Optimised "green solvent" extraction of long-chain menaquinones (Vitamin K2) from wet *Lactococcus lactis* biomass

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ABSTRACT

Menaquinone (vitamin K2) is an essential fat-soluble vitamin for bone and cardiovascular health. Long-chain menaquinones are exclusively synthesised by bacteria, and *Lactococcus lactis* appears promising as a microbial production platform. Menaquinone exists in the bacterial cytoplasmic membrane protected by a thick cell wall, thus requiring efficient cell lysis and extraction for its recovery. In this work, several potential solvents that are ranked as "recommended" for pharmaceutical manufacture were screened for their effectiveness, with the aid of external heating, to simultaneously disrupt cells and extract menaquinone from wet biomass. These results were compared with those obtained by traditional two-step strategies and extraction approaches using dry biomass. Ethanol appeared to be the most effective extractant, and the technique was then optimised using response surface methodology. Under optimal conditions (75 °C, biomass concentration of 0.199 g mL^{-1} and extraction time of 36.8 min), the extraction yields for menaquinone-7 and menaquinone-8 were 6.73 ± 0.17 and 179.6 ± 3.59 μg g^{-1} dry cell weight, respectively. In addition, the use of ethanol as the extraction solvent coupled with microwave heating and bead-beating was investigated. Enhanced extraction was achieved using microwave-assisted extraction at an elevated temperature of 125 °C for 5 min, with a 1.15- and 1.2-fold increase in menaquinone-7 and menaquinone-8 yields, respectively. This work proposes the development of an integrated single-step process for the sustainable and efficient recovery of menaquinone from biomass, with the advantage of using ethanol as a green extraction solvent.

KEYWORDS:

Lactococcus lactis; Long-chain menaquinone; Vitamin K2; Cell lysis; Solvent Extraction

1. Introduction

It has been well established that Vitamin K is essential in blood clotting, and acts as a cofactor for γ-glutamyl carboxylase which is required for a specific γ-carboxylation reaction converting glutamate to γ-carboxyglutamate residues [1]. Vitamin K is categorized into three main groups based on its side-chain structure, with a methylated naphthoquinone ring as the base skeleton. These forms are phylloquinone (vitamin K1, with a phythyl side chain) found primarily in green leafy vegetables; menaquinone (vitamin K2, with an isoprene side chain) derived from animal and fermented foods; and menadione (vitamin K3), a synthetic analogue without a side chain. Menaquinone is a series of prenylated naphthoquinone derivatives characterized by the length of its isoprenoid side chain, and is designated as MK-*n*, where *n* denotes the number of isoprenyl units. The most well-known isoforms are MK-4, -7, -8, and -9; MK-4 originates from animal-based sources such as eggs, meat and liver, whereas long-chain MK-7, -8 and -9, are principally synthesized by bacteria and found in fermented foods.

There is increasing scientific evidence on the health benefits of menaquinone, boosting its demand as a nutritional supplement [2]. It was proposed that menaquinone should be separated from the current recommended daily intake of vitamin K which was based on phylloquinone and normal coagulation requirements, that is 1 μg phylloquinone kg^{-1} body weight [3]. Besides blood clotting, menaquinone has other distinct biological functions in regulating bone metabolism to support strong bones and thereby prevent osteoporosis [4]. The length of the isoprenyl side chain has a dominant effect on its bioavailability, and the medium-length side chains (e.g., MK-7 and MK-8) are better absorbed compared to those with either short (e.g., MK-4) or long (e.g., MK-9) side chains [5, 6]. Additionally, MK-7 and MK-8 have longer plasma half-life times, and induced more complete carboxylation of osteocalcin than phylloquinone and short-chain menaquinone [7]. A deficiency in menaquinone will lead to the development of impaired bone mineralization and increase the risk of bone fractures. This

hypothesis is supported by the findings that significant deficiencies of MK-7 and MK-8 were observed in patients with osteoporotic fractures [8]. Furthermore, clinical studies reported that a low-dose supplementation of MK-7 improved bone strength and reduced age-related decline in bone mineral density in postmenopausal women [9]. Moreover, menaquinone intake is associated with a decreased risk of cardiovascular disease [10]. Because of these therapeutic effects, nutritional additives based on specific or mixed long-chain menaquinones are sought after, especially after their approval by the European Food Safety Authority in 2009 [11].

Long-chain menaquinones can be produced naturally through biosynthesis, or by organic synthesis; the former is more favoured due to its non-toxic nature [12], and several bacteria have been reported to synthesize long-chain menaquinones. They are constituents of the bacterial cytoplasmic membrane, and act as an electron-delivery vector of the respiratory chain due to their ability to mediate electron transport between enzymes [13]. Menaquinones show uniqueness in the length and degree of unsaturation of the polyprenyl chain unit in different bacterial strains, e.g., *Bacillus subtilis* (MK-7) [14, 15], *Escherichia coli* (MK-8) [16] and *Lactococcus lactis* (MK-7, MK-8 and MK-9) [17]. Commercial production of MK-7 is typically through extraction from natto, a popular traditional Japanese soybean product fermented by *B. subtilis* [18]. Natto is rich in menaquinones, ranging from MK-6 to MK-8, in which MK-7 constitutes > 90% of the total menaquinone content, with around 900 µg/100 g natto [19]. Another menaquinone-producing strain, *L. lactis*, also holds great potential as a microbial platform for long-chain menaquinone production. It is not only useful as a dietary supplement (with greater than 230 nmol menaquinones (MK-7, MK-8 and MK-9) g^{-1} dry cell weight (DCW)), but can also be used as a an enhancer of menaquinone in many fermented products due to its wide use as a starter culture in various food fermentation processes [17]. Morishita and co-workers [17] reported that the level of menaquinone in fermented milk by *L. lactis* was close to 250 nmol L^{-1}, and this could be increased to nearly 700 nmol L^{-1} when a

metabolically engineered strain was used [20]. Furthermore, cheese produced by *L. lactis* and enriched in vitamin K2 (up to 110 μg/100 g) is essential in the Western diet [21]. Recent efforts have focused on improving the production of menaquinone in *L. lactis* through the optimisation of growth conditions [22] and metabolic engineering [20].

Since menaquinones exist in bounded forms within cytoplasmic membranes protected by a multi-layered peptidoglycan sacculus, the development of methodologies to recover menaquinones from bacterial cells is essential to obtain the product at high yield, and low cost. Downstream processing of intracellular compounds usually involves a cell lysis pretreatment, can be mechanical (e.g., bead-beating, homogenization, sonication and pulsed electric field) and/or chemical (e.g., acid, base, organic solvent and enzyme), and is accompanied by a solvent extraction step [23-25]. Cell lysis using acid hydrolysis, organic solvents and enzymatic lysis have been reported for *Bacillus natto* [26, 27], *Flavobacterium* [28] and *Actinomycetes* [29]. However, their use for menaquinone extraction from *L. lactis* has not yet been adequately evaluated and optimised. Different cells will have distinct disruption resistances towards different methods due to their differing cell wall compositions, and therefore need tailor-made cell lysis protocols. In addition, more advanced technologies such as microwave- and bead-beating-assisted extraction remain unexplored for the separation of menaquinone from biomass. Furthermore, the separation of menaquinones from ruptured cells often requires the use of significant amounts of organic solvents such as *n*-hexane, chloroform and isooctane [22, 29, 30]. Mixtures of *n*-hexane/isopropanol and chloroform/methanol are often used in liquid-liquid extraction processes to recover menaquinones after cell lysis. Unfortunately, above a certain extent of exposure these solvents are hazardous to the environment or human health [31], so there is a need to look for more sustainable solvents, preferably bio-derived, aimed at replacing these toxic solvents to develop efficient extraction processes.

This work addresses the development of efficient approaches for the recovery of long-

chain menaquinones from the wet biomass of *L. lactis*, starting with a preliminary evaluation of the extraction performance of solvents up to the investigation of integrated biosolvent-based extraction platforms. The ability of nine organic solvents to simultaneously disrupt wet cells and extract long-chain menaquinones at 60 °C was initially evaluated. Furthermore, conventional two-step schemes comprised of cell lysis (lysozyme at different concentrations, sonication, acid heating and repeated freeze-thaw) followed by liquid-liquid extraction using a mixture of *n*-hexane and isopropanol (2:1 v/v) were investigated. Additionally, to validate the disruption capability of solvents towards wet cells, the performance of the same solvent systems to extract menaquinones from freeze-dried and ground biomass was examined. After identifying the most promising approach, an optimisation study using response surface methodology (RSM) based on central composite design (CCD) was carried out to optimise the parametric factors of extraction temperature, biomass concentration and extraction time. The use of ethanol as an extraction solvent coupled with microwave heating (known as microwave-assisted extraction (MAE)) and bead-beating, respectively, was also assessed. The proposed platforms were finally compared and discussed.

2. Experimental section

2.1. Chemicals

Methanol, acetonitrile and isopropanol were of Optima™ LC-MS grade and purchased from Fisher Chemical. Ethanol (≥99.5%), tert-butanol (≥99.5%), ethyl acetate (≥99.7%), *n*-hexane (≥97.0%), chloroform (≥99.8%) and isooctane (≥99.0%) were of HPLC grade and acquired from Sigma-Aldrich. Formic acid (LC-MS grade) was obtained from Thermo Fisher. Ammonium formate (LC-MS grade, ≥99.0%), lysozyme from hen egg white (~70000 U mg^{-1}) and MK-7 (United States Pharmacopeia (USP) reference standard) were obtained from Sigma-Aldrich. MK-8 and MK-9 were supplied by Santa Cruz Biotechnology. Ultra-pure water used was prepared by Millipore Milli-Q water purification system.

2.2. Microorganism and culture conditions

L. lactis, isolated from soybean soaking wastewater (Mr. Bean, Singapore), was used in this work. Batch cultures were carried out in a 20-L stirred-tank bioreactor containing de-Mann Rogosa and Sharpe (MRS) medium at 30 °C and 200 rpm, using the inoculum prepared at 30 °C for 24 h. After 48 h of cultivation, the cellular pellets were collected, and the cell-free supernatants were discarded. The pellets were washed twice with 1× volume of phosphate-buffered saline (PBS) and collected by centrifugation at 3000*g* for 10 min for subsequent studies.

2.3. Menaquinone extraction from wet biomass

Wet *L. lactis* biomass was subjected to different treatments; a solid-liquid ratio of 0.2 g mL^{-1} (mass of wet biomass/volume of solvent or cell lysis solution) was used. All extraction studies were conducted under reduced lighting to avoid decomposition and photo-oxidation of the light-sensitive menaquinones. The cell extracts obtained were measured for the menaquinone (MK-7, -8 and -9) content.

2.3.1. Integrated cell lysis and extraction

Wet *L. lactis* biomass was mixed with the organic solvents. After vortexing at 3000 rpm for 30 s, the samples were homogenized at 60 °C and 300 rpm using a Thermo Scientific™ Reacti-Therm™ Heating/ Stirring Module (Reacti-therm III # TS-18823 Heating/Stirring unit) for 1 h. A preheat time of 5 min was applied for all samples. After homogenization, the samples were cooled to 25 °C followed by vortexing for 30 s at 3000 rpm. The cell extract supernatants were collected by centrifugation at 5000*g* and 25 °C for 10 min using an Eppendorf® Centrifuge 5810/5810R and processed according to the protocols described in the "Determination of menaquinone content" section.

2.3.2. Two-step schemes of cell lysis and extraction

A two-step approach comprised of cell lysis (i.e., enzymatic lysis, sonication, acid heating and repeated freeze-thaw) followed by solvent extraction were investigated. All samples were vortexed at 3000 rpm for 30 s at the beginning and end of the cell lysis procedures. After cell lysis the lysed cells were extracted using a mixed solvent (*n*-hexane and isopropanol -2:1, v/v). 4 × volume of mixed solvent was added to the cell lysate, and the mixture vortexed at 3000 rpm for 30 s. The top layer was collected after centrifugation at 3000*g* for 10 min (Eppendorf® Centrifuge 5810/5810R), and then an equal volume of *n*-hexane was added to the remaining bottom phase. The hexane extraction step was repeated twice, and the three supernatants combined for quantitative measurement.

Enzymatic lysis: wet *L. lactis* cells were re-suspended in lysozyme solution at different concentrations (0.25, 0.5, 1, 5 and 10 mg mL^{-1} in PBS), and incubated at 37 °C and 100 rpm for 1 h using an incubation shaker (Infors HT Ecotron).

Cell lysis by sonication: cell suspensions (prepared in PBS) were sonicated (VCX130, Sonic & Materials) at 24 kHz in a pulse mode of 5 s on/3 s off for 5 min and 10 min, respectively. The samples were immersed in an ice bath during the process to prevent localised heating.

Cell lysis by acid heating: 15% (v/v) HCl solution was added to the wet cells, and heated at 60 °C and 300 rpm for 1 h. A preheat time of 5 min was allowed.

Cell lysis by repeated freeze-thaw: the freeze-thaw method involved freezing the cell suspensions (in PBS) at −80 °C, and then thawing them in a 37 °C water bath. The freeze-thaw protocols were repeated for 4 cycles and the cell lysate then used in solvent extraction.

2.4. Menaquinone extraction from dry biomass

The clean cell pellets were lyophilized at −85 °C under 0.160 mbar for 48 h using a FreeZone Legacy freeze dryer (Labconco). The freeze-dried cells were then ground into fine particles using a mortar and pestle and treated with a series of solvents at a dry biomass concentration of 0.025 g mL^{-1} at 250 rpm and 25 °C for 1 h. Tert-butanol was not included in this test since

it tends to solidify at room temperature. The samples were centrifuged at 12000*g* for 10 min and the supernatants were collected to determine the menaquinone content. In addition, the protocols developed by Collins and co-workers for menaquinone extraction from biomass were performed as described elsewhere [30]. The freeze-dried and ground cells were treated with a mixture of chloroform/methanol (2:1, v/v) at a dry biomass concentration of 0.005 g mL^{-1} and the mixture was stirred continuously at 25 °C overnight.

2.5. Optimisation of the integrated technique

After determining the most promising approach, optimisation was conducted using CCD with Design-Expert® version 13. The independent variables for integrated cell lysis and extraction using ethanol were extraction temperature (°C), wet biomass concentration (g mL^{-1}) and extraction time (min), while yields of MK-7 and MK-8 (µg g^{-1}DCW) were selected as dependent variables. The independent factors were randomized and varied over five levels, as listed in Table S1 in the ESI. A total of 20 treatments were performed and summarised in Table S2 in the ESI. Statistical analysis was performed using one-way analysis of variance (ANOVA) with a confidence level of 95%. Optimisation of the parametric factors was performed using the inbuilt Derringer's desirability function in Design-Expert.

2.6. Alternative integrative platforms based on ethanol as extraction solvent

The use of ethanol, the best extraction solvent, was incorporated with microwave heating to intensify the extraction efficiency. In addition, another alternative approach to replacing heat with a bead-beating operation was explored. The concentration of wet biomass in the studies of alternative techniques was kept constant at 0.199 g mL^{-1} (the optimised value obtained from using ethanol as extraction solvent with external heating -see Table 1).

2.6.1. Microwave-assisted extraction

Wet pellets of *L. lactis* were suspended in ethanol and subjected to different microwave conditions using a microwave digestion system (Multiwave 5000, Anton Paar). The

temperature and duration of heating ranged from 75 to 125°C and 5 to 20 min, respectively, and a static preheat time of 5 min to reach the tested temperature was applied. The supernatants were collected by centrifugation at 10000*g* for 10 min and their menaquinone measured.

2.6.2. Bead-beating-assisted extraction

Wet *L. lactis* cells and ethanol were added to the lysing matrix A (garnet matrix and one 1/4 inch ceramic sphere) and lysing matrix B (0.1 mm diameter glass beads) tubes, respectively, and cooled on ice for 10 min before extraction. Bead-beating was performed using a high-speed homogeniser (FastPrep-24™ 5G, MP Biochemicals) set at a speed of 6.0 m s^{-1} for 30 s, with a 300 s pulse time between each cycle, and 3, 6 and 9 beat-beating cycles were studied. The tubes were centrifuged at 12000*g* for 10 min using a high-speed mini centrifuge (Microspin 12, Biosan) to pellet debris, and the supernatants were collected for menaquinone measurement.

2.7. Determination of menaquinone content

The cell extracts were evaporated to dryness under a gentle stream of nitrogen and reconstituted with appropriate amounts of isopropanol. All samples were filtered with PTFE syringe filters (0.22 μm, 25 mm) before quantitative analysis. Separation and detection of menaquinones was accomplished using an Agilent 1290 Infinity II LC system coupled with an Agilent 6460 triple quadrupole mass spectrometer equipped with jet stream electrospray (AJS ESI) source (Agilent Technologies, Singapore). Chromatographic separation was carried out on an Agilent InfinityLab Poroshell 120 PFP (2.1 mm × 100 mm, 2.7 μm) column with an 11 min gradient elution method. The details of measurement protocols can be found in Text S1 in the ESI. Eluted compounds were detected by the ESI-MS/MS system operating in multiple reaction monitoring (MRM) mode (Table S3 in the ESI).

2.8. Data analysis

LC-MS/MS data were processed in Agilent MassHunter workstation software (version B.07.00). The sample contents of MK-7, MK-8 and MK-9 were determined based on the

calibration curves constructed using linear regression prepared by respective standards dissolved in isopropanol. The limit of quantification (LOQ) was determined as the lowest analyte concentration within a standard solution possessing a signal-to-noise ratio of more than 10 and an accuracy between 80 and 120% [32].

Triplicate independent runs were performed for each condition, and the results were expressed as average ± standard deviation. The yields of menaquinone were expressed as μg per g of DCW. The DCW for each sample was determined by applying the conversion factor derived from freeze-drying known amounts of wet biomass for 48 h. Statistical significance analysis was performed using ANOVA and accompanied by a post hoc Tukey method for all pair-wise multiple comparisons. Significance was set at an alpha level of 0.05.

3. Results and discussion

3.1. Screening of solvent for integrated cell lysis and menaquinone extraction from wet biomass

L. lactis are Gram-positive and their cytoplasmic membrane (where the menaquinones are located) are encased in a thick cell wall principally composed of multiple peptidoglycan layers with attached proteins and glycopolymers like polysaccharides and teichoic acids, and some species even have an additional outer layer of cell wall polysaccharides [33, 34]. Therefore, effective cell disruption and extraction techniques must be developed to recover menaquinones.

The performance of nine organic solvents for the recovery of long-chain menaquinones, in particular MK-7, MK-8 and MK-9 (their chemical structures are depicted in Figure S1 in the ESI), from wet biomass of *L. lactis* after 1 h of stirring at 300 rpm and 60 °C was first screened. As tabulated in Table S4 in the ESI, most of the solvents under study are recommended for the pharmaceutical industry (CHEM21 selection guide [31]), i.e., methanol, ethanol, isopropanol, tert-butanol and ethyl acetate. To facilitate the comparison, hazardous solvents such as isooctane, *n*-hexane and chloroform were also studied since they are frequently used (usually

in a mixture form with a polar solvent) to extract menaquinone. Additionally, conventional two-step approaches using enzymatic lysis, sonication, acid heating and freeze-thaw, followed by a liquid-liquid extraction using a mixture of *n*-hexane and isopropanol were conducted.

The results in Figure 1A show that all polar solvents, except ethyl acetate, were able to extract (at 60 °C) some MK-7 and MK-8 from the wet *L. lactis* biomass. Ethanol and methanol gave the best yields of MK-7, MK-8 and MK-9, although MK-9 was only a very small fraction of the biomass. The high extraction yields with polar solvents can be attributed to their ability to permeabilise into the cellular matrix allowing for the liberation of membrane-bound menaquinones. These results are in good agreement with literature findings that short-chain alcohols solubilise cell wall constituents and dramatically alter the tertiary protein structure of the cell membrane, resulting in cell disruption [35, 36]. More polar and water-miscible solvents, i.e., methanol (the octanol/water partition coefficient, log K_{OW}, = −0.77), and ethanol (log K_{OW}, = −0.31) had a higher diffusivity into the cell membrane and hence liberated the targeted solute [37]. In contrast, more hydrophobic solvents such as ethyl acetate (log K_{OW}, = 0.71), chloroform (log K_{OW}, = 1.75), *n*-hexane (log K_{OW}, = 3.94) and isooctane (log K_{OW}, = 4.46), were not able to efficiently extract menaquinones, even at 60 °C, and extremely low yields of MK-8 were obtained.

On the other hand, given the ability of lysozyme to hydrolyse the glycosidic bonds in the peptidoglycan of bacterial cell walls [38], treatment of wet cells of *L. lactis* with lysozyme at 37 °C for 1 h, particularly at high concentrations of 10 mg mL^{-1}, demonstrated efficient disruption by achieving high yields of MK-7 and MK-8 during liquid-liquid extraction. Nevertheless, this efficiency depends heavily on the high concentration of lysozyme, and this decreased substantially with reducing concentrations ($p < 0.05$). Besides, acid hydrolysis treatment using 15 %(v/v) HCl solution at 60 °C for 1 h resulted in relatively high yields of

MK-7 and MK-8. Other cell lysis techniques, namely sonication and repeated freeze-thaw, achieved moderate to low levels of cell disintegration, with low yields of MK-7 and MK-8.

Interestingly, despite the large variations in MK-7 and MK-8 in this study, MK-8 seems the main homologue found, while trends in the recovery of MK-7 and MK-8 were similar, probably due to their similar nature. From the results obtained in this screening ($p < 0.05$), integrated extraction using ethanol recovered the highest yield of MK-7 (6.41 ± 0.25 μg g^{-1} DCW), however, it achieved a lower yield of MK-8 (167.9 ± 4.01 μg g^{-1} DCW) compared to those from enzymatic lysis (203.7 ± 6.09 μg g^{-1} DCW) and acid heating (184.6 ± 4.86 μg g^{-1} DCW). The MK-7 yields from enzymatic lysis and acid heating were 5.58 ± 0.15 and 5.29 ± 0.08 μg g^{-1} DCW, respectively. Despite the high efficiency of lysozyme lysis of *L. lactis*, the use of large amount of lysozyme involves high cost rendering it uneconomic at industrial-scale [38]. Furthermore, cell disintegration techniques such as enzymatic lysis and acid heating followed by liquid-liquid extraction, although efficient, require the use of hazardous *n*-hexane to separate menaquinones. *n*-hexane is not only of concern due to its high flammability and explosiveness, but it is also neurotoxic to human health [39-41], and therefore substituting this solvent during process development has to be prioritised. Moreover, multiple-step approaches are less favourable due to loss of product, and increased chemical consumption, waste generation and production cost. In contrast, integrated approaches combining cell lysis and extraction in one step simplify product handling and minimise product loss. In view of these considerations, single-step extraction using ethanol was selected as the most promising technique for the recovery of menaquinones from *L. lactis*. Ethanol is a sustainable solvent in the GSK solvent sustainability guide (Table S4 in the ESI) [42], and is renewable [43].

3.2. Comparison to extraction from dry biomass

Most quantification methods for menaquinone in biomass utilize freeze-dried cells, such as the well-established method of Collins and co-workers [30], and the recent approach by Cao et al.

[44]. Freeze-drying enhances the efficiency of solvent extraction because of the formation of large ice crystals in the cells disrupting the cell wall followed by the sublimation of these ice crystals. However, lyophilisation is an energy-intensive process and best avoided in downstream processing. Nonetheless, to validate the effectiveness of the techniques applied to wet cells, the performance of the same solvent systems to extract menaquinones from freeze-dried and mechanically-lysed cells was assessed after 1 h of stirring at 250 rpm and 25 °C. Additionally, the widely used Collins's method for menaquinone extraction from bacterial cells using a mixture of chloroform/methanol (2:1, v/v) at 25 °C for 24 h was conducted for comparison purposes.

The results in Figure 1B show that both MK-7 and MK-8 were readily extracted from ruptured cells by a series of organic solvents, revealing that the polarity of the solvent had minimal impact on the recovery yield of menaquinones when the protective peptidoglycan cell walls are damaged. However, overall, the maximum yields of menaquinones obtained from freeze-dried and ground cells, including the Collins's method, were lower than those optimum yields obtained from wet biomass. Xie et al. [29] also found similar results when applying their lysozyme-chloroform-methanol extraction method on wet Actinomycetes biomass compared to those obtained by Collins's method using freeze-dried cells. This might be due to the loss or degradation of menaquinones during the lyophilization and grinding process, demonstrating that direct extraction from wet biomass is better to obtain a higher product yield.

3.3. Process optimisation for an integrated extraction approach using ethanol

To optimise the integrated extraction process from wet *L. lactis* biomass using ethanol, the influence of three process variables, i.e., extraction temperature, wet biomass concentration and extraction time, were studied using RSM based on CCD (Table S1 in the ESI). This allows for the simultaneous analysis of different process parameters and their interactive effect on the extraction yields of MK-7 and MK-8. The statistical analysis results (Table S5 in the ESI)

indicate that the empirical relationship between the independent variables and extraction yield could be well represented by a quadratic polynomial model (sequential p-value < 0.0001) for both MK-7 and MK-8. The precision of the model was validated by the proximate unity of the coefficient of determination, R^2, being 0.9891 and 0.9918 for MK-7 and MK-8, respectively. In addition, the predicted R^2 are in excellent agreement with the adjusted R^2, with only a difference of 0.0249 and 0.0090 for MK-7 and MK-8, respectively, suggesting that the experimental and predicted values (presented in Table S2 in the ESI) have a high degree of correlation. Lack of fit test also shows that the quadratic functions developed were insignificant relative to pure error, with a small F-value and p-value greater than 0.05 obtained for the MK-7 and MK-8 models. Figure 2 shows the 3D surface charts illustrating the interactive effect of two process variables on the yields of MK-7 and MK-8, while keeping the third variable constant (at zero level in terms of the coded factor). The corresponding contour plots are provided in Figure S2 in the ESI. The effect of process variables on the yields of MK-7 was similar to that of MK-8.

Based on the ANOVA analysis, extraction temperature (A) was found to have the greatest positive linear impact on the extraction yields of MK-7 and MK-8, followed by extraction time (C). On the other hand, biomass concentration (B) exhibited a poor ($p = 0.0172$ for MK-7 model) or insignificant ($p = 0.3964$ for MK-8 model) linear effect on the extraction yields, however, it did exert a strong negative impact through interactions with extraction temperature ($p < 0.0001$ for MK-7 model; $p < 0.0154$ for MK-8 model). Other interactive coefficients (AC and BC) were found to be statistically insignificant ($p > 0.1000$), and all three quadratic coefficients (A^2, B^2 and C^2) were significant ($p \leq 0.1000$) for both models.

It can be seen from the results that a high extraction temperature leads to high yields of MK-7 and MK-8. This can be attributed to higher temperatures promote the rapid diffusion of ethanol into the cell membrane facilitating the breakdown of cell wall constituents and allowing

for the transport of menaquinones out of the cellular matrix. Heat provokes structural change in cells, causing damage to the cell wall and loss of membrane integrity [45], and Teixeira et al. [46] demonstrated that the cell wall of *Lactobacillus bulgaricus* was damaged during heat treatment at 65 °C and above. Efficient solid-liquid extraction of valuable biomolecules from biomass amplified by heat has been reported [37, 47]. Likewise, Jensen et al. [48] extracted phylloquinone and menaquinone from food matrices by heating cheese, pork and hazelnut in boiling isopropanol for 20 min. From Figure S2 in the ESI, a higher extraction temperature above 75 °C promotes higher yields of MK-7 and MK-8, however, considering the boiling point of ethanol at 78.3 °C, the upper limit of extraction temperature was set at 75 °C. Using boiling ethanol for extraction in industry should be avoided due to the highly flammable vapours posing safety risks to the plant.

Numerical optimisation was carried out to maximise the yields of MK-7 and MK-8 with minimum extraction time. The optimised conditions were found to be at 75 °C for 36.8 min and a biomass concentration of 0.199 g mL^{-1}, as presented in Table 1. Under these conditions yields of MK-7 and MK-8 of 6.73 ± 0.17 and 179.6 ± 3.59 μg g^{-1} DCW, respectively, were obtained, in close agreement with the predicted values. Although the yield of MK-9 was not used in the optimisation study, it was found to be 0.709 ± 0.033 μg g^{-1} DCW under the optimised conditions.

3.4. Alternative approaches coupled with microwave heating and bead-beating

To further explore alternatives, the use of ethanol coupled with microwave irradiation and beat-beating was evaluated. MAE was carried out using hermetically sealed pressure vessels equipped with ceramic vessel jackets which can withstand temperatures up to 240 °C. This differs from the optimised extraction technique using external heating which capped the temperature at 75 °C; MAE was operated in a closed-vessel system under pressure at elevated temperatures far above the normal boiling point of ethanol.

As presented in Figure 3A, there was no significant difference in terms of recovery yields obtained using conventional heating and MAE operating at the same temperature, 75 °C for 36 min. On the other hand, MAE at elevated temperatures, in particular at 100 and 125 °C, achieved relatively high yields of MK-7 and MK-8 in a significantly shorter time. The yields achieved using MAE operating at 100 °C for 5 min were comparable with those using external and microwave heating techniques at 75 °C for 36 min. The highest yields of MK-7 and MK-8 in this work were 7.74 ± 0.22 and 220.2 ± 6.50, respectively, using MAE at 125 °C for 5 min. In contrast to solid-liquid extraction with external heating, microwaves disrupt cells by direct generation of heat in the interior of cells [49]. At elevated extraction temperature the moisture within a bacterial cell evaporates rapidly, creating intense intracellular pressure which ultimately ruptures the cell wall [50]. Both heat and mass gradients in MAE work in the same direction, i.e. from inside the cell outwards. In this work, the extraction process was intensified due to the capability of ethanol to solubilise cell wall components, and concomitantly its good microwave energy absorption allowing rapid heat-up (dissipation factor of ethanol = 2500×10^4) [51]. Process acceleration and high extraction yield obtained in the MAE at elevated temperatures can be attributed to the synergism of both cell rupture mechanisms, and also the increased solubility of menaquinone in ethanol at elevated temperatures.

The efficiency of MAE was further confirmed by comparing the results with a vigorous mechanical disintegration technique, namely bead-beating, combined with ethanol as the extraction solvent. In this technique, bacterial cells are ruptured when they are sheared against the solid surfaces of beads during the collision with high-speed spinning beads in the shaking vessel. Two types of lysing matrix particles were used in this work, namely Lysing Matrix A and B. Both displayed high degrees of efficiency in lysing *L. lactis* in ethanol, and there was no significant difference between them in terms of the product recovery yield. Consistent yields were obtained, and after 6 cycles of operation the highest MK-7 and MK-8 yields of 6.55 ±

0.07 and 210.1 ± 1.88 μg g^{-1} DCW were attained, respectively, as shown in Figure 3B. Besides the impact of bead-beating, high-speed agitation in a tridimensional motion in the process also improves cell-matrix wetting and penetration of ethanol for enhanced extraction. Bead-beating is a powerful mechanical lysis method, hence the results obtained could be used as an indication to benchmark the relative lytic efficiency of the techniques studied.

In this work, MAE appears the most efficient in terms of recovery at 125 °C for 5 min. Since elevated temperature was applied to accomplish the extraction, the thermal stability of commercially pure long-chain menaquinones in ethanol was examined to ascertain the viability of this technique. The results, as shown in Figure 3C, are presented in relative values using the original ethanolic menaquinones before heating as the control. It was observed that there was no loss of menaquinones, including MK-7, MK-8 and MK-9, during 60 min of heating at 75 °C. In contrast, significant loss of menaquinones occurred during heating at 100 °C for 45 min and onwards, and even at a shorter time of 30 min when heated at 125 °C in a closed-vessel system. The destructive effect of high-temperature extraction has been reported for bioproducts like phenolic compounds (e.g., epicatechin, resveratrol and myricetin) [52] and anthocyanins [53]. The thermal stability study demonstrated that long-chain menaquinones in ethanol are stable in processing mode (75 °C, up to 60 min; 100 °C, up to 30 min; 125 °C, up to 15 min). Prolonged extraction at elevated temperature is not recommended due to the possible degradation of menaquinones. It is noteworthy that the MK-7, MK-8 and MK-9 yields obtained using the MAE technique were significantly improved compared to those obtained in enzymatic lysis, giving a total of 228.6 μg g^{-1} DCW, equivalent to a specific concentration of 320.7 nmol g^{-1} DCW in which MK-8 is the major homologue. Morishita et al. [17] reported that *L. lactis* produced long-chain menaquinones reaching 230 nmol g^{-1} DCW, while on the other hand, a specific concentration of 125 nmol g^{-1} DCW was attained from *L. lactis* ssp. *lactis* FM03 in recent work by Liu and co-workers [22].

3.5. Comparison of the extraction alternatives

In summary, all three extraction techniques, based on ethanol as the extraction solvent, developed in this work displayed high recovery yields of long-chain menaquinones from wet *L. lactis* biomass. Their operating conditions, as well as the associated benefits and shortcomings, are compared in Figure 4. The MAE approach affords the highest product yield and the shortest total processing time, but the high initial investment cost, complexity in scaling-up and safety aspects are of concern when trying to justify its industrial applicability [54]. However, by taking advantage of recent advances in microwave technology, the design of microwave-assisted extractors for large-scale application is possible, and industrial microwave extractors are currently available [55]. Nevertheless, MAE is still in its industrial infancy, and hazards associated with microwave irradiation and pressurised extraction in a large-scale system should be assessed thoroughly [56]. On the other hand, classical extraction with external heating seems simple and easy to scale-up, although it achieved slightly lower extraction yields, about 87.0 and 81.6 % of those MK-7 and MK-8 recovered by MAE, respectively, over a longer process duration. Detailed cost-benefit analysis has to be performed to evaluate if the economic benefits gained from the improvement in yield and short process time of MAE outweigh the equipment and maintenance cost, and vice versa for the classical extraction technique with external heating. Envisioning the industrial application, the main processes for the production of long-chain menaquinones from *L. lactis* biomass are designed based on solid-liquid extraction intensified with external heating and presented in Figure 5. Please note that the product fractionation and polishing steps were not experimentally investigated in this work, but have been reported previously [28, 57]. The extract obtained in this work had a purity of 74 % (see Text S2 in the ESI for the protocols), and further purification and polishing operations, e.g., chromatography, drying and microencapsulation, are required to prepare the finished product in a pure and stable form for application as food additives and

dietary supplements. The recovered substance, as a microbial-derived food ingredient, needs to gain generally recognized as safe (GRAS) status with scientific evidence before marketing. Microbial production of long-chain menaquinones is promising, but advancement in downstream processing is essential to boost its industrial potential while maintaining the sustainability of the whole process.

4. Conclusions

Enhanced extraction of long-chain menaquinones from wet *L. lactis* biomass was successfully achieved, with the development of efficient extraction alternatives (solid-liquid extraction with external heating, MAE and bead-beating) based on ethanol as the extraction solvent. The integrated platforms proposed allow for cell lysis and extraction of menaquinones to be accomplished in a single-step, and the results obtained were comparable and even superior to those acquired using conventional two-step approaches comprised of enzymatic lysis/acid heating followed by liquid-liquid extraction. These strategies eliminate the complications resulting from the need to adopt individual downstream process units together and avoid the use of hazardous solvents such as *n*-hexane. In addition, with the use of a bio-based and food-grade solvent, namely ethanol, the extraction process is sustainable and safe for pharmaceutical and food products; in this work menaquinones that have potential application as a human supplement and food additive. Further scale-up studies for the extraction approaches with either external heating or microwave heating, whichever is better after a detailed technical, economic and safety feasibility assessment, should be performed for implementation on an industrial-scale.

Acknowledgements

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References

[1] J. Stenflo, P. Fernlund, W. Egan, P. Roepstorff, Vitamin K dependent modifications of glutamic acid residues in prothrombin, Proceedings of the National Academy of Sciences, 71 (1974) 2730-2733.
[2] J.W. Beulens, S.L. Booth, E.G. van den Heuvel, E. Stoecklin, A. Baka, C. Vermeer, The role of menaquinones (vitamin K2) in human health, British Journal of Nutrition, 110 (2013) 1357-1368.
[3] A.C. Akbulut, A. Pavlic, P. Petsophonsakul, M. Halder, K. Maresz, R. Kramann, L. Schurgers, Vitamin K2 needs an RDI separate from vitamin K1, Nutrients, 12 (2020) 1852.
[4] T. Ichikawa, K. Horie-Inoue, K. Ikeda, B. Blumberg, S. Inoue, Vitamin K2 induces phosphorylation of protein kinase A and expression of novel target genes in osteoblastic cells, J Mol Endocrinol, 39 (2007) 239-247.
[5] L.J. Schurgers, K.J. Teunissen, K. Hamulyák, M.H. Knapen, H. Vik, C. Vermeer, Vitamin K–containing dietary supplements: comparison of synthetic vitamin K1 and natto-derived menaquinone-7, Blood, 109 (2007) 3279-3283.
[6] L.J. Schurgers, C. Vermeer, Determination of phylloquinone and menaquinones in food, Pathophysiology of Haemostasis and Thrombosis, 30 (2000) 298-307.
[7] T. Sato, L.J. Schurgers, K. Uenishi, Comparison of menaquinone-4 and menaquinone-7 bioavailability in healthy women, Nutrition journal, 11 (2012) 1-4.
[8] S. Hodges, M. Pilkington, T. Stamp, A. Catterall, M. Shearer, L. Bitensky, J. Chayen, Depressed levels of circulating menaquinones in patients with osteoporotic fractures of the spine and femoral neck, Bone, 12 (1991) 387-389.
[9] M. Knapen, N. Drummen, E. Smit, C. Vermeer, E. Theuwissen, Three-year low-dose menaquinone-7 supplementation helps decrease bone loss in healthy postmenopausal women, Osteoporosis International, 24 (2013) 2499-2507.
[10] J.W. Beulens, M.L. Bots, F. Atsma, M.-L.E. Bartelink, M. Prokop, J.M. Geleijnse, J.C. Witteman, D.E. Grobbee, Y.T. Van Der Schouw, High dietary menaquinone intake is associated with reduced coronary calcification, Atherosclerosis, 203 (2009) 489-493.
[11] J.-L. Bresson, A. Flynn, M. Heinonen, K. Hulshof, H. Korhonen, P. Lagiou, M. Løvik, R. Marchelli, A. Martin, B. Moseley, Vitamin K2 added for nutritional purposes in foods for particular nutritional uses, food supplements and foods intended for the general population and Vitamin K2 as a source of vitamin K added for nutritional purposes to foodstuffs, in the context of Regulation (EC) N° 258/97 [1]: Scientific Opinion of the Panel on Dietetic Products, Nutrition and Allergies, EFSA Journal, 6 (2008).
[12] N. Lal, A. Berenjian, Cis and trans isomers of the vitamin menaquinone-7: which one is biologically significant?, Applied microbiology and biotechnology, 104 (2020) 2765-2776.
[13] M. Kurosu, E. Begari, Vitamin K2 in electron transport system: are enzymes involved in vitamin K2 biosynthesis promising drug targets?, Molecules, 15 (2010) 1531-1553.
[14] C. Liao, H. Ayansola, Y. Ma, K. Ito, Y. Guo, B. Zhang, Advances in Enhanced Menaquinone-7 Production From Bacillus subtilis, Frontiers in Bioengineering and Biotechnology, 9 (2021).
[15] J. Song, H. Liu, L. Wang, J. Dai, Y. Liu, H. Liu, G. Zhao, P. Wang, Z. Zheng, Enhanced Production of Vitamin K2 from Bacillus subtilis (natto) by Mutation and Optimization of the Fermentation Medium, Brazilian Archives of Biology and Technology, 57 (2014) 606-612.

[16] M.K. Kong, P.C. Lee, Metabolic engineering of menaquinone-8 pathway of Escherichia coli as a microbial platform for vitamin K production, Biotechnology and bioengineering, 108 (2011) 1997-2002.
[17] T. Morishita, N. Tamura, T. Makino, S. Kudo, Production of Menaquinones by Lactic Acid Bacteria, Journal of Dairy Science, 82 (1999) 1897-1903.
[18] L. Ren, C. Peng, X. Hu, Y. Han, H. Huang, Microbial production of vitamin K2: current status and future prospects, Biotechnology advances, 39 (2020) 107453.
[19] M. Tarvainen, M. Fabritius, B. Yang, Determination of vitamin K composition of fermented food, Food Chemistry, 275 (2019) 515-522.
[20] C.A. Bøe, H. Holo, Engineering Lactococcus lactis for Increased Vitamin K2 Production, Frontiers in Bioengineering and Biotechnology, 8 (2020).
[21] E. Manoury, K. Jourdon, P. Boyaval, P. Fourcassié, Quantitative measurement of vitamin K2 (menaquinones) in various fermented dairy products using a reliable high-performance liquid chromatography method, Journal of dairy science, 96 (2013) 1335-1346.
[22] Y. Liu, E.O. Van Bennekom, Y. Zhang, T. Abee, E.J. Smid, Long-chain vitamin K2 production in Lactococcus lactis is influenced by temperature, carbon source, aeration and mode of energy metabolism, Microbial cell factories, 18 (2019) 1-14.
[23] C. Zhang, X. Chen, H.-P. Too, Microbial astaxanthin biosynthesis: recent achievements, challenges, and commercialization outlook, Applied Microbiology & Biotechnology, 104 (2020).
[24] G.P. Leone, R. Balducchi, S. Mehariya, M. Martino, V. Larocca, G. Di Sanzo, A. Iovine, P. Casella, T. Marino, D. Karatza, Selective Extraction of ω-3 fatty acids from Nannochloropsis sp. using supercritical CO2 extraction, Molecules, 24 (2019) 2406.
[25] J.M. Martínez, Z. Gojkovic, L. Ferro, M. Maza, I. Álvarez, J. Raso, C. Funk, Use of pulsed electric field permeabilization to extract astaxanthin from the Nordic microalga Haematococcus pluvialis, Bioresource technology, 289 (2019) 121694.
[26] M.-m. Luo, L.-j. Ren, S.-l. Chen, X.-j. Ji, H. Huang, Effect of media components and morphology of Bacillus natto on menaquinone-7 synthesis in submerged fermentation, Biotechnology and Bioprocess Engineering, 21 (2016) 777-786.
[27] Z. Fang, L. Wang, G. Zhao, H. Liu, H. Wei, H. Wang, W. Ni, Z. Zheng, P. Wang, A simple and efficient preparative procedure for menaquinone-7 from Bacillus subtilis (natto) using two-stage extraction followed by microporous resins, Process Biochemistry, 83 (2019) 183-188.
[28] H. Wei, G. Zhao, H. Liu, H. Wang, W. Ni, P. Wang, Z. Zheng, A simple and efficient method for the extraction and separation of menaquinone homologs from wet biomass of Flavobacterium, Bioprocess and biosystems engineering, 41 (2018) 107-113.
[29] F. Xie, S. Pei, X. Lin, Y. Tian, G. Zhang, A rapid and efficient method for the extraction and identification of menaquinones from Actinomycetes in wet biomass, BMC microbiology, 21 (2021) 1-10.
[30] M. Collins, T. Pirouz, M. Goodfellow, D. Minnikin, Distribution of menaquinones in actinomycetes and corynebacteria, Microbiology, 100 (1977) 221-230.
[31] D. Prat, A. Wells, J. Hayler, H. Sneddon, C.R. McElroy, S. Abou-Shehada, P.J. Dunn, CHEM21 selection guide of classical-and less classical-solvents, Green Chemistry, 18 (2015) 288-296.
[32] I.H.T. Guideline, Validation of analytical procedures: text and methodology, Q2 (R1), 1 (2005) 05.
[33] G. Andre, S. Kulakauskas, M.-P. Chapot-Chartier, B. Navet, M. Deghorain, E. Bernard, P. Hols, Y.F. Dufrêne, Imaging the nanoscale organization of peptidoglycan in living Lactococcus lactis cells, Nature communications, 1 (2010) 1-8.
[34] B. Martínez, A. Rodríguez, S. Kulakauskas, M.-P. Chapot-Chartier, Cell wall homeostasis in lactic acid bacteria: threats and defences, FEMS Microbiology Reviews, 44 (2020) 538-564.

[35] R. Baker, R. Kramer, Cytotoxicity of short-chain alcohols, Annual review of pharmacology and toxicology, 39 (1999) 127-150.
[36] J.T.d. Faria, P.F. Rocha, A. Converti, F.M. Passos, L.A. Minim, F.C. Sampaio, Statistical investigation of Kluyveromyces lactis cells permeabilization with ethanol by response surface methodology, Brazilian Journal of Microbiology, 44 (2013) 1067-1074.
[37] C.U. Mussagy, V.C. Santos-Ebinuma, K.A. Kurnia, A.C. Dias, P. Carvalho, J.A. Coutinho, J.F. Pereira, Integrative platform for the selective recovery of intracellular carotenoids and lipids from Rhodotorula glutinis CCT-2186 yeast using mixtures of bio-based solvents, Green Chemistry, 22 (2020) 8478-8494.
[38] Ö. Üstün-Aytekin, S. Arısoy, A.Ö. Aytekin, E. Yıldız, Statistical optimization of cell disruption techniques for releasing intracellular X-prolyl dipeptidyl aminopeptidase from Lactococcus lactis spp. lactis, Ultrasonics sonochemistry, 29 (2016) 163-171.
[39] J.-H. Pan, C.-Y. Peng, C.-T. Lo, C.-Y. Dai, C.-L. Wang, H.-Y. Chuang, n-Hexane intoxication in a Chinese medicine pharmaceutical plant: a case report, Journal of medical case reports, 11 (2017) 1-7.
[40] J.D. Wang, Y.C. Chang, K.P. Kao, C.C. Huang, C.C. Lin, W.Y. Yeh, An outbreak of n-hexane induced polyneuropathy among press proofing workers in Taipei, American journal of industrial medicine, 10 (1986) 111-118.
[41] A. Głuszcz-Zielińska, Occupational N-hexane neuropathy: clinical and neurophysiological investigation, Medycyna pracy, 50 (1999) 31-36.
[42] C.M. Alder, J.D. Hayler, R.K. Henderson, A.M. Redman, L. Shukla, L.E. Shuster, H.F. Sneddon, Updating and further expanding GSK's solvent sustainability guide, Green Chemistry, 18 (2016) 3879-3890.
[43] C. Capello, U. Fischer, K. Hungerbühler, What is a green solvent? A comprehensive framework for the environmental assessment of solvents, Green Chemistry, 9 (2007) 927-934.
[44] S. Cao, X. Du, P. Li, G. Yuan, S. Chen, W. Chen, X. Song, B. Kuang, A chemical screening method for menaquinone-producing strains based on HPLC-UV technology, Journal of microbiological methods, 172 (2020) 105907.
[45] G. Cebrián, S. Condón, P. Mañas, Physiology of the inactivation of vegetative bacteria by thermal treatments: mode of action, influence of environmental factors and inactivation kinetics, Foods, 6 (2017) 107.
[46] P. Teixeira, H. Castro, C. Mohácsi-Farkas, R. Kirby, Identification of sites of injury in Lactobacillus bulgaricus during heat stress, Journal of Applied Microbiology, 83 (1997) 219-226.
[47] J. Benfica, J.l.S. Miranda, E.S. Morais, M.G. Freire, J.o.A. Coutinho, R. de Cássia Superbi de Sousa, Enhanced Extraction of Levodopa from Mucuna Pruriens Seeds Using Aqueous Solutions of Eutectic Solvents, ACS Sustainable Chemistry & Engineering, 8 (2020) 6682-6689.
[48] M.B. Jensen, P.L. Švarc, J. Jakobsen, Vitamin K (phylloquinone and menaquinones) in foods–Optimisation of extraction, clean-up and LC–ESI-MS/MS method for quantification, Food Chemistry, 345 (2021) 128835.
[49] S. Tsubaki, A. Onda, M. Hiraoka, S. Fujii, J.-i. Azuma, Y. Wada, Chapter 7 - Microwave-Assisted Water Extraction of Carbohydrates From Unutilized Biomass, in: H. Dominguez González, M.J. González Muñoz (Eds.) Water Extraction of Bioactive Compounds, Elsevier, 2017, pp. 199-219.
[50] P.C. Veggi, J. Martinez, M.A.A. Meireles, Fundamentals of microwave extraction, in: Microwave-assisted extraction for bioactive compounds, Springer, 2012, pp. 15-52.
[51] V. Mandal, Y. Mohan, S. Hemalatha, Microwave assisted extraction—an innovative and promising extraction tool for medicinal plant research, Pharmacognosy reviews, 1 (2007) 7-18.

[52] A. Liazid, M. Palma, J. Brigui, C.G. Barroso, Investigation on phenolic compounds stability during microwave-assisted extraction, Journal of Chromatography A, 1140 (2007) 29-34.
[53] I. Aprodu, Ș.A. Milea, E. Enachi, G. Râpeanu, G.E. Bahrim, N. Stănciuc, Thermal Degradation Kinetics of Anthocyanins Extracted from Purple Maize Flour Extract and the Effect of Heating on Selected Biological Functionality, Foods, 9 (2020) 1593.
[54] H.-F. Zhang, X.-H. Yang, Y. Wang, Microwave assisted extraction of secondary metabolites from plants: Current status and future directions, Trends in Food Science & Technology, 22 (2011) 672-688.
[55] R. Ciriminna, D. Carnaroglio, R. Delisi, S. Arvati, A. Tamburino, M. Pagliaro, Industrial feasibility of natural products extraction with microwave technology, ChemistrySelect, 1 (2016) 549-555.
[56] Y. Li, M. Radoiu, A.-S. Fabiano-Tixier, F. Chemat, From laboratory to industry: scale-up, quality, and safety consideration for microwave-assisted extraction, in: Microwave-assisted extraction for bioactive compounds, Springer, 2012, pp. 207-229.
[57] H. Wei, L. Wang, G. Zhao, Z. Fang, H. Wu, P. Wang, Z. Zheng, Extraction, purification and identification of menaquinones from Flavobacterium meningosepticum fermentation medium, Process Biochemistry, 66 (2018) 245-253.

Table 1. Predicted and experimental value of response at the optimised conditions for integrated extraction from wet biomass of *L. lactis* using ethanol.

Optimum conditions	Coded level	Actual level
Extraction temperature (°C)	+ 1	75
Biomass concentration (g mL^{-1})	– 0.001	0.199
Treatment time (min)	– 0.1598	36.804
Response	Predicted value	Experimental value
Yield MK-7 (µg g^{-1}DCW)	6.45	6.73 ± 0.17
Yield MK-8 (µg g^{-1}DCW)	182.8	179.6 ± 3.59

Figure captions

Figure 1. Extraction yield of long-chain menaquinones from **(A)** wet biomass of *L. lactis* using integrated and two-step cell lysis and extraction approaches; and **(B)** freeze-dried and ground cells using solvent extraction technique. Error bars indicate standard deviations.

Figure 2. 3D surface plots representing the yields of MK-7 (A-C-E) and MK-8 (B-D-F), respectively, with the combined effects of the extraction temperature (°C), biomass concentration (g mL^{-1}) and extraction time (min) in the optimisation study.

Figure 3. Extraction yield of long-chain menaquinones from wet biomass of *L. lactis* based on ethanol as the extraction solvent coupled with **(A)** microwave heating at different temperature and heating duration; and **(B)** bead-beating using different lysing matrices and bead-beating cycles; and **(C)** relative thermal stability of pure MK-7, MK-8 and MK-9 in ethanol subjected to different heating temperature and duration.

Figure 4. Comparison of extraction strategies developed in this work based on ethanol as the extraction solvent combined with the use of external heating, microwave heating and beat-beating, respectively, for the recovery of long-chain menaquinones from wet biomass of *L. lactis*.

Figure 5. A diagram of the processes developed for the production of long-chain menaquinones from *L. lactis* biomass. Dashed lines in the flow diagram represent the steps that were not performed experimentally in this work.

Figure 1A.

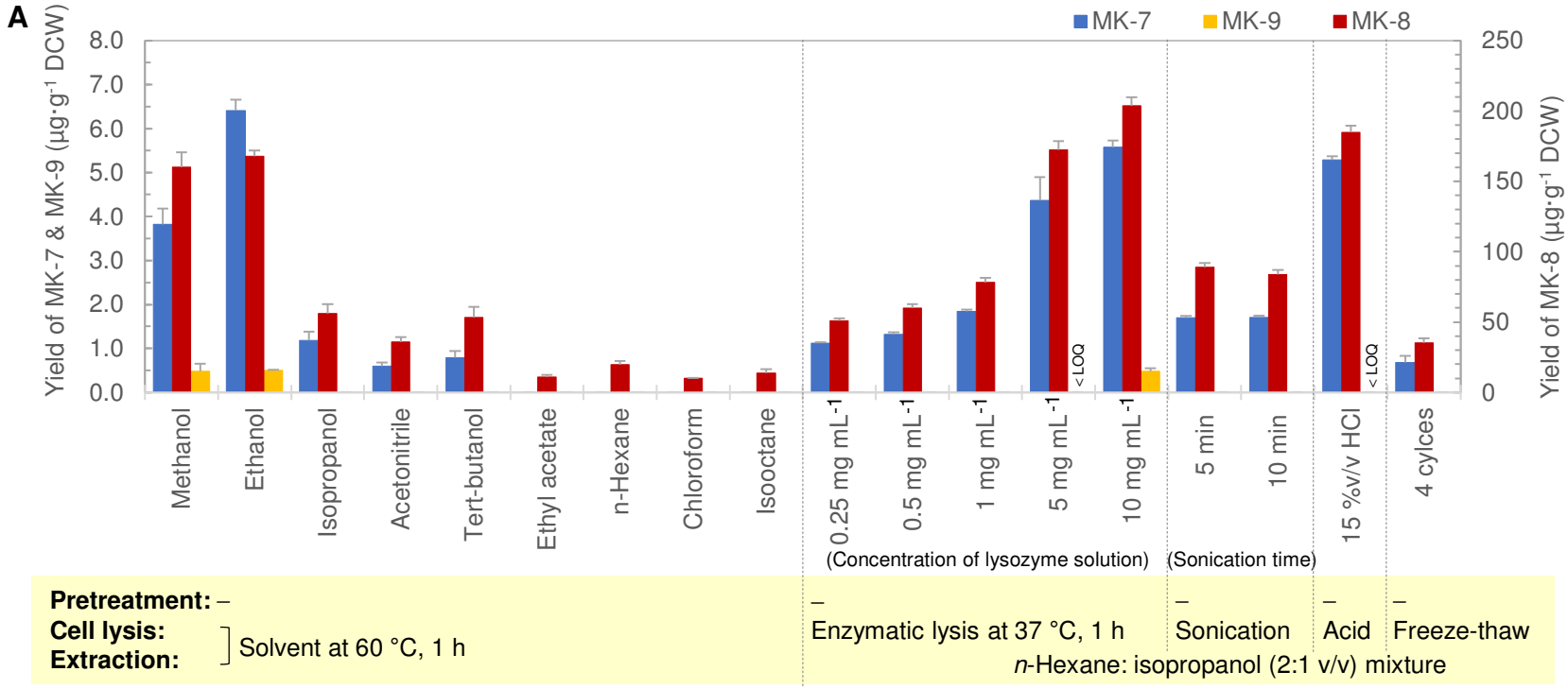

A
MK-7
MK-9
MK-8
Yield of MK-7 & MK-9 (µg·g⁻¹ DCW)
Yield of MK-8 (µg·g⁻¹ DCW)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
250
200
150
100
50
0
< LOQ
< LOQ
Methanol
Ethanol
Isopropanol
Acetonitrile
Tert-butanol
Ethyl acetate
n-Hexane
Chloroform
Isooctane
0.25 mg mL-1
0.5 mg mL-1
1 mg mL-1
5 mg mL-1
10 mg mL-1
5 min
10 min
15 %v/v HCl
4 cylces
(Concentration of lysozyme solution)
(Sonication time)
Pretreatment: –
Cell lysis:
Extraction:
Solvent at 60 °C, 1 h
–
Enzymatic lysis at 37 °C, 1 h
–
Sonication
–
Acid
–
Freeze-thaw
n-Hexane: isopropanol (2:1 v/v) mixture

Figure 1B.

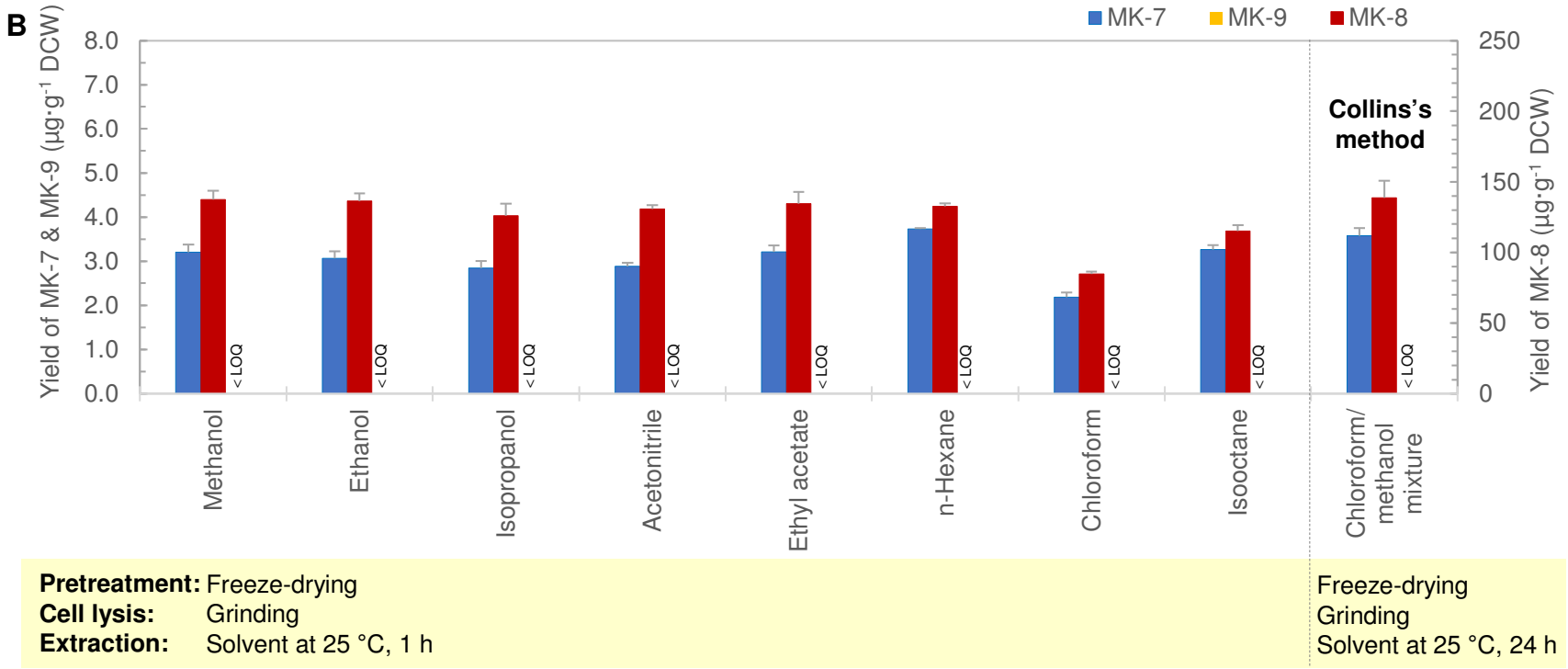

B
MK-7
MK-9
MK-8
Yield of MK-7 & MK-9 (µg·g-1 DCW)
Yield of MK-8 (µg·g-1 DCW)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
250
200
150
100
50
0
< LOQ
Collins's method
Methanol
Ethanol
Isopropanol
Acetonitrile
Ethyl acetate
n-Hexane
Chloroform
Isooctane
Chloroform/ methanol mixture
Pretreatment: Freeze-drying
Cell lysis: Grinding
Extraction: Solvent at 25 °C, 1 h
Freeze-drying
Grinding
Solvent at 25 °C, 24 h

Figure 2.

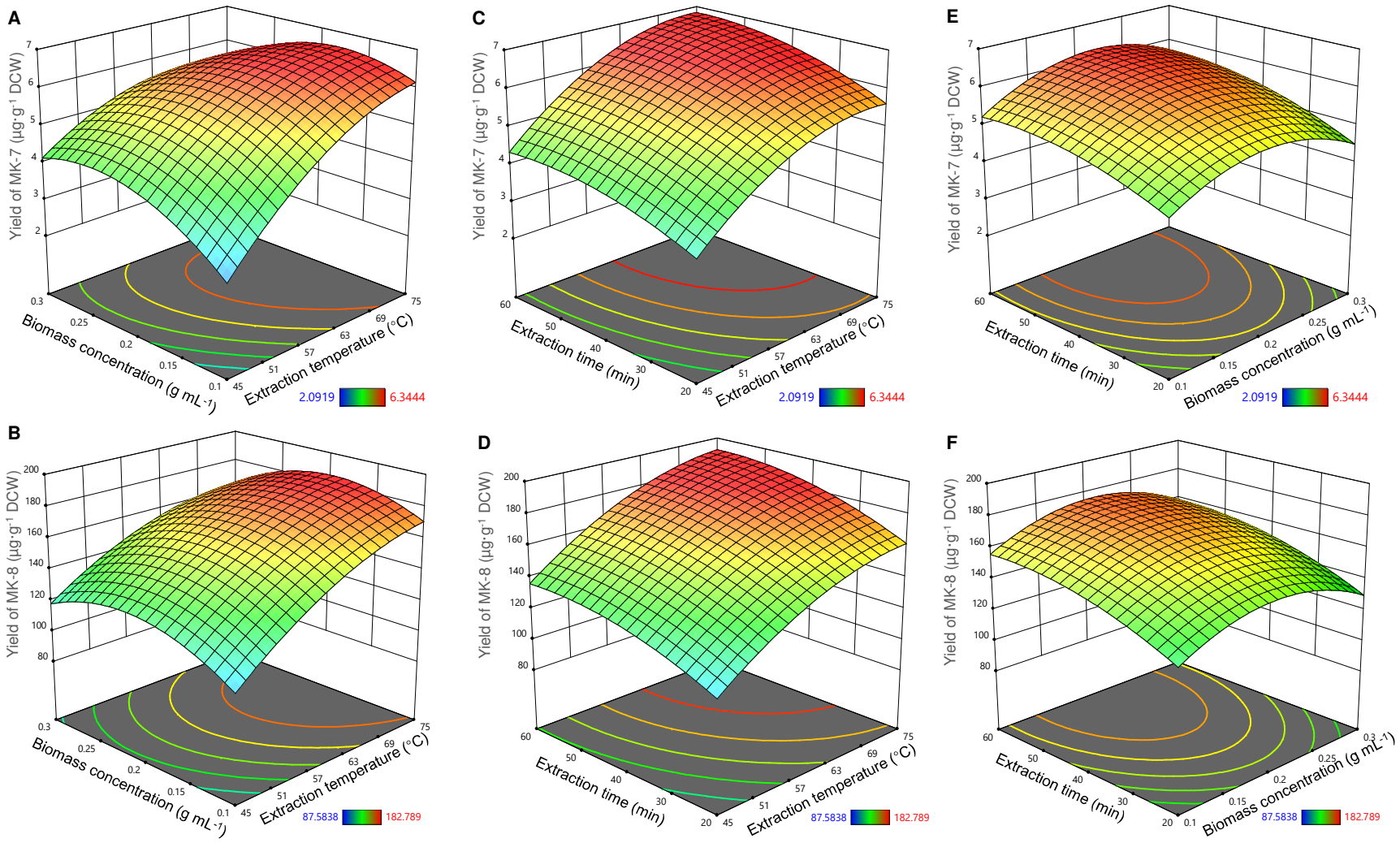

A
Yield of MK-7 (μg·g⁻¹ DCW)
Biomass concentration (g mL⁻¹)
Extraction temperature (°C)
2.0919
6.3444
C
Yield of MK-7 (μg·g⁻¹ DCW)
Extraction time (min)
Extraction temperature (°C)
2.0919
6.3444
E
Yield of MK-7 (μg·g⁻¹ DCW)
Extraction time (min)
Biomass concentration (g mL⁻¹)
2.0919
6.3444
B
Yield of MK-8 (μg·g⁻¹ DCW)
Biomass concentration (g mL⁻¹)
Extraction temperature (°C)
87.5838
182.789
D
Yield of MK-8 (μg·g⁻¹ DCW)
Extraction time (min)
Extraction temperature (°C)
87.5838
182.789
F
Yield of MK-8 (μg·g⁻¹ DCW)
Extraction time (min)
Biomass concentration (g mL⁻¹)
87.5838
182.789

Figure 3.

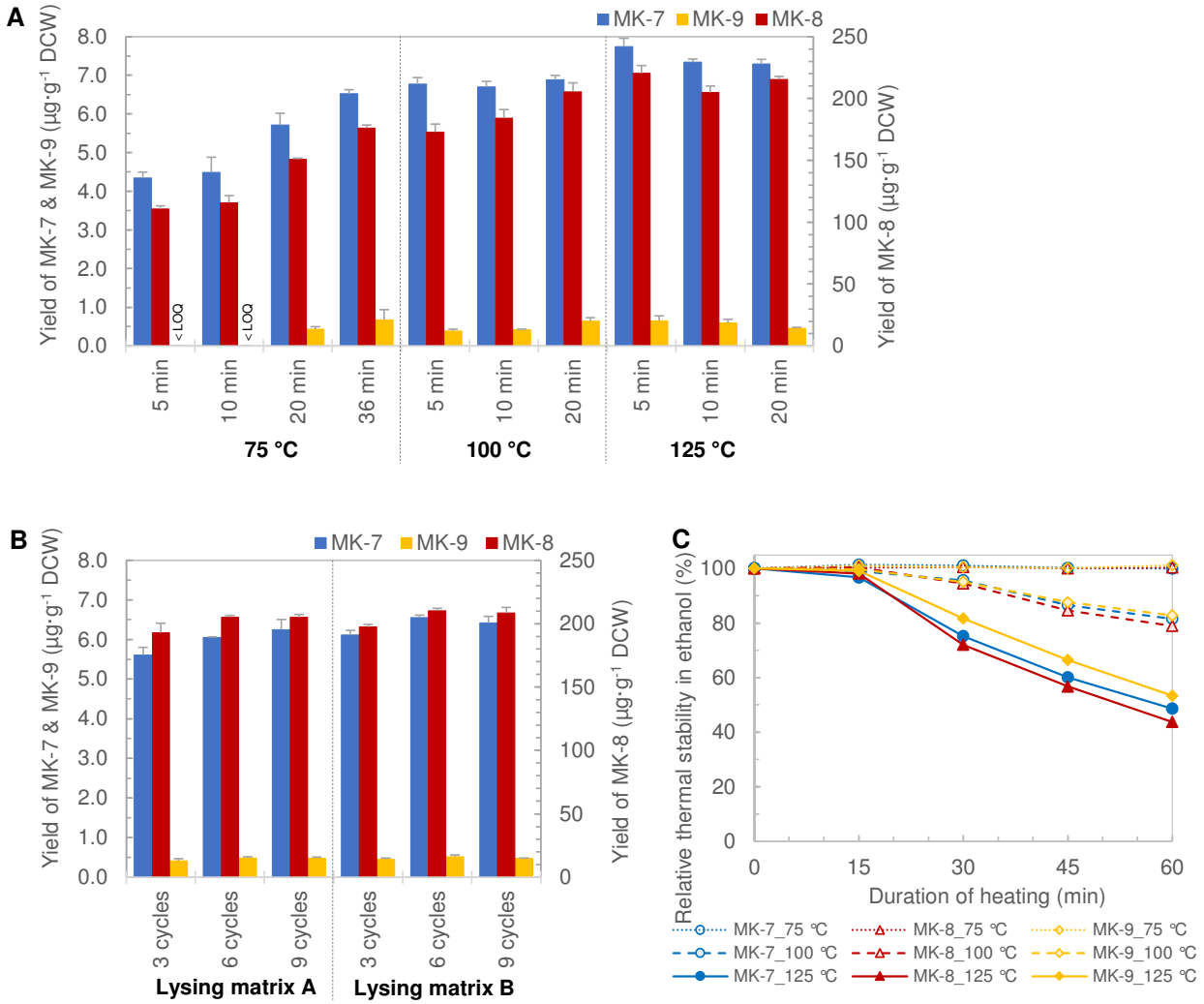

A
MK-7
MK-9
MK-8
Yield of MK-7 & MK-9 (μg·g-1 DCW)
Yield of MK-8 (μg·g-1 DCW)
< LOQ
< LOQ
5 min
10 min
20 min
36 min
5 min
10 min
20 min
5 min
10 min
20 min
75 °C
100 °C
125 °C
B
MK-7
MK-9
MK-8
Yield of MK-7 & MK-9 (μg·g-1 DCW)
Yield of MK-8 (μg·g-1 DCW)
3 cycles
6 cycles
9 cycles
3 cycles
6 cycles
9 cycles
Lysing matrix A
Lysing matrix B
C
Relative thermal stability in ethanol (%)
Duration of heating (min)
MK-7_75 ℃
MK-8_75 ℃
MK-9_75 ℃
MK-7_100 ℃
MK-8_100 ℃
MK-9_100 ℃
MK-7_125 ℃
MK-8_125 ℃
MK-9_125 ℃

Figure 4.

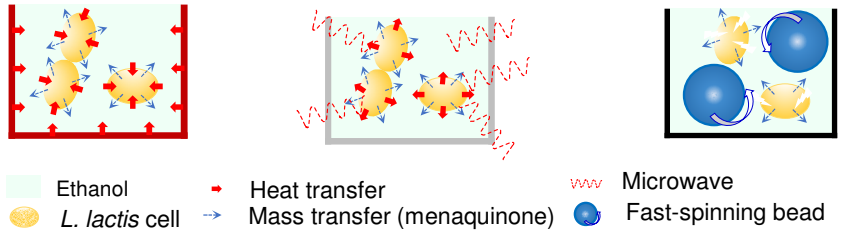
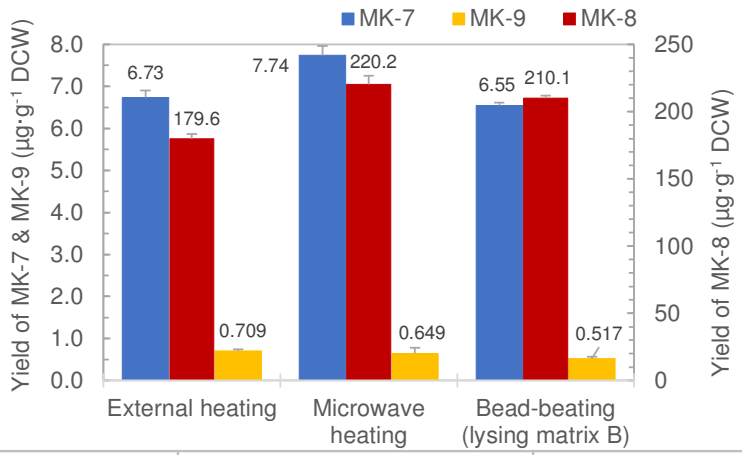
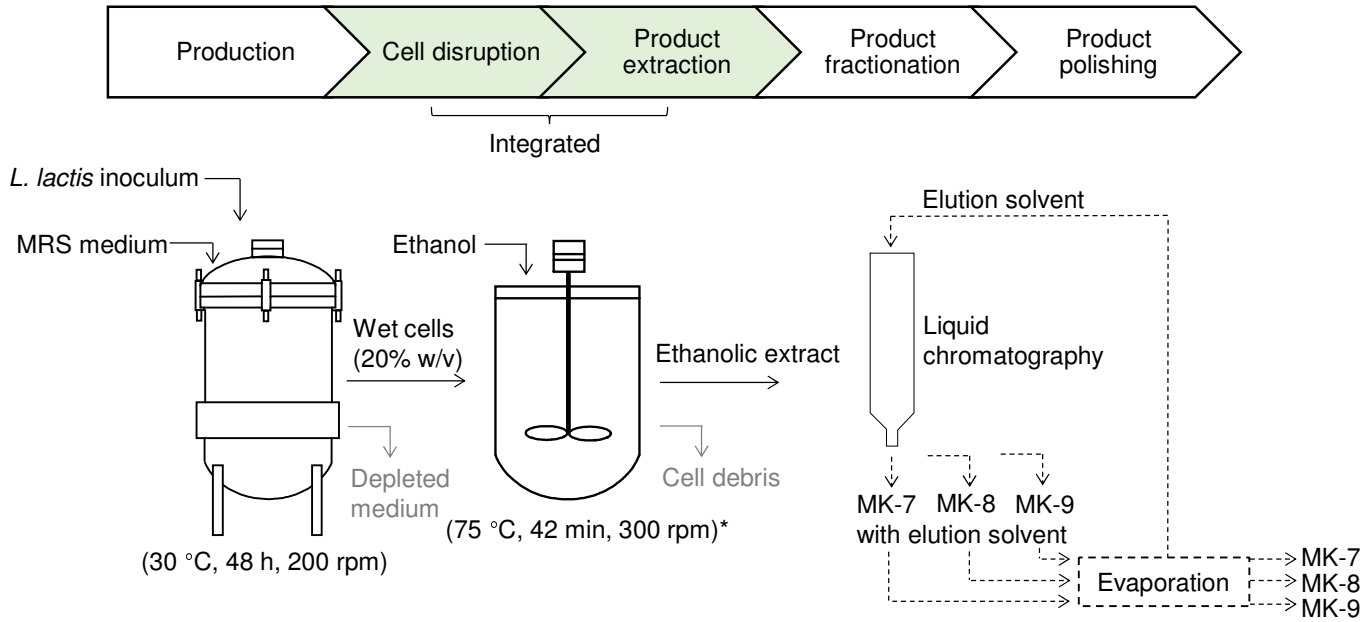
Extraction technique	Ethanol as the extraction solvent coupled with		
	External heating	Microwave heating	Bead-beating
Working principle			
Maximum extraction yield			
	Low	High	Moderate
Extraction temperature	75 °C	125 °C	Not controlled.
Processing time	Preheat: 5 min Operation: 36.8 min Total: 42 min	Preheat: 5 min Operation: 5 min Cooling: 12 min (cool to 75 °C) Total: 22 min	Operation: 6 × 30 s = 3 min Pulse: 6 × 300 s = 30 min Total: 33 min (6 cycles)
Processing volume	Easy to scale up/down	6 – 50 mL filling volume per vessel (based on the equipment' specification)	2 – 50 mL impact-resistant tube (based on the equipment' specification)
Cost	Low	High equipment cost	Moderate equipment cost
Benefit	▪ Simple and easy to scale-up	▪ High product yield ▪ Rapid process	▪ High product yield ▪ Moderate process time
Weakness	▪ Moderate product yield ▪ Slow process	▪ Expensive equipment ▪ Technically complex to scale-up ▪ Pressurized vessel needs safety device to be in place	▪ Difficult to scale-up

Figure 5.



*Can be replaced with the MAE.