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RESEARCH PAPER

Effects of medium components in a glycerol-based medium on vitamin K (menaquinone-7) production by *Bacillus subtilis natto* in biofilm reactors

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Abstract
Menaquinone-7 (MK-7) as the most important form of Vitamin K has been reported to have miraculous benefits such as preventing cardiovascular diseases and osteoporosis along with antitumor effects. Therefore, there have been numerous studies in the past decades to improve MK-7 production via microbial fermentation. Unfortunately, both solid and liquid state fermentation strategies that are utilized for MK-7 production, face fundamental operational and scale-up issues as well as intense heat and mass transfer problems during fermentation. In this regard, biofilm reactors seem to be a practical solution to overcome these issues and enhance the production in agitated liquid fermentation. Therefore, this study was undertaken to utilize biofilm reactors in investigating and optimizing different media components in a glycerol-based medium. Using response surface methodology, the effects of glycerol, yeast extract, and soytone were studied in the fermentation medium on MK-7 production in biofilm reactor. With a composition of 48.2 g/L of glycerol, 8.1 g/L of yeast extracts, 13.6 g/L of soytone and 0.06 g/L of K_2HPO_4, MK-7 concentrations could reach 14.7 ± 1.4 mg/L in biofilm reactors, which was 57% higher compared to the MK-7 concentration achieved in suspended-cell reactors under similar conditions, while glycerol was depleted by the end of the fifth day in biofilm reactors, but glycerol was never depleted in suspended-cell reactors. Evidently, biofilm reactors present a reliable strategy to address the operational issues that occur during MK-7 biosynthesis on an industrial scale production.

Keywords MK-7 · Menaquinone-7 · Vitamin K · Biofilm reactor · *Bacillus subtilis* · RSM optimization

Introduction

Vitamin K was first discovered as a fat-soluble cofactor, essential for blood clotting and avoiding hemorrhages in chickens [1]. Not many years later, it was also discovered that vitamin K exists in two major forms [2]. The plant form, known as phylloquinone, is found abundant in most leafy green vegetables such as spinach and kale [3, 4]. The animal and microbial forms, known as menaquinones have several subtypes (designated MK-1 to MK-15) and include the predominant forms in microbial metabolisms [5]. Among all subtypes, MK-7 stands out with extraordinary benefits for human health; yet, the amount of MK-7 present in our daily diets is very low for such effects [6–10]. Although microbial flora present in human intestines are also able to synthesize vitamin K2, there is no significant absorbance in this way due to poor bioavailability [11, 12]. Thus, the only feasible method to produce vitamin K on an industrial scale is through microbial fermentation [13].

In this fashion, several bacterial strains including *Bacillus subtilis natto* [14], *Bacillus licheniformis* [15] and *Bacillus amyloliquefaciens* [16] in both solid-state fermentation (SSF) and liquid state fermentation (LSF) strategies have been investigated for MK-7 production with *B. subtilis natto* as the dominant strain [16, 17]. However, as pointed out in previous studies, both SSF and static LSF strategies with no robust agitation and aeration, face serious scale-up and operational issues [5, 18]. This is besides the fact that pelicle and biofilm formations that create these issues are beneficial

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for the MK-7 biosynthesis in the bacteria [19]. This situation presents an opportunity to utilize biofilm reactors to keep these benefits and at the same time have robust agitation and aeration.

Biofilm reactors host biofilms that result from migration of cells from planktonic form into biofilm formations through passive immobilization [20]. With suitable microbial strain and support [21], they are able to harness the extraordinary abilities that cells gain in the biofilm colonies [22]. Throughout recent years, production of many value-added products such as enzymes, antibiotics, biofuels, and biopolymers have been enhanced by biofilm reactors [23–26]. Similarly, biofilm reactors seem to be able to enhance MK-7 production in *B. subtilis* as well [27]. Biofilm reactors also require an optimum media to produce MK-7. Different carbon and nitrogen sources have been investigated in past studies [28, 29]. Sato et al. [29] investigated several carbon sources including glucose, mannose, galactose, fructose, sucrose, lactose, maltose, glycerol, mannitol, sorbitol and ribose with *B. subtilis* and the top MK-7 producers were glycerol (45.1 mg/L) and mannitol (42.8 mg/L). Moreover, Berenjian et al. [28] reported that among glucose, glycerol, starch and sucrose, only glycerol as a single carbon source had a significant effect on MK-7 production. Thus, glycerol-based media have been recommended by these studies and have been utilized most commonly, where in the most recent study 12.1 mg/L of MK-7 was produced in biofilm reactors using glycerol [27, 28, 30, 31]. Nevertheless, effects of media components still need to be investigated and optimized for biofilm reactors as well, as non-optimal levels can suppress growth and MK-7 biosynthesis [14]. Therefore, the aim of this study was to determine these optimum levels through statistical optimization techniques as another step towards production of MK-7 more efficiently on an industrial scale.

Materials and methods

Microorganisms and media

Bacillus subtilis natto (NF1) was isolated from commercial natto, as previously described [21]. For biofilm formation on the Plastic Composite Support (PCS), Tryptic Soy Broth (TSB) medium fortified with 10% (w/v) glucose (Tate & Lyle, Decatur, IL, USA) and 0.8% yeast extract (Biospringer, Milwaukee, WI, USA) was used. Main fermentation media consisted of 100 g of soytone (Difco), 35 g of yeast extract (Difco), 45 g of glycerol (EMD Chemicals, Gibbstown, NJ, USA) and 0.6 g of K_2HPO_4 (VWR, West Chester, PA, USA) per liter of deionized water, as concluded in previous studies [27].

Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA, USA) equipped with 2-L vessels were utilized. Sterile 4N sulfuric acid (EMD) and 4N sodium hydroxide (Amresco, Solon, OH, USA) along with antifoam B emulsion (Sigma-Aldrich, Atlanta, GA, USA) were added automatically to maintain pH and suppress foaming as needed. Plastic composite support (PCS) tubes type SFYB (50% polypropylene, 35% soybean hulls, 5% soybean flour, 5% yeast extract, 5% bovine albumin and salts) were manufactured and implemented as described in previous studies [24, 25, 32].

Biofilm formation

For biofilm formations to form on the PCS, bioreactors were set up with grid-like fashion PCS formations and were autoclaved at 121 °C for 45 min containing 1.5 L of DI water, as the working volume of the bioreactors were 1.5 L each. Then, the water was replaced with a sterile medium. After that, the bioreactors were inoculated with 24-h old inoculum prepared in Erlenmeyer flasks (from liquid broth test tubes) with TSB medium grown at 30 °C and 200 rpm with %3 (v/v) inoculation ratios. The medium was refreshed for four times every 48 h as the broth was pumped out of the vessels and sterile-fresh medium was immediately pumped in without any further inoculation; as described in the previous study [33]. At the end of the four fermentation cycles, the fermentation broth was sampled and gram-stained to verify a pure culture. At this time, the biofilm population was assumed mature and vessels were ready to be treated as biofilm reactors.

Experimental design

Effects of glycerol (30–60 g/L), yeast extract (20–50 or 0–10 g/L) and soytone (50–200 or 0–25 g/L) concentrations on MK-7 production were evaluated using two Response Surface Methodology (RSM) Box–Behnken designs with three levels of variables (Tables 1 and 3). Each batch was carried out in 144 h (6 days) at 35 °C, pH 6.58, 200 rpm and 1 vvm aeration as described in our previous studies [27]. Samples were obtained every 12 h before the medium was refreshed for the next set of fermentation experiment and were kept in 4 °C. ANOVA analysis was performed with maximum MK-7 concentration that was observed throughout each fermentation run as the only response (Tables 1 and 3). To validate the effects of the biofilm formations, duplicated suspended-cell fermentation runs were also carried out under same conditions as a control.

Table 1 RSM design including variables glycerol, yeast extract and soytone concentrations (g/L) in predicting MK-7 concentrations (mg/L) in the higher concentration composition medium

Run	Medium component (g/L)			MK-7 concentration (observed) (mg/L)	MK-7 concentration (predicted) (mg/L)
	Glycerol	Yeast extract	Soytone		
1	30	20	125	6.7	6.1
2	60	20	125	5.9	5.4
3	30	50	125	4.7	5.2
4	60	50	125	4.0	4.6
5	30	35	50	11.0	10.4
6	60	35	50	7.7	7.1
7	30	35	200	3.1	3.7
8	60	35	200	5.1	5.7
9	45	20	50	8.5	9.7
10	45	50	50	6.6	6.6
11	45	20	200	3.5	3.5
12	45	50	200	6.0	4.8
13	45	35	125	7.7	6.1
14	45	35	125	3.7	6.1
15	45	35	125	6.9	6.1

Analysis

MK-7 analysis

Fermentation broth sample (3 mL) was mixed with 2:1, v/v n-hexane:2-propanol mixture to extract the MK-7 content [28]. N-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used. The mixture was vigorously shaken using a vortex mixer for 3 min and then the organic phase was separated and evaporated under forced air flow at ambient temperature. Then, dried pellets containing the MK-7 were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH, USA) for 15 min at ambient temperature. After the pellets were completely suspended in methanol, the mixtures were filtered through 0.2 µm PTFE filters (PALL Life Sciences, Port Washington, NY, USA). High-performance liquid chromatography (HPLC) (Waters, Milford, MA, USA) equipped with a 2489 UV/Visible detector and a Supelcosil C18 column (15 cm × 4.6 mm, 5 µm, Supelco Analytical, Bellefonte, PA, USA) was used at 40 °C for the analysis of MK-7 concentration. Methanol (EMD) was used as mobile phase with the flow rate of 1 mL/min. The wavelength of 248 nm was selected for calibration and analysis. The MK-7 calibration curve was linear between 0.1 and 30 mg/L ($R^2 = 0.999$) [21, 34].

Glycerol analysis

Samples of the fermentation broth were centrifuged at 9000×*g* for 5 min (Microfuge 20 Series, Beckman Coulter Inc., Brea, CA, USA) and then filtered through 0.2 µm cellulosic filters (PALL). Then, without dilution, the clear broth was analyzed by HPLC (Waters) equipped with a 2414 Refractive Index detector and an HPX-87H Aminex column (300 × 7.8 mm, 9 µm, Bio-Rad, Hercules, CA, USA) at 50 °C and 410 nm. A 0.05 M sulfuric acid (EMD) solution was used as the mobile phase. Samples were kept at 4 °C during the injections. The glycerol calibration curve obtained from 99.3% pure glycerol (EMD) was linear between 1 and 60 g/L ($R^2 > 0.999$) [27].

Statistical analysis

The effects of glycerol (g/L), yeast extract (g/L), and soytone (g/L) along with the second-order and two-way interaction effects were obtained using Minitab 17.0 ANOVA (Minitab Inc., State College, PA, USA) with full quadratic models and regression analysis with optimal λ transformation. A confidence level of 95% was implemented throughout the analysis procedures to distinguish significant parameters except for the full quadratic models [35, 36].

Results and Discussions

As shown in Tables 1 and 3, the highest MK-7 concentration observed in the Box–Behnken design was 16.9 mg/L. This is fairly higher when compared with previous studies with glycerol-based medium [27]. With ANOVA applied to responses (Tables 2, 4), the predicted values obtained by the models are also depicted in Tables 1 and 3.

Table 2 ANOVA output for MK-7 concentrations (mg/L) versus glycerol, yeast extract and soytone initial concentrations (g/L) in the higher concentration composition medium

Source	*df*	Seq SS	Contribution (%)	Adj SS	Adj MS	*F* value	*P* value
Regression	9	50.6	77.9	50.6	5.6	1.96	0.238
Soytone	1	32.3	49.7	17.9	17.9	6.22	0.055
Yeast extract	1	1.6	2.4	0.07	0.07	0.03	0.879
Glycerol	1	1.0	1.5	0.3	0.3	0.11	0.756
Soytone × soytone	1	2.3	3.6	2.0	2.0	0.69	0.443
Yeast extract × yeast extract	1	1.7	2.6	1.7	1.7	0.59	0.478
Glycerol × glycerol	1	0.05	0.07	0.05	0.05	0.02	0.903
Soytone × yeast extract	1	4.8	7.4	4.8	4.8	1.68	0.251
Soytone × glycerol	1	6.9	10.7	6.9	6.9	2.42	0.181
Yeast extract × glycerol	1	0.002	0.00	0.002	0.002	0.00	0.978
Error	5	14.4	22.1	14.4	2.9		
Lack-of-fit	3	5.6	8.6	5.6	1.9	0.43	0.757
Pure error	2	8.8	13.5	8.8	4.4		
Total	14	64.9	100.0				

Table 3 RSM design including variables glycerol, yeast extract and soytone concentrations (g/L) in predicting MK-7 concentrations (mg/L) in the lower concentration composition medium

Run	Medium component (g/L)			MK-7 concentration (observed) (mg/L)	MK-7 concentration (predicted) (mg/L)
	Glycerol	Yeast extract	Soytone		
1	45	10	25	7.4	9.5
2	60	10	0	9.1	7.8
3	45	0	25	12.5	12.7
4	30	10	25	5.6	5.2
5	30	0	10	3.7	3.8
6	60	10	10	8.8	10.7
7	60	0	25	11.9	11.3
8	60	10	5	8.9	9.7
9	30	5	0	3.7	3.9
10	60	5	25	8.9	10.3
11	60	5	0	5.5	5.7
12	60	10	25	8.9	6.9
13	45	5	10	14.6	13.1
14	45	5	10	16.9	13.1
15	45	5	10	12.7	13.1

Yeast extract

Figures 1 and 2 clearly indicate a significant negative effect of high concentrations of yeast extract on MK-7 biosynthesis. As shown in Fig. 1, increasing yeast extract concentration from 20 to 50 g/L can decrease MK-7 concentration by about 50% (from 10 to 5 mg/L). Although yeast extract is an excellent source of nitrogen for any microbial fermentation due to containing a spectrum of readily metabolized proteins, *B. subtilis* protein synthesis pathways are regulated by these compounds [37]. Also, extracellular MK-7 is biosynthesized and secreted coupled with a protein known as vitamin K2-binding factor (KBF) that renders MK-7 soluble in the broth and accessible to the extracellular matrices [19]. In this fashion, it is understandable that simple nitrogen sources such as yeast extract have a negative effect on MK-7 biosynthesis, especially at rather high concentrations (Fig. 2). Thus, it did not seem enough to just apply the lower level of yeast extract, as the negative effect seemed to be more drastic compared to shake flask fermentations [14, 28]. Therefore, another Box–Behnken design was carried out to explore further lower concentrations of yeast extract compared to the lower level of the original design which was at 20 g/L (Fig. 3). Although, even at these lower concentrations the negative effect did not seem to vanish completely, a level of 8.1 g/L seems to result in the highest amount of MK-7, which is superior to the conditions where yeast extract was eliminated and soytone was the sole nitrogen source (Fig. 4).

Table 4 ANOVA output for MK-7 concentrations (mg/L) versus glycerol, yeast extract and soytone initial concentrations (g/L) in the lower concentration composition medium

Source	*df*	Seq SS	Contribution (%)	Adj SS	Adj MS	*F* value	*P* value
Regression	9	0.09	92.0	0.09	0.01	6.36	0.028
Glycerol	1	0.03	25.2	0.01	0.01	8.98	0.030
Soytone	1	0.007	7.2	0.006	0.006	3.92	0.104
Yeast extract	1	0.0007	0.7	0.008	0.008	5.05	0.075
Glycerol × glycerol	1	0.04	44.3	0.01	0.01	7.84	0.038
Soytone × soytone	1	0.003	3.4	0.007	0.007	4.38	0.091
Yeast extract × yeast extract	1	0.00004	0.04	0.0007	0.0007	0.42	0.545
Glycerol × soytone	1	0.0003	0.3	0.00001	0.00001	0.01	0.930
Glycerol × yeast extract	1	0.004	3.7	0.0008	0.0008	0.51	0.507
Soytone × yeast extract	1	0.007	7.2	0.007	0.007	4.48	0.088
Error	5	0.008	8.0	0.008	0.002		
Lack-of-fit	3	0.007	7.4	0.007	0.002	7.17	0.125
Pure error	2	0.0007	0.7	0.0007	0.0003		
Total	14	0.1	100.0				

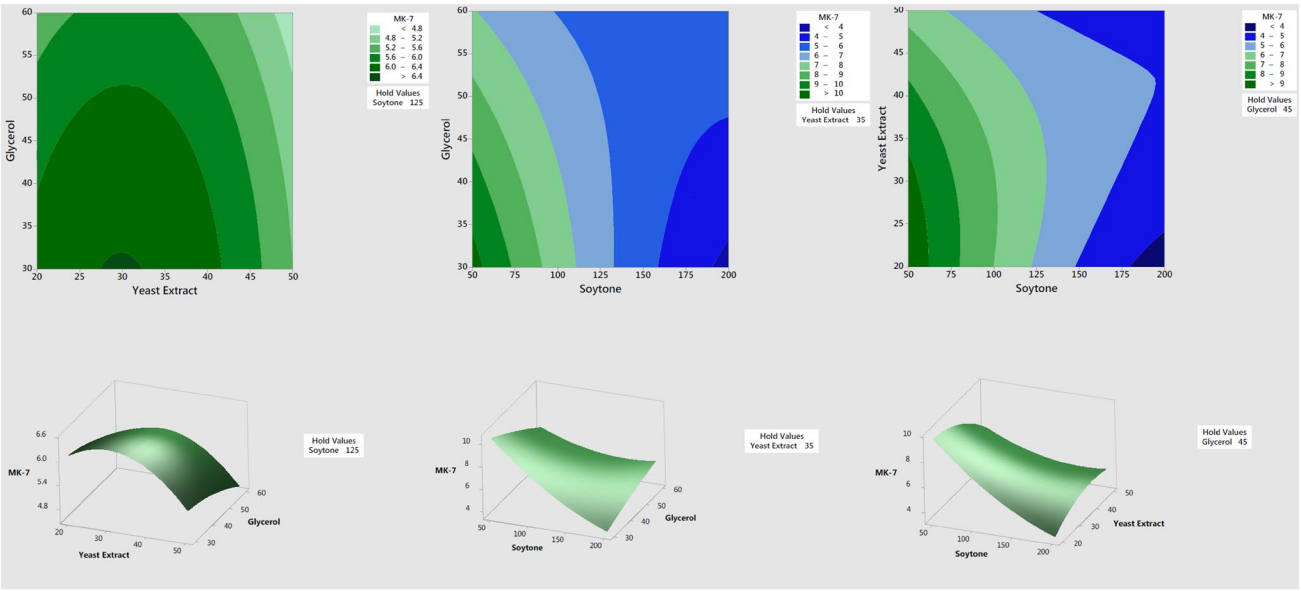

Fig. 1 RSM surface and contour plots for glycerol, yeast extract and soytone effects on MK-7 production in higher concentrations of composition medium in biofilm reactors at 35 °C, 200 rpm and pH 6.6

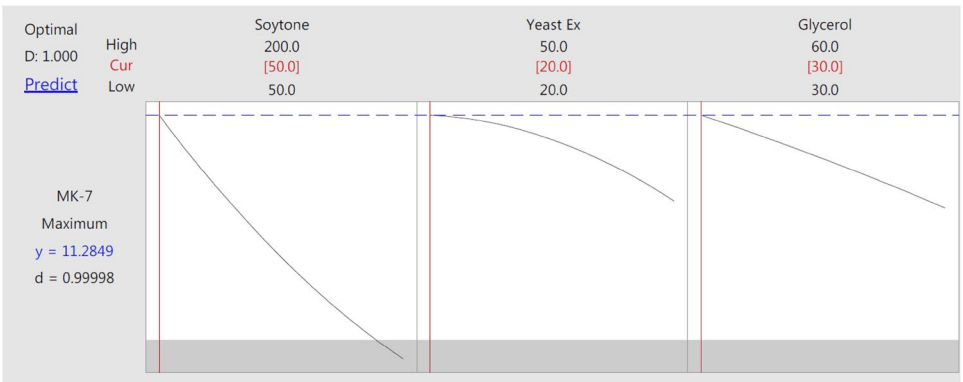

Fig. 2 Optimized concentrations of glycerol, yeast extract and soytone for maximum MK-7 production in higher concentrations of composition medium in biofilm reactors at 35 °C, 200 rpm and pH 6.6

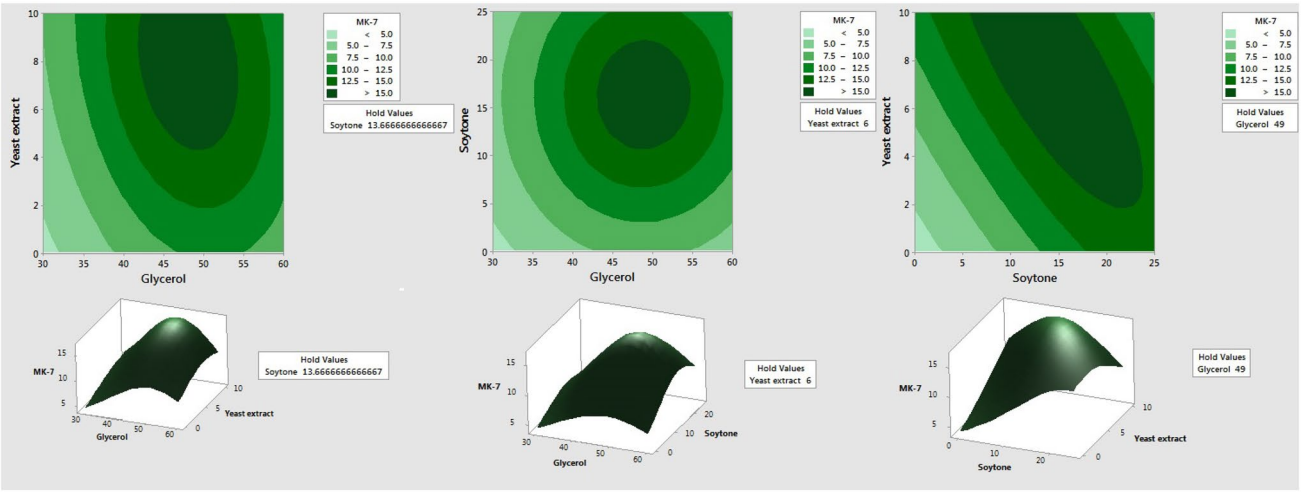

Fig. 3 Effects of combinations with lower concentrations of yeast extract and soytone in the media on MK-7 biosynthesis in biofilm reactors at 35 °C, 200 rpm and pH 6.6

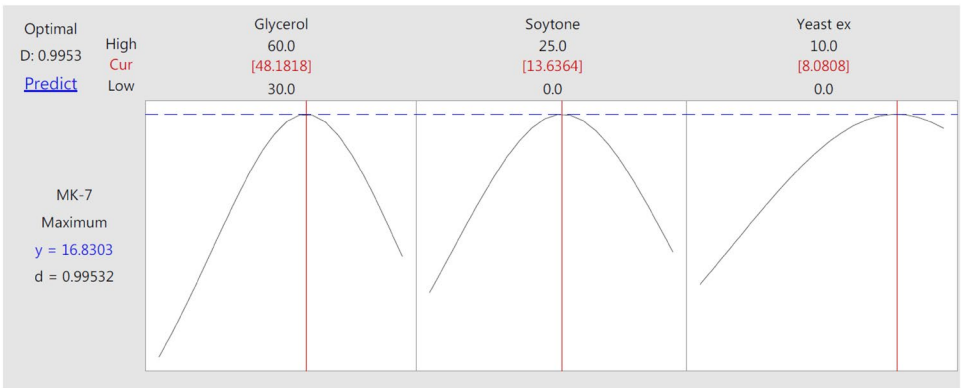

Fig. 4 Optimum concentrations of glycerol (48.2 g/L), yeast extract (8.1 g/L) and soytone (13.6 g/L) for maximum MK-7 production (16.8 mg/L predicted) in lower concentrations of composition medium in biofilm reactors at 35 °C, 200 rpm and pH 6.6

Soytone

Similar to yeast extract, soytone also posed a negative effect on MK-7 synthesis in levels of 50–200 g/L. Naturally, soytone interaction with yeast extract showed comparably significant effects on MK-7 biosynthesis (Table 2), since both are potent sources of amino acids for the fermentation. As Fig. 2 clearly illustrates, the negative effects associated with soytone are even greater than those of yeast extract. Increasing soytone concentrations from 50 to 200 g/L may decrease MK-7 concentration from over 10 mg/L to less than 4 mg/L (over 60% decrease). On one hand, this makes sense since applied soytone concentrations were much higher than yeast extract. But, on the other hand, soytone effects were positive in previous studies in shake flasks and a rather high concentration of 189 g/L was selected for fermentation [28]. However, in biofilm reactors it seems that the negative effects appear since the role of soytone in the fermentation can be played with lower concentrations required. Yet, MK-7 biosynthesis is closely dependent on soy protein products being present in the medium. Either soytone or soybean extracts at rather high concentrations have been a key composition for MK-7 biosynthesis [14, 16, 28, 29]. Soytone is an enzymatic digestion product of soybean proteins and soybean extracts are byproducts of natto manufacturing where soybeans are steamed before being inoculated with *B. subtilis*. Besides MK-7 dependency on KBF, heme expression is also critical, which requires amino acids coming from easily metabolized nitrogen sources such as yeast extract and soytone [14]. Furthermore, *B. subtilis* species are potent spore former strains and sporulation is triggered in them by nitrogen source starvation [37]. Since MK-7 biosynthesis continues until several days in LSF, it is essential to prevent nitrogen starvation and sporulation which would naturally terminate the biosynthesis. That seems to be the vital role that in this case soytone plays and the reason that soytone was favorable at 13.6 g/L, as the second design revealed (Fig. 4).

Glycerol

Glycerol is the primary carbon source in this study and is believed to have effects beyond a mere carbon source for MK-7 biosynthesis. Figure 2 indicates that at levels 30–60 g/L in the more concentrated medium composition in biofilm reactors, glycerol has a constant negative effect. This is in agreement with previous evaluation of glycerol in a similar glycerol-based medium in shake flasks where a similar negative effect resulted in choosing the lower level of glycerol in the central composite design (CCD) at 50 g/L [28]. Similarly, a 5% glycerol content, and not a higher concentration, was concluded to be optimal along with 10% soybean extract being implemented in the shake flasks [29]. Naturally, as Fig. 2 suggests, the lower concentration level of glycerol being 30 g/L is desirable for MK-7 expression. However, in the less concentrated medium composition, 48.2 g/L of glycerol was deemed optimum for MK-7 biosynthesis (Fig. 4). Figure 5 indicates that even when applying 48.2 g/L of glycerol in the medium, glycerol is depleted in the biofilm reactors by the middle of the fifth day (where MK-7 concentration is about 12.7 mg/L), whereas MK-7 concentrations continue to rise until the end of the sixth (to about 14.7 mg/L). Eventually, despite the fact that the metabolic negative effects of glycerol may persist at these concentrations, concentrations lower than 45 g/L are not feasible simply because they cannot sustain the metabolism long enough for the MK-7 concentration to peak.

It is known that in *B. subtilis* strains, the majority of phospholipids consist of phosphatidylglycerol, cardiolipin and phosphatidylethanolamine. Also, it has been reported that glycerol addition at different stages of cell density significantly influences the phospholipids' composition of cell membranes and as MK-7 is a membrane-associated compound, one possibility is that its biosynthesis could be affected by the effect of glycerol addition on cell membranes [30].

Moreover, glycerol has been found to have a suppressing effect on biopolymers' production in bacteria such as poly(hydroxyalkanoate) in *Pseudomonas corrugate* [38] and ε-poly-L-lysine in *Streptomycetaceae* [39] and decrease the fermentation viscosity, which might boost the mass transfer and stimulate the uptake of extracellular substrates. The difference between the MK concentrations and cell growth rates in media with and without glycerol addition may be due to this medium composition phenomenon. Or as an alternative explanation glycerol might similarly suppress the production of glycopeptide biopolymers in *B. subtilis* strains and since extracellular MK is presumed to have a role in extracellular matrix carboxylation made of these biopolymers, it indirectly induces the MK expression.

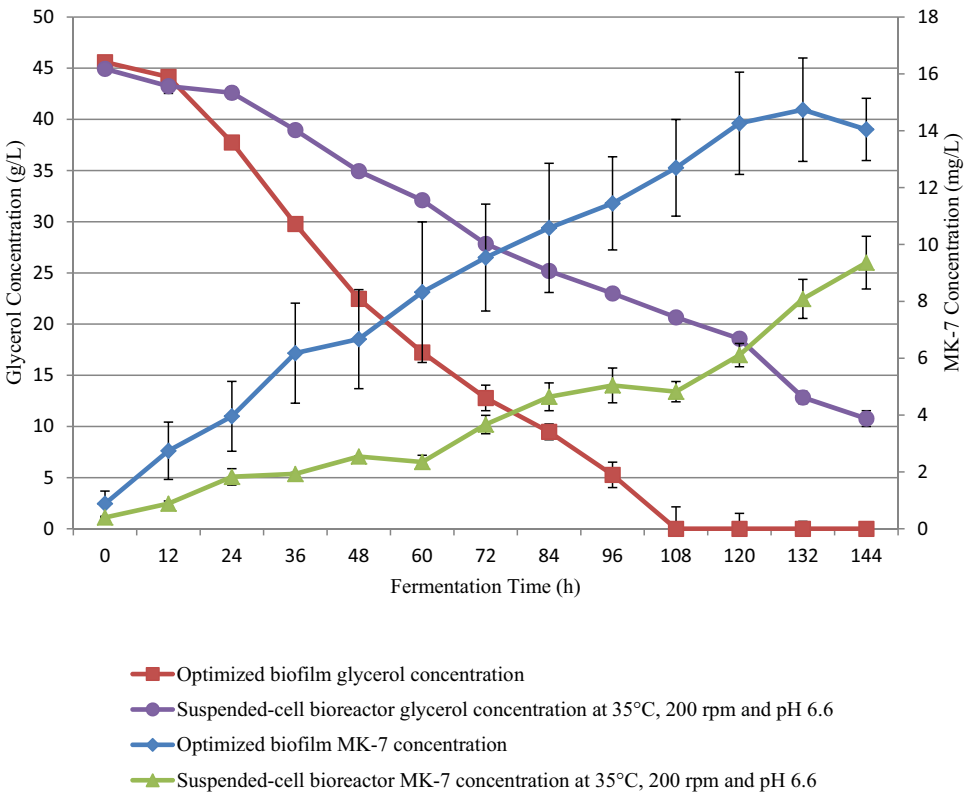

Fig. 5 MK-7 and glycerol concentrations in biofilm reactors versus suspended-cell reactors with optimized medium composition of 48.2 g/L glycerol, 8.1 g/L yeast extracts, 13.6 g/L soytone and 0.06 g/L K_2HPO_4 at 35 °C, 200 rpm and pH 6.6

Optimum conditions in biofilm reactors

After the application of ANOVA on the second design, the MK-7 biosynthesis model was obtained:

$$\begin{aligned}[\text{MK-7}]^{-0.5} = {} & [1.478] - [\,0.0386]\,\text{Glycerol} - [0.01872]\,\text{Soytone} - [0.0441]\,\text{Yeast extract} \\ & + \left[0.000381\right]\text{Glycerol} \times \text{Glycerol} + \left[0.0004\right]\text{Soytone} \times \text{Soytone} \\ & + [0.00107]\,\text{Yeast extract} \times \text{Yeast extract} - [0.000011]\,\text{Glycerol} \times \text{Soytone} \\ & + \left[0.000265\right]\text{Glycerol} \times \text{Yeast extract} + \left[0.001029\right]\text{Soytone} \times \text{Yeast extract}. \end{aligned} \quad (1)$$

Equation 1 indicates the full quadratic statistical model that can explain the effects on MK-7 biosynthesis. The model has a fairly accurate capability in predicting the MK-7 concentrations within this range ($R^2 > 0.91$) with an insignificant lack-of-prediction effect ($P > 0.125$). By plotting the values for MK-7 concentration predicted by the model versus the observed values in the design, a precision of 84.3% can be attained (data not shown). The optimizer software suggests a highest concentration of MK-7 at 16.8 mg/L with 48.2 g/L glycerol, 13.6 g/L soytone and 8.1 g/L yeast extract applied. With these concentrations applied, however, MK-7 concentrations were as high as 14.7 ± 1.4 mg/L (87.5% accuracy).These concentrations were also 57.4% higher than those achieved in suspended-cell reactors where MK-7 concentrations could not exceed 9.4 ± 0.9 mg/L without the effects of biofilm formations. As Fig. 5 shows, there is a clear gap between MK-7 biosynthesis in biofilm reactors and suspended-cell reactors with same conditions. In biofilm reactors, MK-7 concentrations peak at concentrations above 14 mg/L by the 132th h, whereas in suspended-cell reactors concentrations cannot even reach a peak by the end of the sixth day. This is due to the fact that glycerol is much more efficiently taken up and metabolized by the biofilm formations in biofilm rectors where the entire glycerol content is depleted within 108 h; and as such efficient infrastructures do not exist in suspended-cell reactors, glycerol concentrations in them do not even go below concentrations at 10 g/L at the end of the 144 h period. Since in both biofilm reactors and suspended-cell reactors, MK-7 concentration follow an almost constant increase until the end of the 6 day period, since concentrations in the biofilm reactors are significantly higher (14.7 ± 1.4 mg/L compared to 9.4 ± 0.9 mg/L), biofilm reactors give higher productivities. On the other hand, glycerol is a waste byproduct of biodiesel production and quite abundant and cheap. Therefore, glycerol recycling does not make economic sense in this case and therefore suspended-cell reactors do not really present any higher yields. Furthermore, the higher productivity achieved in biofilm reactors seem to be much more imperative when considering operational costs. This is once again evidence of how biofilm reactors can boost the MK-7 biosynthesis for all the medicinal applications of it [40, 41].

Conclusions

Using two Box–Behnken designs and RSM analyses, effects of glycerol, soytone and yeast extract were investigated on MK-7 biosynthesis in biofilm reactors. Full quadratic model with $R^2 > 0.91$ was obtained to predict the effects at optimum conditions. Results indicated that in a medium composition of 48.2 g/L glycerol, 8.1 g/L yeast extracts, 13.6 g/L soytone and 0.06 g/L K_2HPO_4, MK-7 concentrations could reach 14.7 ± 1.4 mg/L in biofilm reactors (which was 57.4% higher compared to suspended-cell reactors) and glycerol was depleted by the end of the fifth day. Under similar conditions in suspended-cell reactors, MK-7 concentration could not go beyond 9.3 ± 0.9 mg/L and glycerol levels were not depleted. This was once again a sure sign how biofilm reactors can improve MK-7 production in LSF strategies. For future studies, it may be possible to further remedy the negative effects caused by carbon source inhibitions by implementing a fed-batch strategy for glycerol in the medium.

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