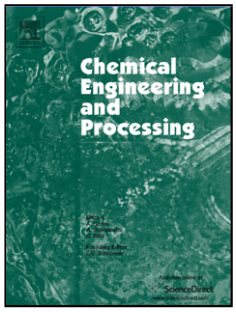

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Effects of a combined processing technology involving ultrasound and surfactant on the metabolic synthesis of vitamin K_2 by *Flavobacterium* sp. M1-14

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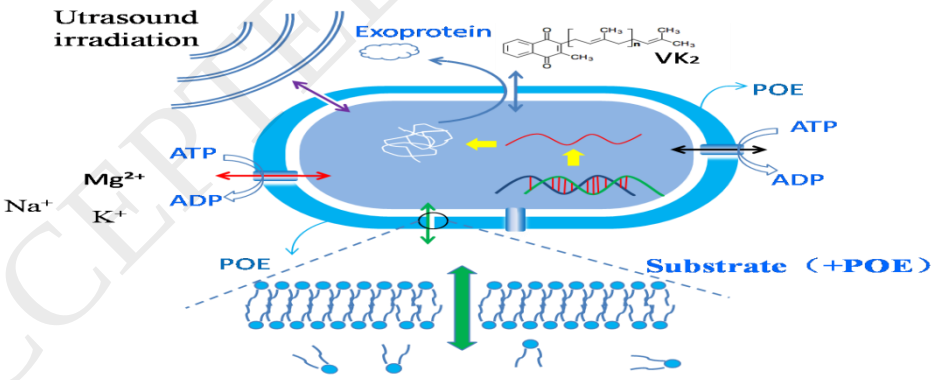
Graphical abstract



Highlights

- Ultrasound and surfactant significantly increased extracellular VK_2 production up to 30.03±1.42 mg/L.

- Promoted cell esterase activity and membrane permeability.
- Ultrasound can reduce damage to the cell membrane induced by POE alone.

Abstract:

Vitamin K_2 (VK_2) is a microbial product that has several applications in the pharmaceutical sector, and its yield is very important for commercial processes. This study analyzed the effects of a single surfactant, with or without ultrasound treatment, on the metabolic synthesis of VK_2 by *Flavobacterium* sp. M1-14. The surfactant combined with ultrasound altered the membrane permeability of *Flavobacterium*sp.M1-14, as shown by scanning electron microscopy and flow cytometry (FCM). The optimum conditions for VK_2 production were as follows: amplitude 80% of total power (130W), sonication time of 40 s, and continuous repetitive ultrasound treatment repeated three times, and the yield of extracellular VK_2 reached 30 mg/L when 1% polyoxyethyleneoleyl (POE)was added to the medium. Scanning electron microscopy studies revealed that the mechanism by which the surfactant combined with ultrasound altered the membrane permeability involved both cell membrane pore formation through stable cavitationand solubilization of the membrane. Based on the double-staining results, we concluded that combined ultrasound with surfactant treatment could promote esterase activity, enhance cell membrane permeability, and reduce the damaging effects of surfactant alone applied to the cells.

Key words: Ultrasound; Surfactant; Flow cytometry; Transmission electron microscopy; Vitamin K_2

1. Introduction

Vitamin K is fat soluble and occurs naturally in two forms, vitaminK_1 and vitaminK_2 (VK_2). VitaminK_1 is the major dietary source of vitamin K and naturally exists in green plants, algae, and photosynthetic bacteria, whereas VK_2 is found in chicken egg yolk, butter, cow liver, certain cheeses, and fermented soybeans[1,2]. Epidemiological studies conducted in Japan and Europe suggested an association between poor VK_2 (but not vitamin K_1) intake and increased postmenopausal bone loss, arterial calcification (notably in diabetic patients), end-stage renal diseases, and cardiovascular diseases during normal aging, as well as an increased risk of bone fracture[3,4]. Bacterial fermentation is an economical and practical method for the production of VK_2, and many studies have mostly focused on *Flavobacterium* spp. and *Bacillus subtilis*[5,6].

In previous studies, surfactants have been added to the culture to improve production, and low concentrations of surfactants have been reported to increase membrane permeability and thus extracellular productivity by removing the restriction of intracellular diffusion of target metabolites. For example, Kim et al. demonstrated that addition of 1.0% Tween-80 remarkably increased the production of 12,13,17-THOA by *Bacillus megaterium*ALA2 in a time-dependent manner, and the production peaked at 48 h after the addition of substrate and Tween-80. The maximum 12,13,17-THOA

production was about 6.4 times higher than that noted in the control, representing almost the same productivity as that in flask under optimized culture conditions[7]. Recently, ultrasound has received increasing attention in biology for both fundamental studies and potential applications[8,9]. The use of low-intensity ultrasound as a method to improve microbial productivity by enhancing membrane permeation (aphonophoretic effect) on cells has been widely reported, and low-frequency ultrasonic irradiation has been reported to accelerate substance exchange inside and outside the cell[10]. As ultrasound may cause damage to the cell and disrupt the cell membrane, all kinds of molecules are therefore easily transported through the cell membrane[11,12]. Dai et al. found that low-frequency ultrasound resulted in a 5-fold increase in the production of riboflavin by *Ecemothecium ashbyii* during submerged fermentation, when compared with that observed in the control[13]. Furthermore, Ju et al. reported similar enhanced production of gentamicin by *Micromonospora* spp. following ultrasonication at 25 kHz, which minimized the mass transfer resistance of the compact cell wall of *Micromonospora* spp. and increased gentamicin production from 38.3% to 75.8%[14]. Similarly, the production of fibrinolytic enzyme by *Bacillus sphaericus* in submerged fermentation was increased from 110 U/mL to 201 U/mL by ultrasonic stimulation[8]. In addition , Liu et al. developed an efficient ultrasound-stimulation strategy for improving the hairy root growth and caffeic acid derivatives (CADs) biosynthesis in hairy root cultures of *Echinacea purpurea*[15].

However, to date, the simultaneous use of surfactants and ultrasound during

microbial fermentation process has not yet been reported, except in the study by Sindhu et al., who found that surfactant-assisted ultrasound pretreatment of sugarcane tops improved enzymatic release of sugars[16]. The present study was conducted to develop an efficient and optimized ultrasound treatment strategy along with polyoxyethy-leneoleyl(POE) addition for enhanced biosynthesis of VK_2 by *Flavobacterium* sp. M1-14. The fermentation process parameters associated with ultrasonication, such as ultrasound power, irradiation duration, were examined and optimized with 1% POE in the culture medium. Scanning electron microscopy was performed to understand the structural changes in the bacterial cell morphology after application of ultrasound treatment and POE. In addition, FCM combined with a double-staining method was used to estimate the esterase activity and membrane permeability of the cells.

2.Methods

2.1 Microorganism and cultivation method

Flavobacterium sp. M1-14 (Gram-negative bacterial cells) was preserved in our laboratory(Institute of Technical Biology and Agriculture Engineering, Key Laboratory of Ion Beam Bioengineering, Hefei). The strain was inoculated onto beef extract-peptone agar slants (3 g/L beef extract, 10 g/L peptone, 5 g/L NaCl, and 20 g/L agar; pH 7) at 37 °C for 24 h then stored at 4 °C. Subsequently, the strain on the agar slants was inoculated into fresh seed medium (20 g/L glycerol, 33 g/L peptone, 3 g/L $K_2HPO_4{\cdot}7H_2O$, 4.5 g/L NaCl, 0.3 g/L $MgSO_4{\cdot}7H_2O$, and 1.5 g/L yeast extract; pH 7.2) in 250-mL Erlenmeyer flask with a working volume of 50 mL and incubated at 22 °C

and 300 rpm for 24 h. Then, a 10-mL aliquot of pre-inoculum was transferred to a 500-mL shake flask containing 100 mL of fermentation medium (20 g/L glycerol, 33 g/L peptone, 3 g/L $K_2HPO_4{\cdot}7H_2O$, 4.5 g/L NaCl, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 1.5 g/L yeast extract, and 10 g/L dry powder of corn plasm; pH 7.2) and incubated at 37 °C and 250 rpm for 9 days. After the 9 day fermentation, biomass accumulation was measured and estimated as dry cell weight (DCW). In brief, 25 mL of the fermentation broth were collected and centrifuged at 15,000 rpm for 15 min and the supernatant was discarded. The wet cells were washed with sterile distilled water and freeze-dried to a constant weight.

2.2 Screening of surfactants and optimization of surfactant concentration

To investigate the influence of surfactants on the formation of extracellular VK_2 metabolites and to screen the optimal surfactant, 0.5% of surfactant, including the anionic surfactant sodium dodecyl benzene sulfonate (SDBS), Triton-100 and POE, and nonionic surfactant Tween-80, were respectively added to the fermentation medium. Subsequently, the concentration of the optimal surfactant was optimized.

2.3 Ultrasound treatment

The fermentation broth was treated in an ultrasonic crushing device (Model VCX130, NEWTOWN, CT, USA) operating at a frequency of 20 kHz and a maximum rated power input of 130W(Fig. 1). The output power of the generator could be selected from 20% to 100%, and the ultrasonic probe was inserted 1.5 cm below the fermentation broth surface. To maintain the temperature of the fermentation broth, the

flask was placed in a glass containing 200 mL of cold water. The ultrasound probe was previously sanitized with 75% ethanoland subsequently exposed to ultraviolet light for 15 min. Ultrasonic devices were operated in a bioclean bench to ensure an aseptic environment. For the course of the experiment, different ultrasonic powers (from 20% to 100%) and durations of ultrasound (from 20 s to 100 s) were applied and their effects were investigated. All the process parameters were optimized by single-factor experimental design by varying a single factor at a time and keeping the other factors constant[8]. Based on these single factors, repetitive ultrasound treatment was applied.

2.4 Extraction and measurementof VK2

A total of 25 mL of the fermentation broth were pipetted out from the 500-mL shake flasks and centrifuged at 15,000 rpm for 15 min. Then, the supernatant was transferred to another tube, and 10 mL of *n*-butanol were added to it and shaken in a shock bed (HZ-2211K,China) at 230 rpm for 5 h. Subsequently, the supernatant of the organic reagent was collected and the VK_2 content was analyzed by HPLC (Shimadzu Essentia LC-6,Japan) using a Shim-pack VP-ODS reversed-phase column (250 L × 4.6 mm, Shimadzu) with detection at 248 nm (typical UV absorption of VK_2) and flow rate of 1 mL/min with a mobile phase of methanol with 20% (v/v) dichloromethane.

2.5. Determination of surface tension and extracellular protein

Modified Wilhelmy hanging plate method (Sigma 703,Finland) was used for the measurement of surface tension of the fermentation broth. Detailed experimental operation steps can be found in the instruction manual. The extracellular protein was

detected by using Modified Lowry Protein Assay Kit (Sangon Biotech,China) according to the manufacturer's instructions.

2.6 Scanning electron microscopy

After 9 days of fermentation, the cells were centrifuged, washed twice with sterile water, placed on coverslips, and dried naturally. Then, the cells were diluted with 2.5% glutaraldehyde, fixed for 2-4 h at 4°C, washed thrice with phosphate buffer, and subjected to gradient ethanol dehydration (30%, 50%, 70%, 90% ethanol once, and 100% ethanol twice, each for 15 min. After dehydration, the cells were metal-sprayedand observed under scanning electron microscope (FEI-Sirion 200 field emission scanning electron microscope,USA).

2.7 Staining procedure and flow cytometric measurement

Application of double staining with FDA (Calbiochem,Darmstadt, Germany) and PI (Sigma Aldrich, St Louis,MO, USA) was used for FCM analysis. Nonirradiated stained cells and cells treated at 85 °C for 15 min and subsequently stained with FDA or PI, respectively, were used as controls to define negative and positive histogram regions. Moreover, a nonstained, nontreated control was employed to determine the auto-fluorescence of cells. Control, POE treated or ultrasound+ POE treated cells were initially incubated with 50 μM FDA at 37 °C for 15 min to allow intracellular enzymatic conversion of cFDA to carboxyfluorescein (cF). After this labeling, the cells were centrifuged (7880 g, 5 min, 10 °C) and resuspended in 1 mL PBS buffer (pH 7.0). Then 30 μM PI in an ice bath were added, and the mixture was incubated for 10 min in the

dark to allow the labeling of membrane compromised cells. Following incubation with PI, samples were placed on ice in the dark until analysis (maximum of 1 h).

Analysis of microbial cells was performed on a flow cytometer (BD FACSCalibur, BD Biosciences, USA). Scatter and fluorescence signals of individual cells passing through the laser zone were collected as logarithmic signals. Green fluorescence of cells stained with FDA was collected in the FL1 channel (525±15 nm), whereas red fluorescence of cells labeled with PI was collected in the FL2 channel (620±15 nm). Flow rates and cell concentrations of the samples were adjusted to maintain acquisition at 200 microorganism cells per second. A total of 20,000 events were registered. Trials were replicated at least three times with three samples for each short wave ultraviolet dose. The data were analyzed using FlowJo version vX.0.7 (TreeStar Inc., Ashland, OR, USA).

2.8 Data analysis

Three independent cultures were run and analyzed for each set of experiments. All data were expressed as mean ± SD, statistical analysis was performed using Microsoft Excel 2007, and a p value of<0.05 was considered statistically significant.

3.Results and discussion

3.1 Screening of surfactants and optimization of surfactant concentration

Surfactants that alter cell membrane permeability and increase the target product during fermentation have previously been reported. For example, Reddy and Reddy [17] reported that adding the surfactants Triton X-100, CHAPS, Tween-80, and sodium

taurocholate to *Clostridium thermosulfurogenes* SV2 culture individually resulted in a marked increase in the yields of thermostable *β*-amylase and pullulanase. In this study, different types and concentrations of surfactants were optimized initially, and the results are shown in Fig.2. 0.5% POE exerted the best effect on VK_2 leakage among the different types of surfactant. After optimizing the concentration of POE, 1% POE was chosen, which yielded 15.95±0.4 mg/L extracellular VK_2. The dry biomass reached its highest value of 12.988±0.64 g/L when 1% POE was added to the culture, which implied that the surfactant could significantly improve the biomass during the fermentation process ($p < 0.05$). We can conclude that an appropriate surfactant accelerates the growth of the microorganism and improves the target product yield.

3.2 Effect of ultrasound treatment on the growth of Flavobacterium sp. M1-14

In general, Gram-positive bacteria are more resistant to ultrasound compared to Gram-negative bacteria, possibly because Gram-positive bacterial cells possess a thick and more robust cell wall due to the cross-linking of peptidoglycan and teichoic acid[18]. Ultrasound may have positive or negative effects on bacterial cell performance depending on the level of sonoporation. Sonoporation can be defined as the formation of transient cavities or pores in the cell membrane due to sonication[19]. Next, we explored the effect of ultrasound on *Flavobacterium* sp. M1-14, which is affected by various ultrasound parameters (e.g. sonication time and ultrasound power).

The ultrasonic power supply converts 50/60 Hz line voltage to high frequency electrical energy(Fig.1). This high frequency electrical energy is transmitted to the

piezoelectric transducer within the converter, where it is changed to mechanical vibrations. The vibrations from the converter are intensified by the probe, creating pressure waves in the liquid. This action forms millions of microscopic bubbles (cavities), which expand during the negative pressure excursion, and implode violently during the positive excursion. As the bubbles implode, they cause millions of shock waves and eddies to radiate outwardly from the site of collapse, and generate extremes of pressures and temperatures at the implosion sites. Although this phenomenon, known as cavitation, lasts but a few microseconds, and the amount of energy released by each individual bubble is minimal, the cumulative amount of energy generated is extremely high. The sudden and violent collapse of huge numbers of microbubbles generates powerful hydromechanical shear forces in the bulk liquid surrounding the bubbles[20]. The collapsing bubbles disrupt adjacent bacterial cells by extreme shear forces, rupturing the cell walls and membranes.

Cavitation results in localized high temperatures and pressures in the fermentation broth. At high temperatures, lipids in the cytoplasmic membrane decompose, resulting in holes within the membrane, through which intracellular materials leak to the aqueous phase[21]. Ultrasonic intensity(UI=P/A; P= power input (kW); A = surface area of the probe in cm^2) is defined as the power supplied to the fermentation broth per unit of converter area and is expressed as kW/cm^2. Ultrasonic intensity therefore reflects the power-generating capacity of the converter. The higher the amplitude, the higher is the ultrasonic intensity that the system will be able to produce[22,23]. The ultrasonic dose

(UD=PT/V，J/L; T= sonication time(s); V= volume of fermentation broth (L)) relates to the energy supplied per sample volume unit[24].

The effect of sonication time (20-100s) on *Flavobacterium* sp. M1-14 is shown in Fig.3A. In this ultrasonic time zone, the amount of extracellular production of VK_2 increased first and then decreased gradually with increasing sonication time. The yield of extracellular VK_2 reached a maximum of 19.47±0.76 mg/L under the ultrasonic conditions of 20 kHz and 130 W for 40 s (on for 3 s and off for 4 s). The biomass concentration slightly increased at short sonication times and attained its maximum of 13.94±1.96 g/L at 40 s. Longer ultrasonication times did not enhance the accumulation of biomass. Pitt and Ross[25] proposed that higher degrees of irreversible sonoporation can lead to leakage of cellular contents because of physical disruption and/or alternation of the cell membrane lipid bilayer causing lipid peroxidation and eventually leading to cell death. From the above formula UD=PT/V, it can be seen that under constant P and V conditions, UD is proportional to T, so the ultrasound dose increases with increasing ultrasonication time. From this experimental result and previous work, we know that the dose of ultrasound should be controlled during the experiment to prevent the accumulation of bacteria.

The effect of percentage of ultrasonic power (130 W, from 20% to 100%) on *Flavobacterium* sp. M1-14 is plotted in Fig.3B.The yield of extracellular VK_2 increased with the increasing percentage of ultrasonic power when the ultrasonic power was below 80%, and the most effective percentage of ultrasonic power was 80%. The yield

of extracellular VK_2 reached 24.88±2.56 mg/L (55.99% higher than that noted in the control) under the ultrasonic conditions of 20 kHz for 40 s (on for 3 s and off for 4 s). However, a further increase in the ultrasonic power had no significant effect on the yield of extracellular VK_2. The DCW under optimal conditions reached 14.66±0.25 g/L, which was not significantly higher than that of the control. This result was different from the phenomenon observed by Dai et al. [26], in which the biomass of *Saccharomyces cerevisiae* increased by 127.03% under optimum ultrasonic conditions, such as frequency 28 kHz, power 140 W/L, and ultrasonic time 1 h, when it was cultured to the lag phase. Therefore, the biomass in this study is not considered.

Similarly, as can be seen from the above formula UI=P/A, under conditions where A is constant, UI is proportional to P, so the intensity of ultrasound increases as the ultrasonic power increases. In the above experiment, with the increase in ultrasonic power, more microbubbles will be instantaneously generated in the fermentation medium. When these microbubbles burst instantaneously, huge pressures and high temperatures will be generated locally, thus changing the cell membrane. The morphology of the cell will be well displayed in SEM results. Due to the ability of the cells themselves to withstand sonication, we speculated that there a small amount of near-microbubbles would be lysed. Therefore, when the ultrasonic power continues to increase during the experiment and the duration of the ultrasound is too long, it will affect accumulation of the biomass of the bacterium *Flavobacterium* and affect the yield of VK_2. Determining the appropriate ultrasound dose(UD) and ultrasound intensity(UI)

is very important for this process.

3.3 Effect of multiple ultrasound treatments on extracellular VK_2 production

Chu et al.[27]reported that the cumulative time of ultrasonic treatment plays an important role in gentamic in production from *Micromonospora echinospora*. In order to explore the effect of multiple ultrasonic treatments on VK2 production, the effect of continuous repetitive ultrasound irradiation (1-6 times) on the extracellular production of VK_2 was investigated under pre-optimal conditions. The extracellular production of VK_2 significantly improved, reaching 30.03 ± 1.42 mg/L, which was 1.88-fold higher than the control(Fig.4). It was hypothesized that POE is toxic to cells and that a small amount of POE could damage the cell membrane. Moreover, the use of low-frequency (10-60 kHz) ultrasound to enhance various biotechnological processes has received increasing attention over the past decade as a rapid and reagentless method[28]. Therefore, a combination of POE and ultrasound can obtain a good leakage effect of target product from inside cell.

Thus, the optimal conditions to achieve maximum VK_2 production were as follows: three ultrasound treatments of each culture, addition of 1% POE, sonication time of 40 s, and 80% total power(130 W; on for 3 s and off for 4 s;UI=0.092kW/cm^2, UD=124.8J/L). This strategy, with optimized production process and low operation cost, could solve the problem of separation and purification of products synthesized within the cells and could have wide potential for industrial applications.

3.4 Effects of ultrasound treatment on surface tension and extracellular protein

Previous studies have shown that the solvent surface tension influences the cavitation phenomena. The addition of surfactant to an aqueous solution facilitates cavitation. It increases the solvent viscosity, increases the rate of microbubble formation and the stability of bubble. If the natural cohesive forces acting in the liquid are lower, then they will suppress the negative pressure in the expansion or rarefaction cycle. Therefore to increase the cavitation threshold the natural cohesive forces need to be increased by increasing the viscosity of liquid. Leong et al have done related research show that the growth rate of bubbles is higher in surfactant solutions when compare to water, and makes a bubble more shape stable in the presence of surfactant[29,33]. Table1 lists the surface tensions of different media following different treatments. The order of surface tension values was as follows: Tap water> Sterilized water> Sterilized basic medium> Sterilized leakage medium>Basic medium (without POE and ultrasound treatment) cultured for 9 days > Leakage medium(with POE and ultrasound treatment) cultured for 9 days. The decrease in the surface tension of the leakage medium reduced the interfacial resistance of the surface of the bacteria and surface of the fermentation liquid. Moreover, the addition of a surfactant also promotes cavitation, which in turn induces more perforations in the cell membrane, thus promoting the transport of nutrients and metabolites out of the cells.

The results of extracellular protein production are shown in Fig.5. It can be clearly observed from the figure that combined application of surfactant and ultrasound achieved maximum extracellular protein production, reaching 176.71±29.52 mg/L,

which was significantly higher than that noted in the control ($P < 0.05$). A similar effect was observed by Dai et al. who found that the membrane permeability of *Saccharomyces cerevisiae* was enhanced by ultrasound, resulting in the augmentation of extracellular protein, nucleic acid, and fructose-1,6-diphosphate (FDP) contents[26].This experiment also compares extracellular VK_2 under three different treatments, and the results show that the optimal effect is accomplished when surfactant is combined with ultrasound.

3.5 Synergistic effect of surfactant and ultrasound on the cell membrane morphology of *Flavobacterium* sp. M1-14

The morphological appearance of cells subjected to different treatments was studied using scanning electron microscopy, and the results are illustrated in Fig.6. Untreated *Flavobacterium* sp. M1-14 displayed typical long rod shapes with smooth and intact surfaces as a sign of morphological integrity (Fig.6A,B). However, remarkable morphological changes were observed after the cells were subjected to POE treatment (Fig.6C,D), when they exhibited irregular shapes and phospholipid bilayers partly solubilized by POE, which is in agreement with previous results for the same or similar systems[34,35]. Cells exposed to ultrasound in the presence of POE displayed multiple surface pores(Fig.6E,F), and dimple-like craters of various sizes appeared on the membrane surfaces, part of which presented repaired traces. These results were similar to those of Lentackeret al.[19], who reported that all experiments performed at the single cell level indicated that direct contact between a microbubble and the cell

membrane is required to induce pore formation by stable cavitation. Besides, from Fig.6E and 6F, it can be clearly seen that small numbers of bacteria have been ruptured into pieces under the synergistic effects of ultrasound and surfactants, which we speculate may correspond to some cell disruption near the ultrasound probe or microbubbles. It may be that a location too near the probe or microbubbles expose the cells to a very high cavitation strength that exceeds the range that a cell can withstand. Further more, surfactants also have the effect of dissolving cell membranes, and may also induce a small amount of cell lysis at the end of the growth curve. It is noted that the cells in Fig.6C and 6D exposed to of the POE conditions alone display a smaller cell size and more lysed cells or dissolving cells. These results suggest that cell damage induced by POE could be partially reversed by sonication treatment. We speculate that a POE molecule attached to the cell membrane surface is shed and redissolves into the solution due to the vibration and thermal effects of ultrasound. During the cell self-repair function, the reversible damage caused by POE is repaired and gradually membrane integrity is recovered. However, due to the cavitation effect of ultrasound, part of the cell membrane is also perforated, which is beneficial for the exchange of intracellular and extracellular substances and extracellular VK_2 accumulation.

3.6 Esterase activity and membrane permeability

A combination of fluorescein diacetate (FDA) and propidium iodide (PI) can be used in double staining procedures. FDA, used for the evaluation of enzymatic activity and membrane integrity, is a lipophilic, nonfluorescent precursor that readily diffuses across

membranes. In the intracellular compartment of metabolically active cells, FDA undergoes hydrolysis of its diacetate groups by unspecific esterases to form fluorescein (F), a method that is widely used for the assessment of nonspecific enzymatic activity in cells. In contrast, PI is anucleotide-binding probe, positively charged, that is widely used for the assessment of membrane integrity and supposed to enter only cells with damaged membranes. Following the loss of membrane integrity, PI diffuses into the cell and stains the DNA to produce red fluorescence[36,37]. Fig.7 shows dual parameter density plots of the green fluorescence (Y-axis) and the red fluorescence (X-axis), monitoring the ability of *Flavobacterium* sp. M1-14 cells to accumulate and retain F as an indicator of esterase activity and uptake PI to assess membrane permeability. The subpopulations were identified based on their differential staining characteristics with PI and cF: the unstained area (cF negative, PI negative, quadrant 1) (lower left quadrant) most likely corresponded to debris or lysed cells attributable to ultrasound treatment; cF-stained cells (cF positive, PI negative, quadrant 2) (lower right quadrant) had high esterase activity and intact membranes; PI and cF double-stained cells (cF positive,PI positive, quadrant 3) (upper right quadrant) contained cells that experienced sublethal injury with residual esterase activity and compromised membranes. this stressed or injured subpopulation has been denoted as “viable but not cultivable cells” because they have metabolic activity but they cannot be detected by conventional cultivation assays; PI-stained cells (cF negative, PI positive, quadrant 4) (upper left quadrant) had inactivated esterase and damaged membranes[38-40].

These results revealed different esterase activities and membrane permeabilities in the four quadrants corresponding to differently treated cells. From quadrant 1 of all plots, there was a fraction of debris or lysed cells with damage incapable of growing in a culture medium after POE treatment. In agreement with our findings in Fig.6, in the field of view, there are many cell membranes that have dissolved, so that the cell membrane has changed from a full, rounded rod into a flat, cupped, irregular shape. In quadrant 2, viable cells show a gradual decrease from 88.7% to 26.3%, indicating that more cells have changed their physiological state under combined ultrasound and surfactant treatment. There is an increase in the number of *Flavobacterium* sp. M1-14 cells in quadrant 3(cF positive, PI positive,) of the plot. These results indicate that esterase activity and membrane permeability of the cell were promoted after the combination of surfactant and ultrasound, and this approach was superior to surfactant treatment of the cells alone. Wang et al. reported that ultrasound could change the conformation of enzymes to accelerate the contact between enzyme and substrate. Thus, the biological activity of enzymes is promoted. Furthermore, ultrasound can alter the characteristics of substrates, and reactions between enzymes and substrates. It even assists in providing an optimal environment for the reactions[41,42]. It should be noted that the cF and PI simultaneously stained cells predominated (53.1%) in plot C, while they accounted for a minor proportion in plot A (3.17%) and fewer in plot B (33.6%). The cF-negative, PI-positive population (quadrant 2, dead cells) made up 0.2%, 4.25%, and 6.98%, respectively. Based on these results, we conclude that ultrasound with POE

treatment can markedly alter esterase activity and membrane permeability and reduce the damaging effects of surfactant treatment alone, in accordance with the results for the cell membrane morphology of *Flavobacterium*sp. M1-14. The discovery of this phenomenon will have great significance for the application of this combined processing technology in the fermentation industry.

4.Conclusion

The present study showed that surfactant (POE) in combination with ultrasound can be used to improve VK_2 production by *Flavobacterium*sp. M1-14. Under the optimized surfactant and ultrasound conditions, VK_2 production significantly increased to 30.03±1.42 mg/L, which was 1.88-fold that when POE alone was used to treat the cells. In addition, low-intensity ultrasound and POE enhanced cell membrane permeability, evaluated by extracellular proteins and the surface tension of the fermentation broth. Morphologic analysis by scanning electron microscopy showed that the synergistic effect of ultrasound and surfactant significantly changed the morphology of the cell membrane surface, which was beneficial for the exchange of intracellular and extracellular substances and extracellular VK_2 accumulation. Profiles of the population obtained using double staining techniques used in this work have enabled further corroboration that this combined processing technology involving ultrasound and surfactant also results in significant promotion of cellular enzyme activity and the membrane permeability of *Flavobacterium* sp. M1-14.

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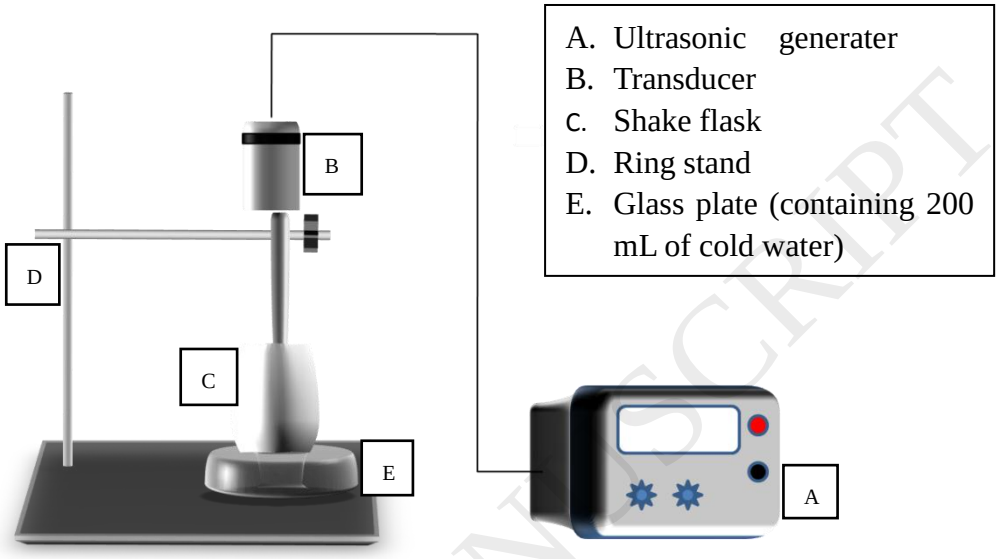
Figures and captions

Fig.1.



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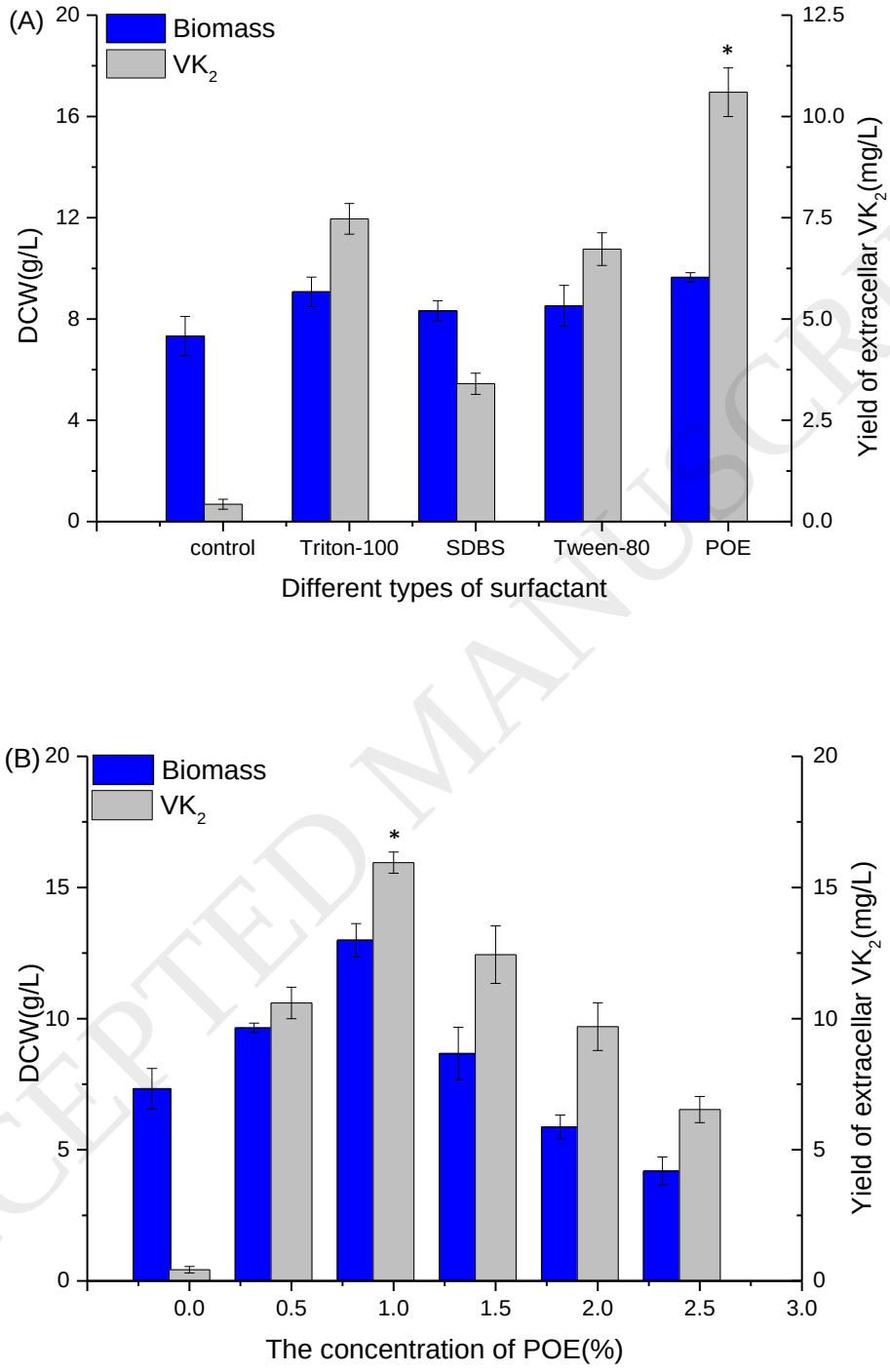

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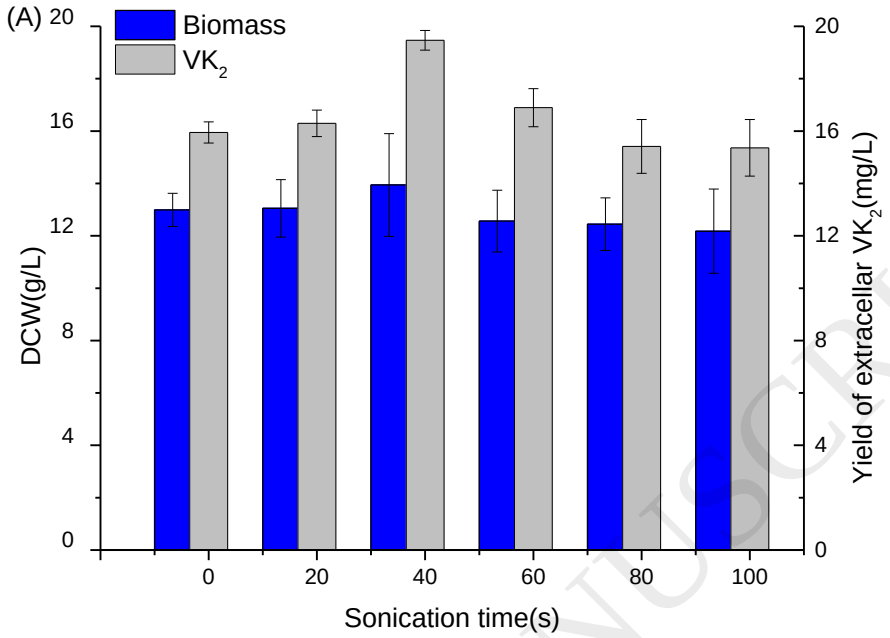

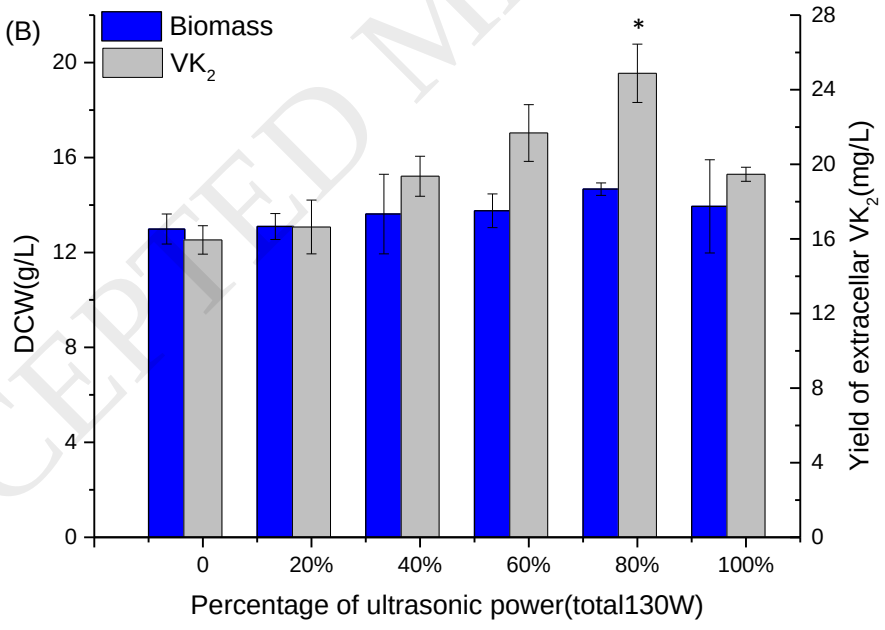

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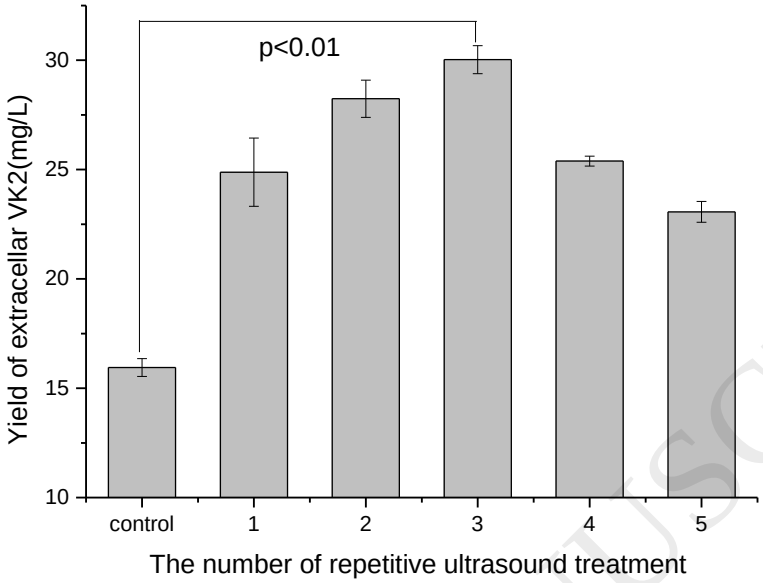

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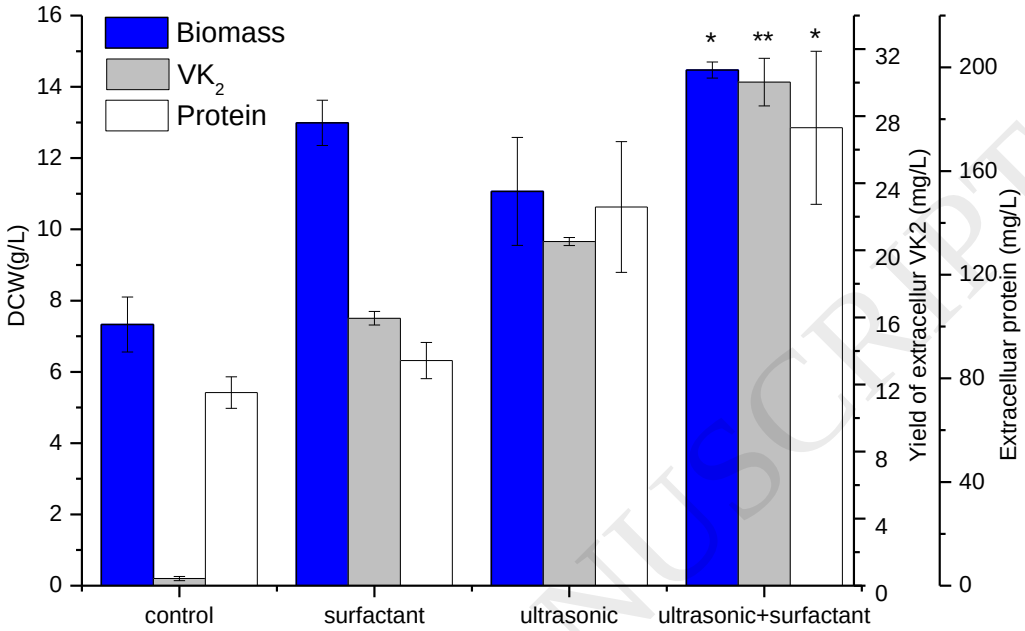

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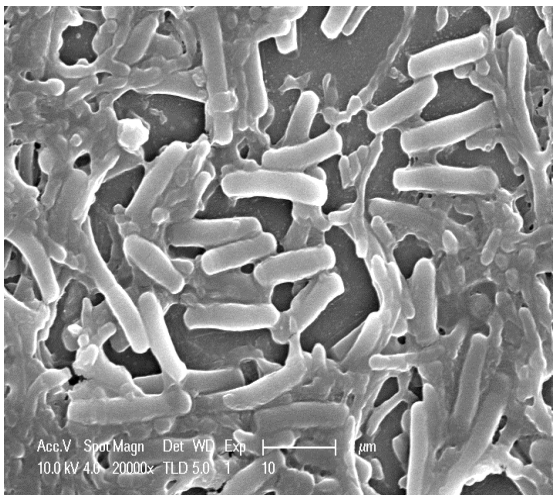

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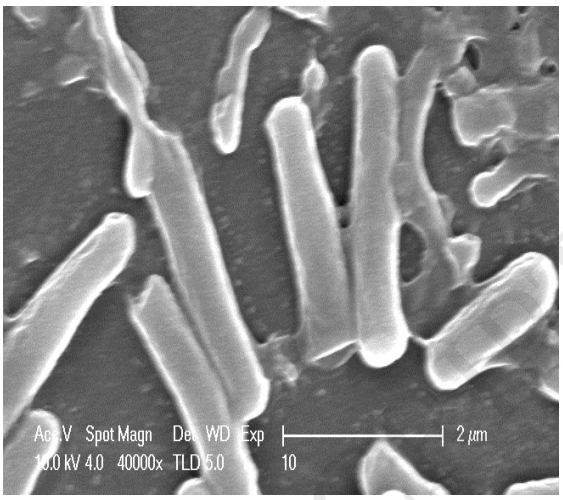

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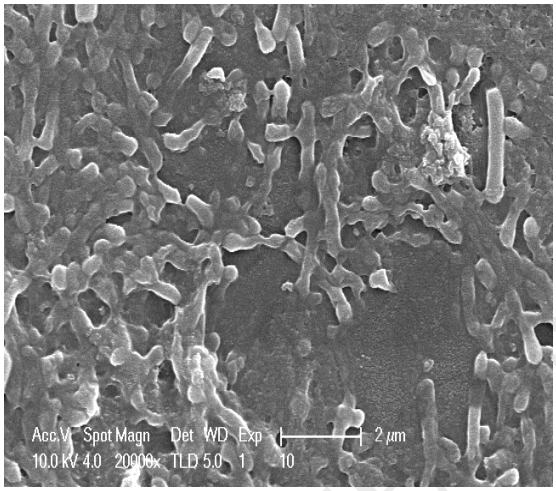

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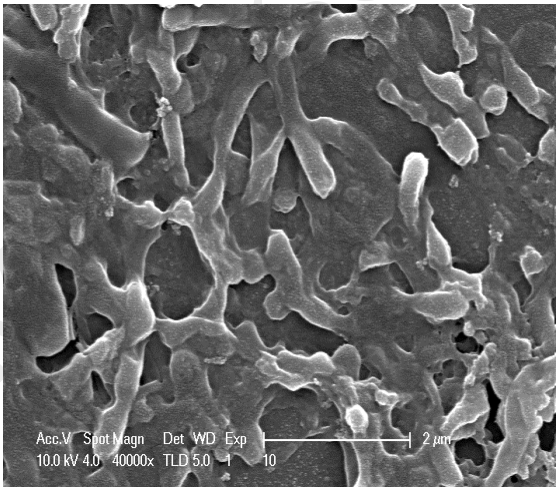

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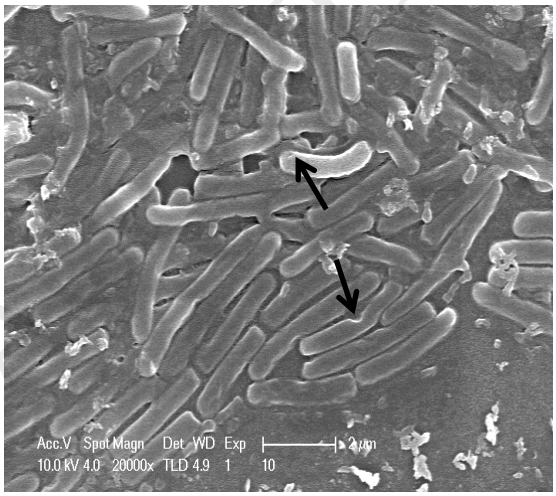

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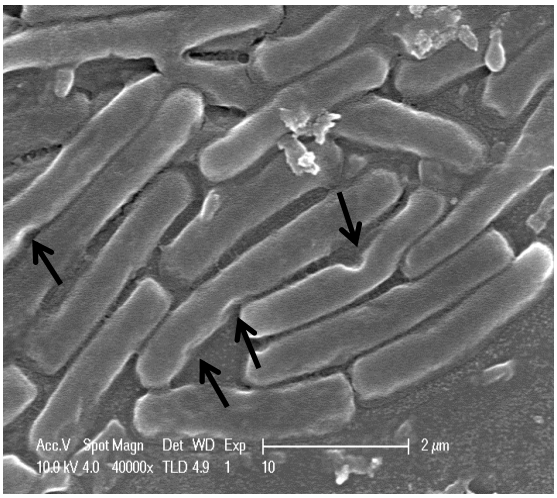

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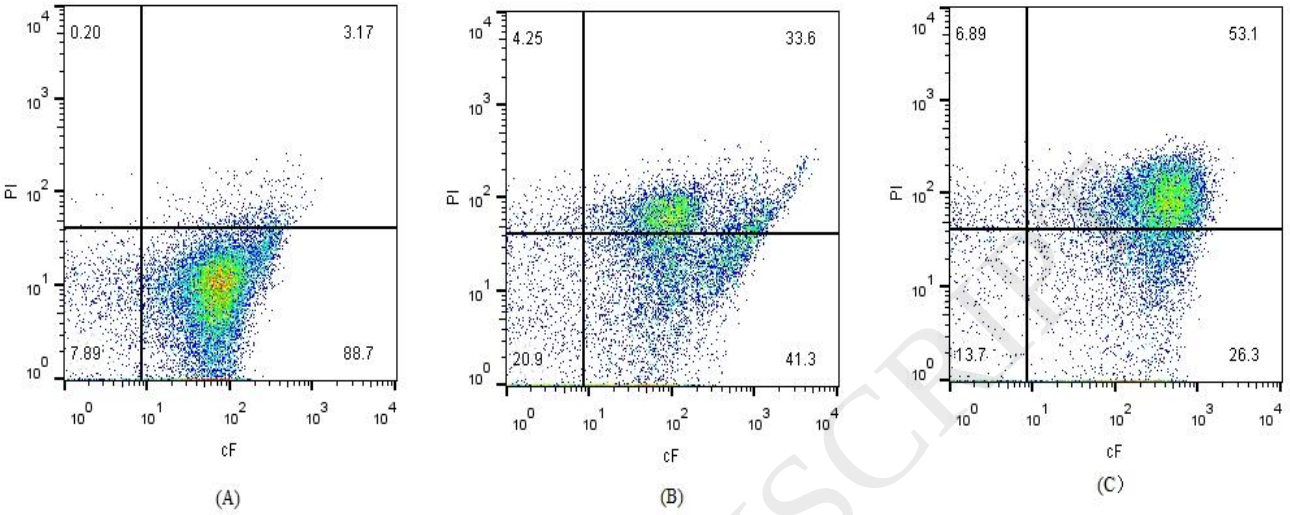

Figure 1. Ultrasound treatment apparatus.

Figure 2. Screening of surfactants and optimization of surfactant concentration.(A) 0.5% of different types of surfactant solutions were added to 100 mL of the fermentation broth, respectively. (B) Selection of optimal POE concentration. Vertical bars indicate standard deviations from the mean (n = 3). *Indicates statistical significance ($P < 0.05$) compared to the control.

Figure 3. Ultrasound treatment of the fermentation broth with different ultrasound parameters. 100-mL aliquots of the fermentation broth were subjected to ultrasound treatment (on for 3sand off for 4 s). (A)Sonication times: 20,40,60,80, and 100s. (B)Percentages of ultrasonic power (total 130W). Vertical bars indicate standard deviations from the mean (n = 3). *Indicates statistical significance ($P < 0.05$) compared to the control.

Figure 4. Effect of continuous repetitive sonication on extracellular VK_2 production. A total of 100 mL of the fermentation broth was subjected to 80% ultrasound power for 40 s (on for 3s and off for 4s).Vertical bars indicate standard deviations of the mean (n = 3). Statistical significance ($P < 0.01$) compared to the control.

Figure 5. The extracellular protein and VK_2 measured under optimal conditions. *Indicates statistical significance ($P < 0.05$) compared to the control.** Indicates statistical significance ($P < 0.01$) compared to the control. Vertical bars indicate standard deviations from the mean (n = 3).

Figure 6. Scanning electron micrographs of the cell membranes following different

treatments. (A,B)Untreated bacteria; (C,D) +POE; (E,F)+US+POE. US stands for ultrasound treatment under optimal conditions.

Figure 7. Fluorescence density plots of *Flavobacterium M1-14* in response to staining with FDA and PI after different treatments under optimal conditions. (A)Untreated bacteria; (B)+POE；(C)+US+POE. US stands for ultrasound treatment under optimal conditions.

Table 1 Surface tension of different media

Medium	Surface tension(mN/m)
Tap water	71.3±1.27
Sterilized water	66.9±0.42
Sterilized basic medium	46.5±0.96
Sterilized leakage medium	38.4±0.31
Basic medium cultured for 9days	41.6±0.54
Leakage medium cultured for 9days	34.6±1.08

Vertical bars indicate standard deviations from the mean (n = 3).The leakage medium(POE+US)was cultured for 9 days under optimized conditions. US represents ultrasound treatment under optimal conditions.