



Fast and reliable voltammetric determination of menaquinone (vitamin K₂) produced in vitro by *Bacillus subtilis* cultures

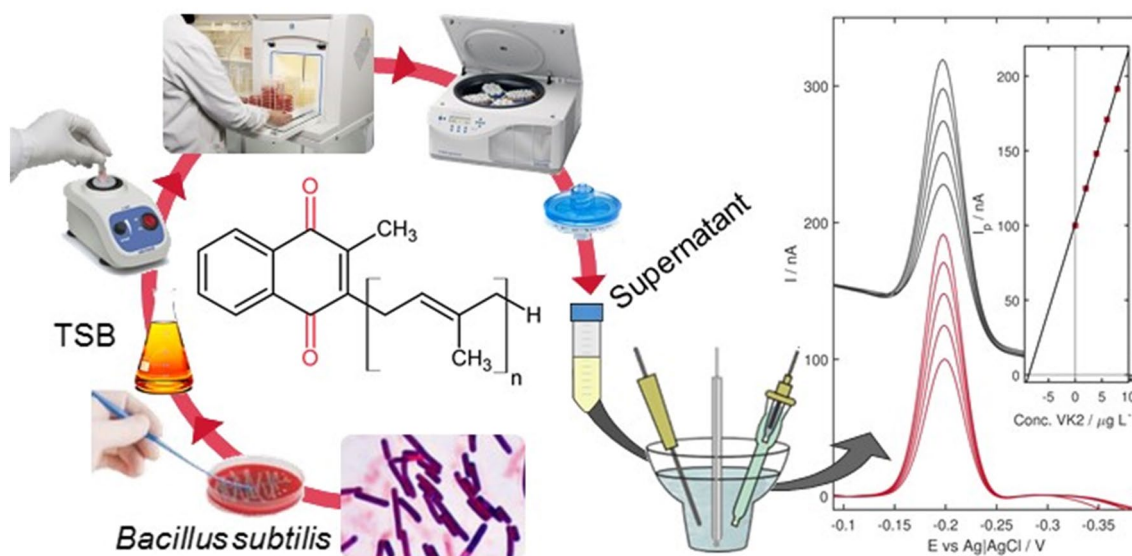
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Abstract

Vitamin K₂ (VK₂) is one of two natural forms of vitamin K, necessary for the proper functioning of organisms. Currently, it is sourced from chemical synthesis, nonetheless, it is worth reaching out to its natural sources. The aim of the study was to devise a voltammetric procedure for the determination of vitamin K₂ (VK₂) produced by bacteria in order to find the strain that exhibits the highest efficiency of VK₂ production. *Bacillus subtilis*, isolated from traditional Japanese food (Nattō), was chosen as a model strain. Employment of the Controlled Growth Mercury Drop Electrode (CGMDE) was crucial, as it is the only electrode that allows performing the measurement on the surface specifically renewed directly before, thus minimizing the influence of interferences. This new method was successfully applied for VK₂ determination in supernatant samples. Developed procedure is robust and easily adjustable for the variety of biological matrixes. Strong differences in VK₂ production depending on cultivation time were observed, but no direct correlation between the VK₂ concentration in the breeding medium and the cultivation time was found. The devised protocol will be used in further measurements with different bacteria species that have the ability to produce VK₂ and settle in the human intestine.

Graphical abstract



Keywords Vitamin K₂ · Menaquinone · Voltammetry · Microbiology · Microbial fermentation · *Bacillus subtilis*

1 Introduction

Vitamin K is a group of fat-soluble vitamins with similar structural composition. It is one of the essential vitamins in the human body, responsible mainly for the blood coagulation process and control of calcium binding in bones and other tissues. Vitamin K is an essential cofactor necessary for the production of clotting factors II, VII, IX, and X in humans and has recently been found to be an essential factor for many other proteins in the body [1–4]. Vitamin K occurs in two natural forms: vitamin K₁ (known as phylloquinone) and vitamin K₂ (menaquinone) [2–6]. Vitamin K₁ is found mainly in green vegetables, such as lettuce, spinach, cabbage, and green fruits, like kiwi and avocado [3]. It is also recommended as oral administration for newborns in order to treat and prevent bleeding caused by vitamin K deficiency [7, 8]. Vitamin K₂ (VK₂) is a group of nine vitamers with a different number of isoprenyl units in its side chain [1]. It is found mainly in fermented products, such as curd, cheese, sauerkraut, nattō, egg yolk, butter, and beef liver [5]. In the human body, vitamin K₂ is produced by some species of anaerobic bacteria inhabiting the human intestinal system that are capable of synthesizing vitamin K₂ with long chains, from MK-6 to MK-10 [2].

Vitamin K deficiency may be associated with severe health problems, such as bone fractures and vascular calcification; therefore, it is important to maintain its proper blood level [1]. More clinically relevant areas are being investigated including cancer that is associated with vitamin K deficiency [2, 6]. In order to prevent deficiencies, vitamin K₂ is widely used as dietary supplements or drugs utilized in the food, pharmaceutical, and healthcare industries. Currently, the production of MK-7 homologue often used in dietary supplements is gradually changing from traditional chemical synthesis to microbial fermentation [2]. There are a few bacterial strains that exhibit abilities of vitamin K₂ synthesis, including *Bacillus*, *Lactococcus*, or *Escherichia* species [2, 9–12] and the most commonly studied bacterial strains from *Bacillus* group are *Bacillus subtilis* nattō, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* [13–21].

The problem of highly sensitive determination of vitamin K₂, in particular without any preliminary extraction, at low costs and within a short time, is still a popular topic for researchers due to the above-mentioned important role of it in the human organism. Nowadays, for the quantitative determination of vitamin K₂ produced in bacterial cultures, chromatographic techniques are commonly used [22–25]. However, despite their advantages, these methods are characterized by a very long analysis time and expensive equipment and also always require the extraction

step, which uses large amounts of harmful reagents. As an alternative, in this work we propose the voltammetric method that was applied for the quantitative determination of vitamin K₂ produced by the culture of *Bacillus subtilis*. In our opinion, voltammetric techniques are definitely more attractive due to their sensitivity, often not possible to obtain using HPLC methods, simplicity, low costs of the equipment and single analysis, short time of analysis (approx. 20–30 min), and reduction of the reagents used during the measurements and sample preparation step, which leads to adapting the method to the green analytical chemistry standards [26–29].

The presented paper is the first part of long-term interdisciplinary studies concentrated on preventing from VK₂ deficiency by settling the large intestine by bacteria species with the ability to synthesize VK₂. The aim of this study was to characterize *Bacillus subtilis* culture for vitamin K₂ production effectivity for different times of cultivation using the stripping voltammetric method. This work presents a new methodology for vitamin K₂ determination directly in bacterial culture medium (supernatant without pretreatment), which will be used in the following experiments with different bacteria species. The Controlled Growth Mercury Drop Electrode has been used in order to achieve satisfying, high repeatability, precision, and sensitivity during measurements in the presence of complex organic matrix. The use of CGMDE is necessary because of its unique properties of providing excellent repeatable working surface in the desired size at the 0.2% level. Another indisputable advantage of the presented procedure is the reduction of analysis time of VK₂ determination, comparing to other analytical methods, such as HPLC. Measurement of the sample is very quick, of about 1–2 min for single voltammogram registration, which in the case of standard addition method with 3–4 additions sums up to 20–30 min of sample analysis. It is a very important factor during an analysis of many samples. The new procedure was successfully applied for the determination of vitamin K₂ in numerous supernatant samples of *B. subtilis* culture with a complex biological matrix.

2 Materials and methods

2.1 Chemicals and reagents

All chemicals used were of analytical reagent grade. Menaquinone (VK₂) and all other reagents used to prepare the supporting electrolyte solutions, including methanol (for HPLC, ≥ 99.9%), acetic acid (≥ 99.5%), sodium acetate (anhydrous, ≥ 99%), and sodium perchlorate (≥ 98%), were purchased from Sigma-Aldrich. The tryptic soy broth (TSB)

medium was obtained from Biocorp, Poland. The solutions were prepared in quadruple distilled water. All experiments were carried out at room temperature (22 ± 1 °C).

2.2 Apparatus and software

During the cultivation time, bacterial strains were placed in the incubator (Bolarus) at a temperature of 37 °C. Samples were shaken with the mechanical shaker (Biosan) and centrifuged in the laboratory centrifuge (Eppendorf).

Voltammetric measurements were performed on the M20 multipurpose electrochemical analyzer coupled with the M164 electrode stand (both *mtm-anko*, Poland) and equipped with the EAPro 1.0 software. All measurements were performed using the three-electrode cell, including the Controlled Growth Mercury Drop Electrode (CGMDE, 1.2 mm^2) as a working electrode, the double-junction silver chloride reference electrode (Ag/AgCl/3-M KCl/2.5-M KNO_3), and a platinum wire as the auxiliary electrode.

2.3 Bacterial strain and culture conditions

Bacillus subtilis subsp. *subtilis* (*Bacillus natto*) was from the ATCC collection. The strain was incubated in Tryptone Soya Broth (TSB) medium (Biocorp, Poland) at 30 °C overnight under aerobic conditions.

Two types of vessels with different capacitances—Erlenmeyer flasks (capacity of 0.1 L) and measuring cylinders (capacity of 1 L)—were chosen for *B. subtilis* cultivation in

order to check the influence of the availability of adherence surface for bacteria on vitamin K_2 production.

One hundred-milliliter Erlenmeyer flasks, 3 for each measuring day, were filled with 50 mL of the medium. The inoculum was prepared by picking eight colonies of $\geq 1 \text{ mm}$ in diameter from 24-hold cultures and suspending the material in sterile TSB. Flasks prepared with bacterial culture were vortexed for 15 s and then statically incubated at 37 °C for the appropriate time (72, 96, 120, 144, 168, 192, 216, 240, 264, 288, and 312 h). For the second experiment, 1-L elongated cylinders were used as a vessel for *B. subtilis* cultivation. Cylinders were filled with 500 mL of TSB medium and statically incubated at 37 °C for the appropriate time (72, 120, 168, 192, 240, 288, and 312 h). Flasks and cylinders were preserved from the light in order to avoid the process of VK_2 degradation. The inoculum in each cylinder flask was prepared by picking 80 colonies of *B. subtilis* strain. To increase the availability of the adherence surface, measuring cylinders were placed horizontally into the incubator. Schematic illustration of the procedure is shown in Fig. 1.

2.4 Preparation of the sample for voltammetric measurements

For each day of measurements, 3 flasks with *B. subtilis* cultivation were prepared. After taking out of the incubator, flasks were shaken in order to pre-homogenize the cultivation. 100 μL of each culture was transferred to 900 μL of 0.9% NaCl to perform the CFU measurements (procedure described in Sect. 2.5). The content of the flasks was

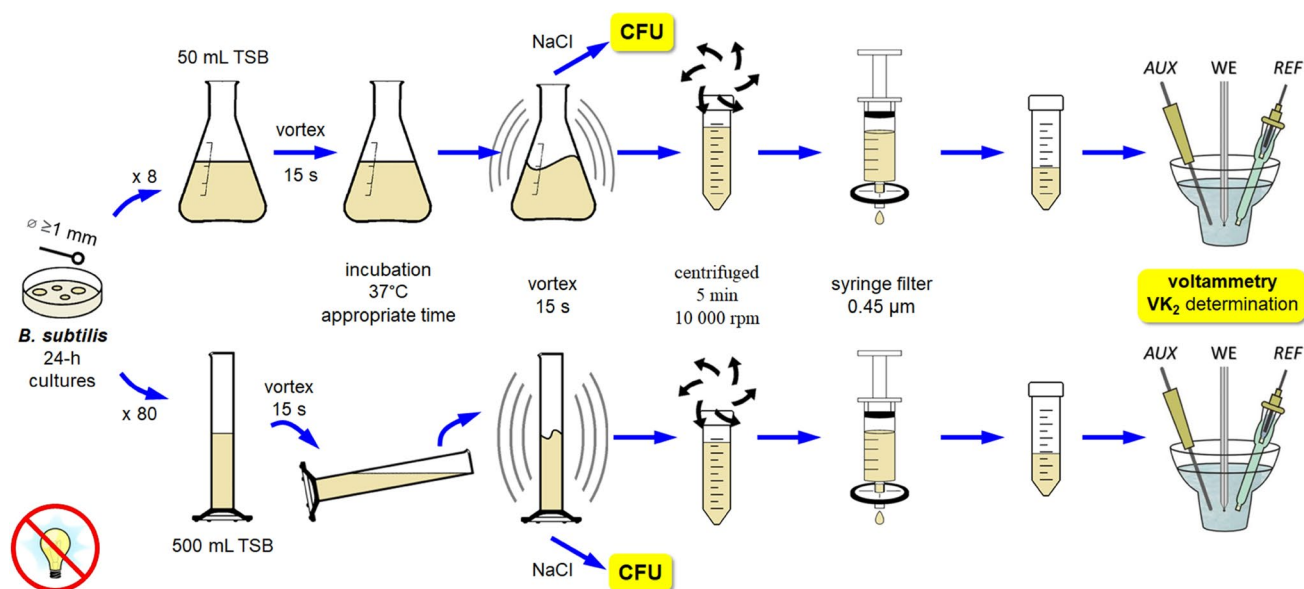


Fig. 1 Schematic diagram of the developed procedure for the preparation of a supernatant sample

transferred to 50-mL sterile centrifuge tubes, one for each culture. Tubes were centrifuged for 5 min with a speed of 10,000 rpm. After the process was finished, the obtained supernatant was filtered to another sterile centrifuge tubes using the PES syringe filter with a pore size of 0.45 μm (Biosens). The amount of filtered supernatant was about 20 mL. For the voltammetric analysis, 50 μL of supernatant was used without other preparation procedures. During all the stages of sample preparation and analysis, samples were preserved from the light in order to avoid the process of VK₂ degradation.

2.5 Procedure of CFU examination/determination of growth rate

The number of colony-forming units (CFU) was measured for each *B. subtilis* culture examined for VK₂ content. For this purpose, the amount of 100 μL of the culture was transferred to 900 μL of 0.9% NaCl and vortexed in order to achieve full homogenization of solution. Then, the series of tenfold diluted solutions with a lowering culture concentration in NaCl were prepared and planted on a standard size Petri dish with TSB agar. The CFU number was read from the dish by counting visible single *B. subtilis* colonies.

2.6 Determination of vitamin K₂ content

The content of vitamin K₂ in supernatant samples was analyzed using the differential pulse voltammetric (DPV) method. Every day, a fresh stock standard solution of Vitamin K₂ (500 $\text{mg}\cdot\text{L}^{-1}$) was prepared by dissolving the dry pure substance in ethyl alcohol and then was stored in an amber volumetric flask in the fridge at 4.0 °C. All diluted solutions of VK₂ were prepared shortly before the measurements from the stock solution. DPV experiments were conducted in the supporting electrolyte containing 70% (v:v) of methanol and 0.04-mol·L⁻¹ acetate buffer (pH 3.8) with a total volume of 5 mL. In order to ensure the appropriate electrolytic conductivity and reduction of the capacitive component of the recorded current, the appropriate weight of 0.122 ± 0.001 g of sodium perchlorate was added directly to the cell with the supporting electrolyte. The solution was deoxygenated with argon for 3 min before measurements

and for 15 s between each repetition of the voltammogram. DP voltammograms were recorded in the potential range from -0.04 to -0.4 V in both anodic and cathodic directions. The optimized experimental parameters for DPV technique were as follows: potential step $E_s = 2$ mV, pulse amplitude $dE = 30$ mV, and pulse period $t_{\text{imp}} = (t_w + t_s) = 10$ ms; in each case it was assumed that $t_w = t_s$ (waiting time = current sampling time). Accumulation step, the key parameter for SV methods, was performed before both cathodic (accumulation potential $E_{\text{acc}1} = -0.04$ V, $t_{\text{acc}1} = 1$ s) and anodic scans ($E_{\text{acc}2} = -0.4$ V, $t_{\text{acc}2} = 50$ s). For reassuring the repeatability and reproducibility of the CGMDE surface at the 0.2% level, the temperature in the room where the measurements were performed was kept constant at 22 ± 1 °C. The voltammetric cell, made of transparent PYREX borosilicate glass, was wrapped with aluminum foil during the measurement in order to protect the VK₂ against decomposition by exposing it to light.

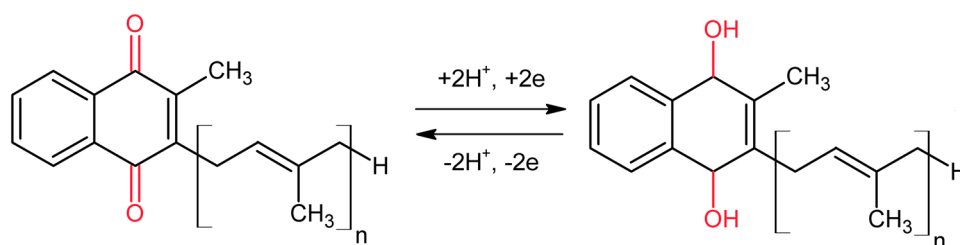
3 Results

3.1 Optimization of experimental conditions

The aim of the optimization is to study the influence of individual parameters on the recorded signal of VK₂ oxidation and, on its basis, find the most suitable conditions for the determination of VK₂ in complex matrices. The current peak corresponding to the reversible reduction of menaquinone to its hydroquinone form (Fig. 2) was exploited as the analytical signal. The starting point for the optimization are the conditions reported by Takamura [24] and Jedlińska [23]. In this work, the optimization was performed in the supporting electrolyte enriched with VK₂ to give the concentration of 2 $\mu\text{g L}^{-1}$, both in the presence and absence of 50- μL TSB breeding medium.

In both cases, the optimal conditions were consistent with the previously reported values in Sect. 2.6 which was of utmost importance to ensure the highest sensitivity and guaranteed the excellent repeatability and reproducibility of the method (Fig. 3A). Both in the absence and presence of the breeding medium, the relationships reported in [23] were conserved. However, due to the introduction of a biological

Fig. 2 The general reaction of menaquinones (n the number of isoprenoid units)



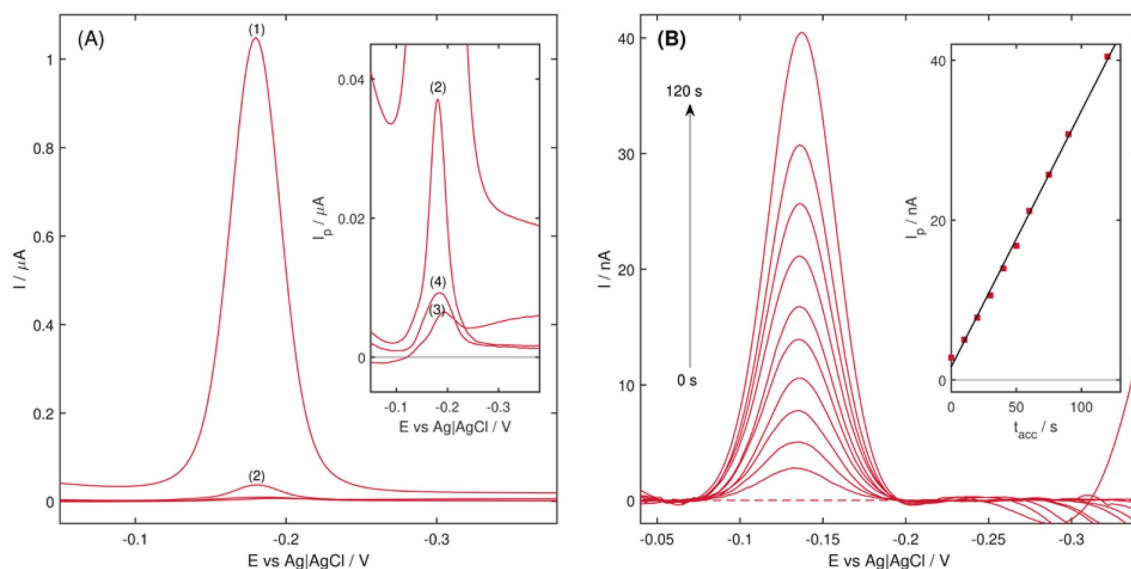


Fig. 3 The influence of DP parameters: **A** pulse period (t_{imp}) and **B** accumulation time (t_{acc}) on the recorded signal of vitamin K₂ oxidation. Experimental conditions as reported in Sect. 2.6. Tested values

of t_{imp} : (1) 10, (2) 20, (3) 30, and (4) 40 ms, where $t_{imp} = t_w + t_s$ and $t_w = t_s$. Inset in (A): the magnification of curves registered for higher pulse period

matrix, the peak height was lower than that in the plain supporting electrolyte, which translates into lesser sensitivity. As described in [23], significant improvement in repeatability and reproducibility was obtained when the cathodic scan preceded the anodic one.

Further enhancement was achieved by preceding the oxidation step by the accumulation of VK₂ in the mercury droplet at the potential $E_{acc} = -0.4$ V. As can be seen in Fig. 3B, the peak current increased linearly with the extension of accumulation time t_{acc} . This provides a simple way to diminish the influence of the interferences present in the difficult biological matrices by diluting the sample and thus decrease their concentrations. At the same time, to counteract for lower VK₂ concentration in the cell, the accumulation time can be extended, thus obtaining a distinguishable signal of appropriate height. To sum up, by adjusting the volume of the sample taken for the analysis and the accumulation time, the presented procedure can be easily adapted for the quantitative determination of VK₂ in the variety of matrices.

3.2 Stability and reproducibility of the procedure

Stability of the designed procedure was examined by recording thirty consecutive voltammograms in supporting electrolyte under optimized conditions. No significant difference in the registered curves was observed, thus the background stability of the system is excellent. In twenty successive

measurements carried out in supporting electrolyte spiked with 20-mg L⁻¹ VK₂, slight shift (4%) was observed. Five repetitions of VK₂ determination at the level of 0.5 mg L⁻¹ and 20 mg L⁻¹ are characterized by RSD of 8.3 and 4.5%, respectively. Over 2 weeks, for 3–7 h of measurements per day, the signal sensitivity fluctuates by about 15%.

3.3 Analytical performance

For validation purposes, the series of DP AdSV voltammograms for VK₂ concentrations ranging from blank to 16 μg·L⁻¹ were recorded under the optimized conditions using the accumulation time of 30 s. Supporting electrolyte spiked with 50 μL of TSB medium, obtained after 312 h of breeding according to the procedure described in Sect. 2.3 without introducing any bacteria, was used as blank. Subsequently, a polynomial background of the 3rd degree was subtracted from every registered curve (Fig. 4A). This resulted in a well-defined, symmetrical peak of small half-width, whose height is proportional to VK₂ concentration in the entire tested range, as depicted in the inset in the respective figure. The calculated basis sensitivity and correlation coefficient were equal to (4.487 ± 0.016) nA·L·μg⁻¹ and 0.9991, respectively. The LOD and LOQ, computed as the standard deviation of the intercept divided by the slope and multiplied by 3 and 10, respectively, were equal to 0.09 μg·L⁻¹ and 0.30 μg·L⁻¹. The obtained figures of merit were compared with the values reported for voltammetric procedures of VK₂ determination which utilize distinguishable construction

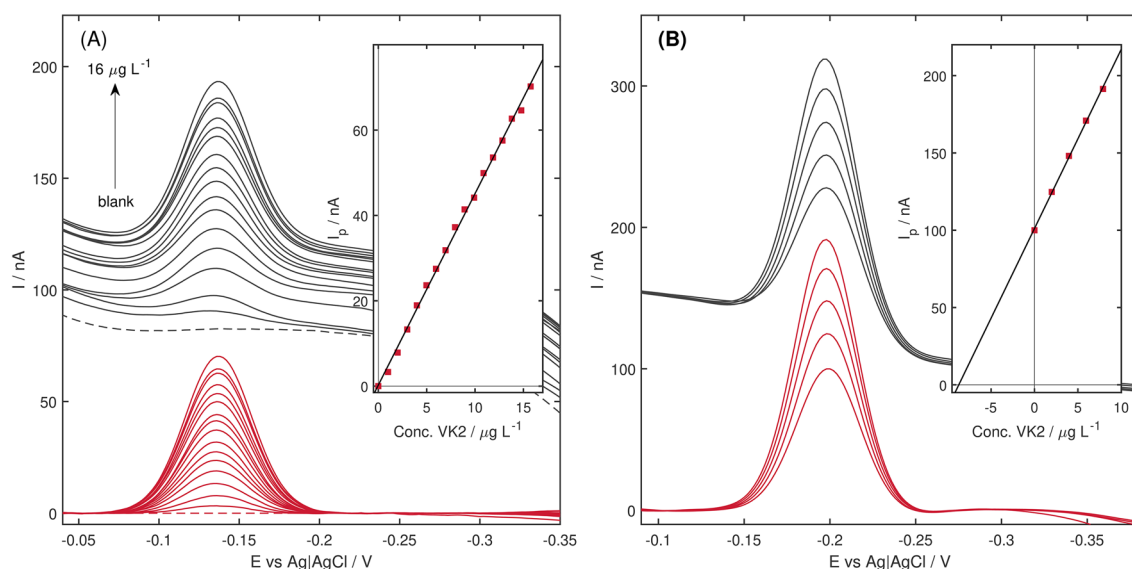


Fig. 4 **A** DP AdSV voltammograms recorded at CGMDE for concentrations of vitamin K₂ in the range from blank to 16 μg·L⁻¹. **B** Determination of VK₂ produced by *Bacillus subtilis* bacteria after 240 h of breeding. Inset: corresponding calibration plots. Gray lines represent

the experimental curves, red—voltammograms after respective background subtraction, and dashed—curve recorded for blank. (Color figure online)

of working electrodes. Furthermore, the relative standard deviation of peak current for 6-μg·L⁻¹ VK₂ was equal to 1.3% ($n=5$), proving excellent short-term stability and repeatability. Based on the above-mentioned characteristics, we concluded that the devised protocol which utilizes the Controlled Growth Mercury Drop Electrode will ensure the highly sensitive, reliable, and reproducible determination of VK₂.

3.4 Analysis of the real samples

The above-described procedure was applied for the determination of VK₂ produced by *Bacillus subtilis* under *in vitro* conditions. The anodic voltammograms registered after injection of the sample and four subsequent addition of 1-μg·L⁻¹ VK₂ standard solution were submitted to the subtraction of polynomial background (Fig. 4B). Based on the obtained standard addition plot, the concentration of VK₂ in the tested specimen was computed. No other signals were observed in the tested potential range, indicating that the proposed strategy is selective toward VK₂.

To examine the effect of cultivation time on vitamin K₂ production, *B. subtilis* was cultivated in TSB medium and the VK₂ levels were measured daily using the proposed method. The averaged results are presented in Fig. 5A. Furthermore, the influence of the shape of the laboratory vessels used to perform the cultivation on the amount of

produced VK₂ was investigated (Fig. 5B). During the first 10 days of breeding, the amount of produced vitamin K₂ in vessels increased, reaching the maximum value of 1822 μg L⁻¹ (2×10^8 CFU mL⁻¹) and 238 μg L⁻¹ for cylinders. From that point on, a decrease in VK₂ concentration has been observed, reaching the values of 1050 μg L⁻¹ and 176 μg L⁻¹ for vessels and cylinders, respectively, at the end of measuring cycle (312 h). The maximum value of the CFU/mL for vessels and cylinders was obtained at the time of 216 h (6.1×10^8 CFU mL⁻¹) and 168 h (2.5×10^8 CFU mL⁻¹), respectively.

Strong differences in specific concentrations of produced vitamin K₂ depending on incubation time were observed in course of the experiment. As presented in Fig. 5A, higher values of determined VK₂ concentration generally correspond to lower CFU mL⁻¹ numbers and vice versa. This proves that VK₂ can act as a nutrient for *B. subtilis*, stimulating its growth and number, since otherwise a strict correlation between the VK₂ concentration and the breeding time would have been observed.

In general, the values obtained for the elongated cylinder were one order of magnitude lower than those obtained for a cultivation conducted in the Erlenmeyer flask. This statement applies to both the concentration of VK₂ as well as the number of bacteria. Based on that, we suppose that one of the key factors here is how well the bacteria are dispersed in the medium and to what amount of the nutrients they have access to.

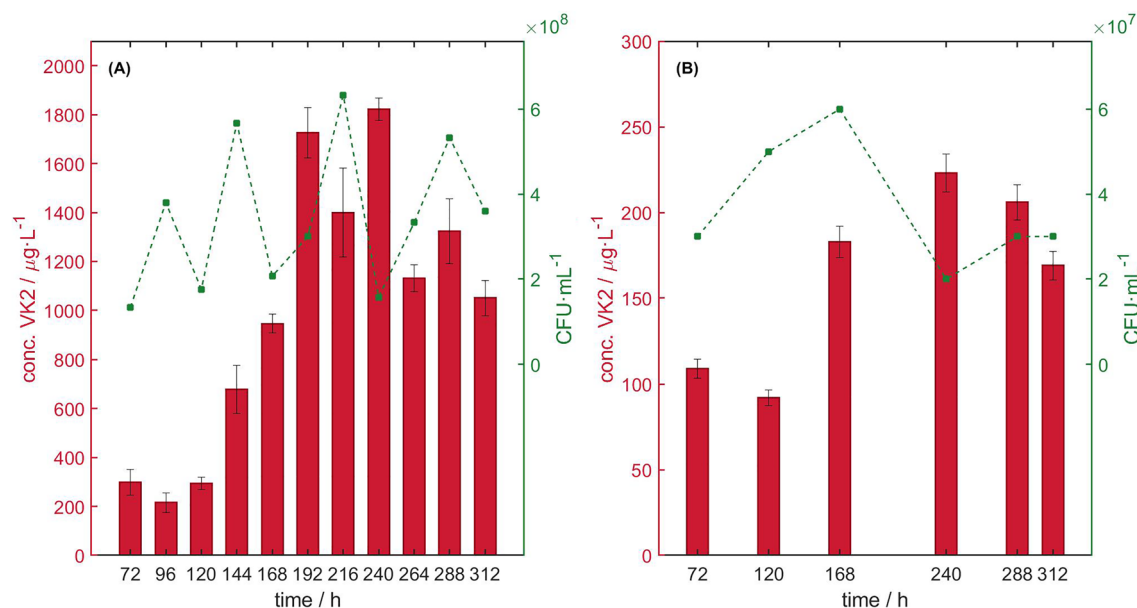


Fig. 5 Changes in the amount of produced vitamin K₂ and the number of bacteria present in the cultivation (CFU) with breeding time for the cultivation performed in the **A** Erlenmeyer flasks and **B** cylinders

4 Conclusion

In this work, we focused on the simple bacteria strain, *Bacillus subtilis*, to devise a procedure for proper bacteria cultivation, separation of the breeding into fractions, sample preparation, and determination of vitamin K₂ produced under in vitro conditions. For the latter, we proposed the employment of voltammetry with the utilization of the Controlled Growth Mercury Drop Electrode, due to the short analysis time, simplicity of the measurement system, and the low cost of the required equipment. The conditions reported in the literature for vitamin K₂ (VK₂) determination in synthetic solutions were adopted for the biological samples in our focus. As mentioned above, this procedure can be easily adjusted for a variety of systems. We believe that this method could be applied for routine analysis of post-cultivation medium, including in particular other bacteria strains, without special sample pretreatment.

The in-depth investigation conducted for the *B. subtilis* indicated no strict correlation between the vitamin K₂ concentration in the breeding medium and the cultivation time, meaning that this vitamin is not only produced by this particular bacteria strain but also acts as its nutrient. *B. subtilis* can produce up to 1822- μg VK₂ per 1 L of the breeding medium. Moreover, considering the obtained results, the production of vitamin K₂ by *B. subtilis* isolated from Nattō in the *in vitro* conditions is strongly dependent on the type and shape of the vessel. Higher values of VK₂

concentrations were observed in the vessels, characterized by definitely smaller adherence surface in comparison to horizontally placed cylinders.

Author contributions JS, RP, and GW performed the laboratory section of the study. JS, RP, JL, and KJ participated in results analysis and manuscript preparation. BB and MS contributed to conception and design of the project and were involved in data analysis and interpretation of results. All authors read and approved the manuscript.

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Declarations

Competing interests The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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