



Original Article

Influence of physical, chemical and inducer treatments on menaquinone-7 biosynthesis by *Bacillus subtilis* MTCC 2756

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Abstract

Effects of physical and chemical treatment on nutrient mobility, their utilization for menaquinone-7 (MK-7) biosynthesis; growth of microbial cells has been investigated in the present research. *Bacillus subtilis* MTCC 2756 fermented medium was supplied with 1-naphthol and hypoxanthine resulted in a significant increase in MK-7 production. Ultrasonication, electric shock, heat shock, and tween 80 were used for inducer uptake by *Bacillus subtilis* and menaquinone-7 production. Induction of *Bacillus subtilis* (at 16 hours of fermentation) using 1-naphthol (2 mg/ml), along with tween 80 (0.1%) was found to increase the MK-7 production by 3 fold i.e. 14.4 µg/ml as compared to the untreated fermentation medium. The ultrasonicated (ultrasonic power 33 W, treatment time 4 min and frequency 36 KHz) microbial cells yielded higher biomass and 2.5 fold increase in the MK-7 production i.e. 10.3 µg/ml than control. 1-naphthol along with physical or chemical treatment is required for maximum MK-7 production by *Bacillus subtilis*.

Keywords: menaquinone-7; 1-naphthol; hypoxanthine; ultrasonication; tween 80

1. Introduction

Members of the vitamins K family have a common methylated naphthoquinones ring structure with a variable side chain length of 4 to 13 isoprene units. The compounds in this series are referred to as MK-n, where n denotes the number of isoprene units (Shearer and Newman, 2008). Several researchers have demonstrated that menaquinone-7 (MK-7) consumption significantly reduces the risk of bone fractures (Truong and Booth, 2011) and cardiovascular disorders by reducing the calcification in arteries and other soft tissues (Theuwissen *et al.*, 2012). Menaquinones are produced by *Bacillus subtilis* and *E. coli* participate in the electron transport chain required for respiration (Lee *et al.*, 1997; Azarkina and Konstantinov, 2002; Berenjian *et al.*, 2014).

Bacillus subtilis is safe for use and potential to produce a wide range of menaquinones i.e. menaquinone 4, menaquinone 5, menaquinone 6 menaquinone 7 and menaquinone 8 (Sato *et al.*, 2001b). Menaquinone-7 produced by submerged and by solid state fermentation process using this bacteria (Beulens *et al.*, 2009). Fermentation conditions, medium components and operating processes are important factors that show a major effect on menaquinone production (Sato *et al.*, 2001a; Berenjian *et al.*, 2011, 2012, 2013, 2014).

During menaquinone-7 biosynthesis, the isoprene side chain and quinone skeleton (1, 4-naphthoquinone) is dependent on the presence of carbon sources such as glucose, fructose and glycerol in the fermentation media. The presence of mono, di and polysaccharides and glycerol in the fermented medium can be used in glycolysis process by *B. subtilis* strains which enhance the production of MK-7 (Sonenshein *et al.*, 2002). In menaquinone-7 biosynthesis, chorismic acid, shikimic acid, 1-naphthol and hypoxanthine; are act as inducers for biosynthesis of MK-7 by the microbial cells

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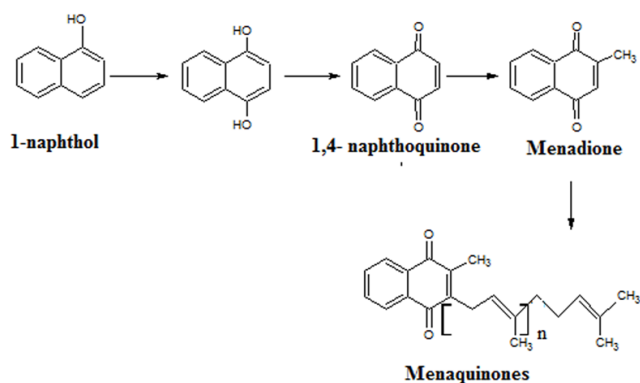


Figure 1. Biosynthetic pathway of menaquinone-7 by *Bacillus subtilis*.

(Leistner *et al.*, 1967) (Figure 1). However, the uptake of inducers by the microbial cell is a challenge.

Cellular uptake can be altered by physical and chemical methods like ultra sonication, electric shock, heat shock and by surfactant treatments to the microbial cells.

The present research work focussed on increasing the cellular synthesis of MK-7 with inducers (1-naphthol and hypoxanthine). The effects of chemical (Tween 80) or physical (ultrasonication, heat shock, electric shock) treatments on cellular uptake of inducer was studied for Enhancement of menaquinone-7 biosynthesis by *B. subtilis*.

2. Materials and Methods

2.1 Soybean, chemicals

Menaquinone-7 was purchased from the MEDLEY Pharmaceutical LTD, India. 1-naphthol and hypoxanthine were purchased from Hi-media Laboratories, India. All other microbiological media, chemicals, reagents used were of analytical grade and were purchased from Merck (Mumbai, India). Soybean variety (Bragg-Kh-09) was obtained as a gift sample from the Pulse Laboratory of Indian Agricultural Research Institute (IARI), New Delhi, India.

2.2 Bacterial strain and cultivation

Bacillus subtilis MTCC 2756 was procured from a Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The strain was maintained on nutrient agar at 4°C and subcultured at every 30 days interval. Seed culture of *B. subtilis* MTCC 2756 was prepared by inoculating the bacterial cells to the sterilized medium (soybean powder 20%, sodium chloride 0.5% and distilled water (up to 100 ml) adjusted to pH 7.0 using 0.1N HCl or NaOH). Culture flasks were incubated at 37°C for 16 hours in a shaker incubator at 180 rpm (Hu *et al.*, 2010). Fermentation was carried out in liquid medium 10 g soy powder, 0.5 g yeast extract, 0.05 g K₂HPO₄, 5 ml glycerol, volume made up to 100 ml of water. Sterilized medium was

inoculated with 5% v/v of *B. subtilis* seed culture and incubated at 37°C for 24 hrs at 180 rpm.

2.3 Determination of bacterial biomass and menaquinone-7

During the fermentation process, samples were collected at pre-determined time intervals for estimation of *B. subtilis* cell density (monitored at λ_{\max} 610 nm) and MK-7 production. Menaquinone-7 was extracted from fermented medium using chloroform, and then reconstituted with acetonitrile. The extracted MK-7 was analysed by HPLC system (Schimadzu Japan), using a 250 mm x 4.6 mm ID Lichrosper® 100 C18 column containing 5 mm sized particles and a 20 ml loop injector. The HPLC was carried out with gradient elution at a flow-rate of 1.2 ml/min with the following system: A: acetonitrile, B: Mixture of water and methanol (1:1) acidified to pH 3.0 by ortho-phosphoric acid. The system was maintained at 80% B for 3.5 min and then increased to 100% in 4.5 min and held for 2 min and at the end of 10 min, the system was recycled back to 80% B. The MK-7 was measured at λ_{\max} 248 nm by using UV detector. All the measurements were taken in triplicate, and the average was used for data analysis.

2.4 Effect of inducers on MK-7 production

Individually, 1-naphthol and hypoxanthine were used as inducers and mixed with different concentrations in sterilized soy based medium to observe its utilization by microbial cells and effects on MK-7 production. 1-naphthol (stock solution 10 mg/ml = 69.36 mM) and hypoxanthine (stock solution 73.45 mM) were added to the growing cells in different concentrations of 0.2, 0.5, 1, 1.5, and 2 mg/ml. 1-naphthol and hypoxanthine were dissolved in dimethylsulphoxide and distilled water, respectively, and sterilized through a 0.45 μ m membrane filter. Sterilized solutions of inducers were added to the fermented medium with bacterial cells in late log phase (i.e., after 16 hrs of fermentation) and incubation was continued for next eight hours at 37°C at 180 rpm. After complete fermentation (24 hrs), the change in MK-7 concentration was analysed using HPLC method as described above. The bacterial growth was also monitored in the fermented medium to assess bacterial cell viability.

2.5 Determination of 1-naphthol and hypoxanthine

High performance thin layer chromatography (HPTLC) was performed to analyse 1-naphthol. Mobile phase was a mixture of toluene: ethyl acetate in the ratio of 9.5: 0.5(v/v). Precoated silica gel aluminium plates 60F-254 (20×10 cm × 200 mm, Merck, Germany) was used. Linear ascending development was carried out in 10×10 cm twin through-glass chambers (Camag, Switzerland). The optimised chamber saturation time was 5-20 min at room temperature 25°C and at 60% relative humidity. The samples were spotted with Camag 100 ml syringe using a Linomat V (Camag, Muttentz, Switzer-

land) sample applicator. The chromatogram was developed for 20 min. TLC plates were dried in hot air oven for 10 min to get sharp bands. Then, the plates were then scanned at 300 nm on the densitometric mode of CAMAG scanner and analysed by win CATS software (version 1.2.0).

Hypoxanthine was analysed by high performance liquid chromatography (Farthing *et al.*, 2007).

2.6 Extraction and estimation of inducers

Soy based fermented medium was dispersed with chloroform and centrifuged at 3000 rpm to separate microbial cells. The chloroform layer was separated to estimate the unutilised 1-naphthol present outside the bacterial cells.

For the estimation of unutilised 1-naphthol present inside the bacterial cells, the cells (pellet) were mixed with chloroform. The bacterial cells were then ruptured via ultrasonication (Vibracell, Sonics, U.S.A.) for 10 min to extract the unutilised naphthol. The mixture was centrifuged at 3,000 rpm to separate the organic layer (containing 1-naphthol). 1-naphthol present both inside and outside the bacterial cells was quantified by HPTLC method described above.

2.7 Effect of Physical and chemical treatment on microbial cells

1-naphthol was added to the bacterial cells during their stationary phase growth and various treatments were given (each at one time) such as electric shock, ultrasonication, heat shock, tween 80 to alter the permeability of bacterial cells for enhanced permeation of inducer (1-naphthol) during fermentation.

Ultrasonication was performed from 4 min to 16 min, on the fermentation medium at 36 kHz in an ultrasonic bath (PCI Analytical, Mumbai, India) at 25°C. Electric shock treatment was given by power supply unit (Consort EV215) at 100 V AC for different time periods varying from 2 sec to 8 sec. Heat shock treatment was performed by increasing the temperature of the fermentation medium up to 80°C for 10 min followed by immediate cooling to room temperature in cooling water. Chemical treatment was given by adding varying concentration of tween 80 (0.05 to 1%) to the fermentation medium.

Various controls were used to compare the bacterial cell viability and MK-7 pre- and post- treatments. Fermented soy based medium harvested at 14 hrs of incubation without any treatment (Control A). Fermented soy based medium harvested at 24 hrs of incubation without any treatment (Control B). Fermented soy based medium after 14 hrs of incubation, 1-naphthol was added to the medium and harvested at 24 hrs of incubation without any treatment (Control C).

The viable bacterial cells count was performed after each treatment and expressed as colony forming unit (CFU). The effect of each treatment on MK-7 production was studied by quantifying MK-7 by HPLC method. Also, the amount of

unutilised 1-naphthol was quantified by HPTLC method.

3. Results and Discussion

3.1 Bacterial cell mass and MK-7 production

Change in bacterial cell growth and MK-7 concentration in fermentation medium were measured every two hour-interval. We observed that the MK-7 production and microbial cell growth had a similar trend as shown in Figure 2. The majority of MK-7 was produced during log phase and only 22 % amount of total MK-7 generated during stationary phase. The MK-7 concentration increased significantly during log phase, and reached to a maximum of 1.9 µg/ml at 48 hrs during the liquid phase fermentation.

3.2 Effect of inducers on MK-7 production

1-naphthol uptake by microbial cells was quantified by HPTLC method and the HPTLC chromatogram of standard 1-naphthol has been shown in Figure S1. Chromatogram of 1-naphthol (10, 25, 50, 100, 200, 500, 1000, 2000 ng) is shown in Figure S2. 1-naphthol and hypoxanthine (0.5 to 2.0 mg/ml) were added to the medium at 16 hours of fermentation as inducers, and the ability of *B. subtilis* cells to utilize these inducers for MK-7 biosynthesis was investigated. The fermentation was further continued for 8 hr, and final MK-7 concentration was determined. The increase in MK-7 production was statistically significant ($p \leq 0.05$), when 1-naphthol concentration was increased from 0.2 to 2 mg/ml in the fermentation media; however, as the concentration of 1-naphthol further increased beyond 2 mg/ml, the increase in MK-7 production was not significant. Therefore, 2 mg/ml 1-naphthol was used in all subsequent fermentations. The MK-7 production pattern was linear with 1-naphthol concentration, and reached to 12.5 µg/ml which was about three-fold higher than the positive control (4.9 µg/ml).

The relationship between inducers (1-naphthol, hypoxanthine) uptake by microbial cells and their effect on MK-7 production has been shown in Figure 3. When hypoxanthine

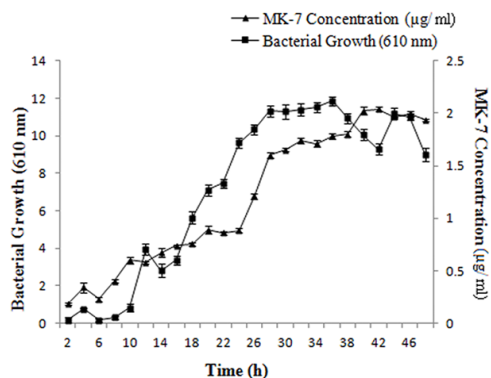


Figure 2. *Bacillus subtilis* growth and Menaquinone-7 (MK-7) level in fermentation medium under control experiment without inducers (1-naphthol & hypoxanthine).

concentration was increased from 0.2 mg/ml to 1 mg/ml in the fermentation media, MK-7 production increased from 4.8 to 5.8 $\mu\text{g/ml}$. Hypoxanthine showed a lesser effect on MK-7 production as compared to 1-naphthol. Results show that increasing the concentration of hypoxanthine up to 1 mg/ml resulted in higher MK-7 production as compared to control and any further increase in hypoxanthine concentration caused a decrease in MK-7 production (Figure 3).

1-naphthol as a carbon source in the fermentation medium had a major effect on MK-7 production. When 1-naphthol was present in the medium, a sequential pattern of 1-naphthol consumption was observed. MK-7 concentration continued to rise after depletion or consumption of 1-naphthol from the fermentation medium. Peak concentrations of MK-7 were obtained after most of the 1-naphthol was consumed, suggesting that 1-naphthol contributed to MK-7 production.

The addition of hypoxanthine was expected to encourage the microbial growth. This was evident from the significant increase in MK-7 biosynthesis after hypoxanthine addition. The increased MK-7 production at the low concentration of hypoxanthine (and above 1 mg/ml) was likely due to the increased bacterial cell growth and biomass. All subsequent fermentations were conducted using 1-naphthol, as an inducer, in soybean based fermentation medium. Further research would include effect of different physical and chemical treatment on microbial viability; 1-naphthol uptake by microbial cells and their effect on MK-7 biosynthesis.

3.3 1-naphthol uptake after physical treatment and MK-7 production

1-naphthol was added to *B. subtilis* fermented medium at 16 hrs of fermentation and different physical treatments (ultrasonication, heat shock, electric shock), and chemical treatment (Tween 80) were given to increase the mobility of 1-naphthol into the microbial cells and incubation was further continued for an 8 hr- period. 1-naphthol uptake by microbial cells and the MK-7 concentration was determined in fermentation medium and simultaneously viable bacterial cells were also counted in percent colony-forming unit (% CFU).

3.3.1 Effect of ultrasonication on MK-7 production by *B. subtilis* cells

Ultrasound, or sound frequency >20 kHz is useful in enhancing metabolic productivity of microbial, plant and animal cells. Ultrasonication is generally associated with the cell damage, but some researchers have proven beneficial effects of controlled sonication on live cells. Ultrasound has the potential for enhancing the dissolution of substrate, gas-liquid oxygen transfer rate, mass transfer within and, outside a cell (Pitt and Ross, 2003). The stimulative effect of ultrasonication *Lithospermum erythrorhizon* cells (Lin and Wu, 2002) have been reported for the synthesis of secondary metabolites.

When the fermented medium was subjected to ultrasonication for 4 and 8 min, a significant reduction in CFU (74 to 50%) was observed. Further increasing the sonication time up to 16 min decreased the viable cell count from 50 to 43%. In our study, low power (33 W) sonication for 4 min enhanced the accessibility of 1-naphthol up to 1204 ng/ μL into the microbial cells, which led to the increase in 1-naphthol consumption by viable cells (74%, in comparison to un-sonicated medium) and subsequently increased MK-7 production (10.3 $\mu\text{g/ml}$) at the end of the process (Figure 4).

The improved utilization of 1-naphthol after sonication was attributed to increased access of dissolved 1-naphthol to microbial cells. Previous studies have shown that using low frequencies of sonication indicates a decrease in microbial cell numbers and increasing ultrasonic exposure time resulted in increased microbial permeation (Duckhouse *et al.*, 2004). Ultrasonication was involved in increasing accessibility of low solubility substrate like 1-naphthol to the microbial cells by altering cell permeability and this indirectly

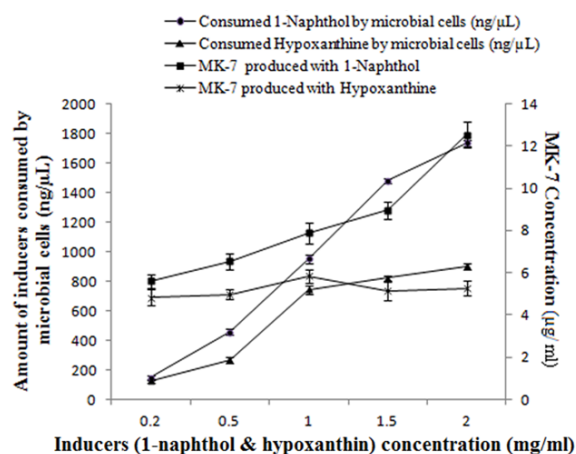


Figure 3. Relationship between Menaquinone-7 (MK-7) production and Inducers (1-naphthol & hypoxanthine) uptake at different inducers concentrations (0.2, 0.5, 1, 1.5, 2 mg/mL) by treated *Bacillus subtilis* cells.

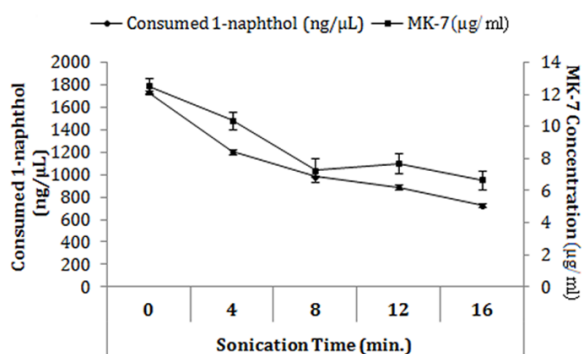


Figure 4. Effect of 36 kHz ultrasonication on 1-naphthol uptake and menaquinone-7 (MK-7) production by *Bacillus subtilis* cells when treated for 0 min, 4 min, 8 min, 12 min, and 16 min.

enhanced MK-7 biosynthesis with decreased number of microbial cells in comparison to the untreated fermentation medium.

3.3.2 Effect of electric field on MK-7 production by *B. subtilis* cells

Electric shock and heat treatment also affect shape, membrane structure and growth of the microbial cells. Electric shock applied to the fermentation medium containing microbial cells had a strong impact on bacterial viability (Szumski *et al.*, 2011).

The electric field (100 V, 100 W, 10 mA) was applied to microbial cells for a specified time period decreased the number of viable cells compared to the control samples. The electric shock for 2 min decreased the number of viable cells to 40 %; 58 % decrease after 4 min and 72 % decrease after 6 min of electric shock application. As microbial cell numbers were decreased, MK-7 production was also decreased to 28% at 8 min of electric shock treatment; with a corresponding decrease in 1-naphthol consumption by the *B. subtilis* cells (Figure 5).

3.3.3 Effect of temperature on MK-7 production by *B. subtilis* cells

The heat shock response occurs when an environmental stress is imposed on the organism. The response comes from the application of a mild heat stress to the fermented medium, only 5 to 10°C higher than the usual preferred growth temperature. However, the heat treatment response, which can be more suitably considered of as an adaptive response, consists of a temporary alteration in the metabolism- of the organism (Hasan and Shimizu, 2008). after treating the fermented medium at different temperatures. Microbial cells were treated with different temperature ranging from 50°C to 80°C and it was observed that viable bacterial cells significantly decreased to 17% in comparison to the reference sample (without heat shock treatment). The number of viable cells varied from 55 to 17% at an increased temperature range of 50 to 80°C. The maximum concentration of MK-7 observed was 6.5 µg/ml in heat treated experiment (Figure 6). The menaquinone -7 concentration is only 33% higher than untreated fermentation medium and 47% lower than the 1-naphthol treated fermented sample (without any physical and chemical treatment).

3.3.4 Effect of surfactant on MK-7 production by *B. subtilis* cells

Tween 80, a non-ionic surfactant, is used in the bacterial cultures to enhance the bioavailability of substrates and to alter the permeability of cell membrane (Brown *et al.*, 1999). It was postulated that the presence of surfactant in fermentation medium facilitates the nutrient and oxygen uptake by the microorganisms.

Microbial cell numbers were increased from 4×10^6 to 9×10^7 CFU/ml, when treated with 0.05% concentration of surfactant (Tween 80), followed by an immediate rise in CFU of 5×10^9 followed by a reduction in CFU to 1×10^6 , with high concentration of surfactant. The inclusion of non-ionic surfactant (Tween 80) to the fermentation medium along with 1-naphthol increased cell growth and menaquinone-7 production as well. The MK-7 production increased to 14.4 µg/ml (15%), when fermented medium was treated with 0.1% non-ionic surfactant (Tween 80) (Figure 7). The cell viability was enhanced, reaching 5×10^9 CFU at 24 hrs with 0.1% Tween 80 in fermentation medium and keeping the cells alive throughout the fermentation period. Addition of surfactant into fermented medium has shown a substantial effect on the transport of substrate into the bacterial cells and thus, increasing the metabolites (MK-7) biosynthesis.

The productivity of MK-7 reported by Berenjin *et al.*, (2014) is 226 µg/ml under optimized fermentation conditions after a five day-fermentation. Further, the productivity of *B. subtilis* can further be increased by treating high yielding

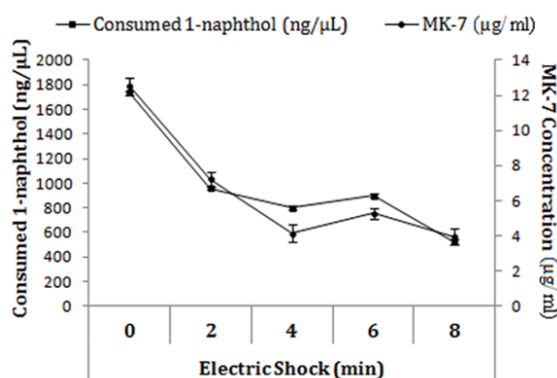


Figure 5. Effect of electric shock (100 V, 100 W, 10 mA) on 1-naphthol uptake and menaquinone-7 (MK-7) production by *Bacillus subtilis* cells when treated for 0 min, 2 min, 4 min, 6 min, and 8 min.

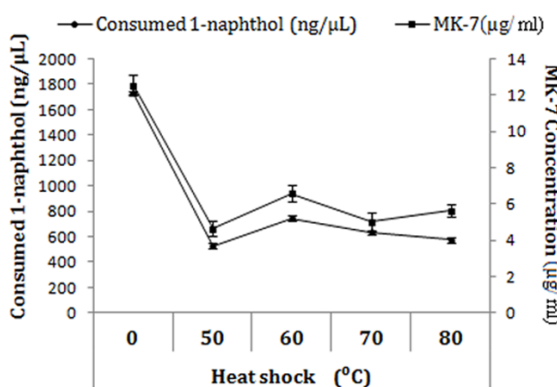


Figure 6. Effect of heat shock temperature (0°C, 30°C, 60°C, 70°C, 80°C) on 1-naphthol uptake and menaquinone-7 (MK-7) production by *Bacillus subtilis* cells when treated for 10 min.

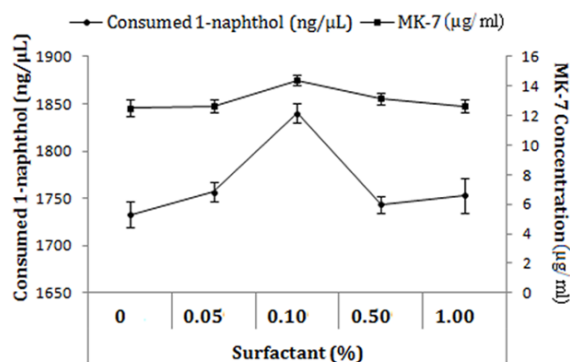


Figure 7. Effect of surfactant (tween 80) concentration (0%, 0.05, 0.1%, 0.5%, 1%) on 1-naphthol uptake and menaquinone-7 (MK-7) production by *Bacillus subtilis* cells.

strains with inducers and different physical and chemical treatments.

4. Conclusions

Among the various physical treatments studied in this research, ultrasonication was found to be the best to alter the cell permeability for the low solubility substrates and maximizing the production of the menaquinone-7. Chemical treatment with addition of Tween 80 to the *B. subtilis* fermentation medium was found to increase the yield of MK-7 with improved accessibility to 1-naphthol by the microbial cells in the fermentation medium.

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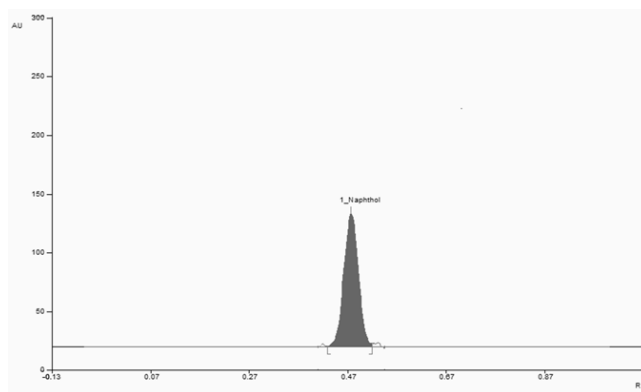


Figure S1. High performance thin layer chromatogram of standard 1-naphthol (50 ng) load developed with mobile phase toluene: ethyl acetate (9.5: 0.5 v/v) at 300 nm.

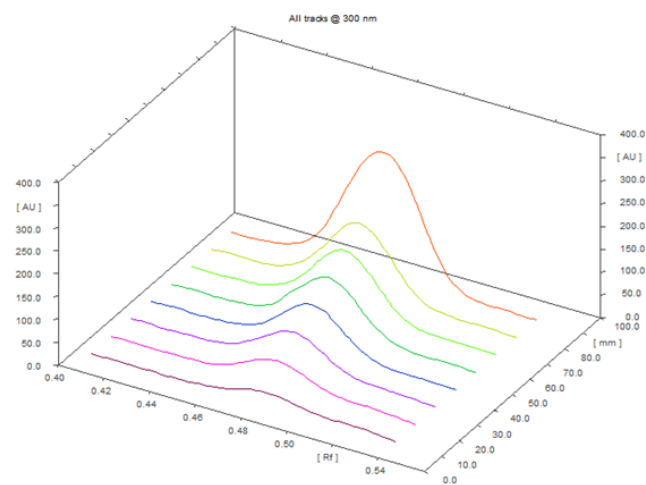


Figure S2. High performance thin layer chromatogram of 1-naphthol (10, 25, 50, 100, 200, 500, 1,000, 2,000 ng).

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