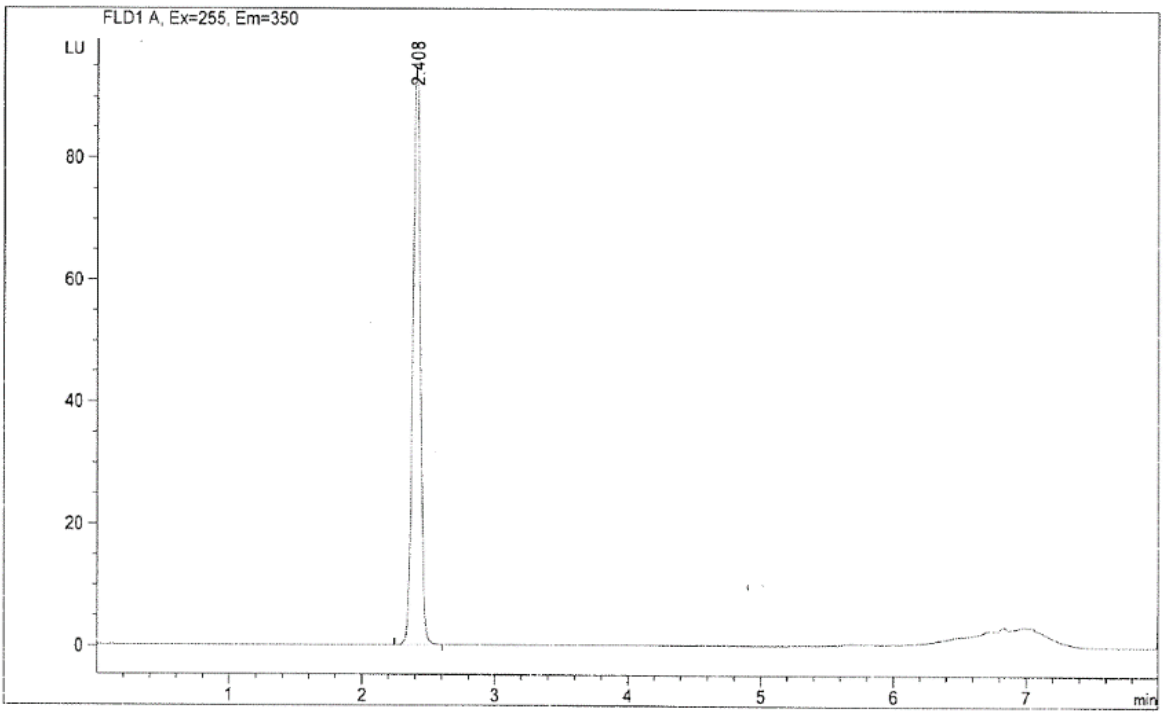

Figure 3. Chromatogram of reference solution

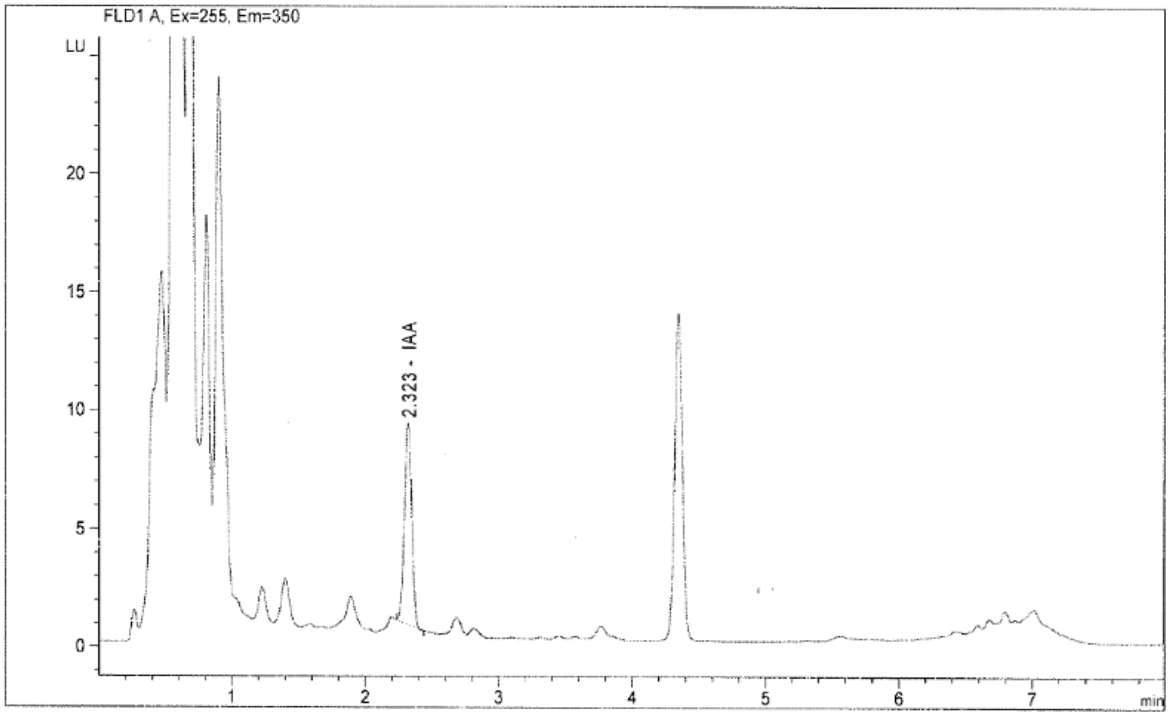

Figure 4. Chromatogram of test solution of *B. amyloliquefaciens* IMV A4 culture

Conclusions

A fast and reliable HPLC method for IAA determination was developed and validated. This method allows for the rapid screening of multiple bacterial strain isolates for IAA production and optimization of growth conditions for it. The method was used to analyze IAA concentration in two bacterial cell culture samples and was found to perform adequately. It was also determined that *B. amyloliquefaciens* IMV A4 is an IAA producer and produces greater IAA concentrations than reference strain *B. subtillis* 26D.

Disclosure

The corresponding author is also an employee of JSC «Kyivmedpreparat» and used its laboratory facilities and equipment in preparation of this article.

Conflict of interest

The authors state no conflict of interest.

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