Bioprocess Biosyst Eng (2018) 41:195–204
DOI 10.1007/s00449-017-1857-0

RESEARCH PAPER

Optimization of *Bacillus subtilis natto* growth parameters in glycerol-based medium for vitamin K (Menaquinone-7) production in biofilm reactors

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Received: 21 June 2017 / Accepted: 16 October 2017 / Published online: 8 November 2017

Abstract Menaquinone-7 (MK-7) is the key form of vitamin K used as a dietary supplement and its production revolves around *Bacillus subtilis natto*. Current fermentation strategies, which suggest static fermentations without aeration and agitation, can be problematic for large scale MK-7 production due to biofilm formation. The use of biofilm reactors, therefore, is proposed in the present study, which could utilize both agitation and aeration without interrupting MK-7 secretion. In this study, biofilm reactors were constructed using the selected plastic composite support (PCS) and *B. subtilis natto* strain for MK-7 production. Using response surface methodology (RSM), optimum growth parameters including temperature, pH, and agitation were determined in a glycerol-based medium. Results were presented in a statistical model ($R^2 = 0.90$), leading to optimum growth conditions of temperature (35 °C), agitation (200 rpm) and pH (6.58). Model-predicted MK-7 concentration was validated and MK-7 concentration of 12.09 mg/L was produced in the biofilm reactor. The obtained concentration was 58% higher as compared to the suspended-cell culture (7.67 mg/L). The results of this study will provide a critical step towards improved industrial scale production of MK-7.

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

Introduction

Since 1935, when Henrick Dam's studies on a fat soluble anti-hemorrhagic factor led to the discovery of vitamin K, numerous studies have been carried out until today to characterize different types and better understand the effects and metabolism of it [1]. It did not take scientists long to discover that this vitamin has two major forms. The one that had smaller molecular weight, was less effective in treatment of symptoms and was mostly found in certain vegetables was called vitamin K1. The other one with typically larger molecular weight, was more effective and was mostly found in animal sources such as fish meals and liver oils was called vitamin K2 [2]. The K1 type, also known as phylloquinone, has a direct role in photosynthesis in plants, and therefore, is abundant in most green leafy vegetables as a food source for vitamin K [3]. Phylloquinone has a singular form and animal cells are capable to convert it to K2 subtypes [4]. K2 types are main storage forms in animals and have several subtypes that are characterized by the number of isoprenoid residues in their side chains, which is four residues in MK-4 and seven residues in MK-7 [5].

Later on, scientists discovered that many bacterial strains can produce K2 types especially long-chain subtypes (MK-7 to MK-11) as they play the same role of electron transports as other quinones in respiration [6]. This discovery opened a new window to produce other MK types through

Chemical compound studied in this article: Menaquinone-7 (PubChem CID: 5287554); glycerol (PubChem CID: 753); glucose (PubChem CID: 79025); dipotassium hydrogen phosphate (PubChem CID: 24450); sulfuric acid (PubChem CID: 1118); sodium hydroxide (PubChem CID: 14798); *n*-hexane (PubChem CID: 8058); 2-propanol (PubChem CID: 3776); methanol (PubChem CID: 887).

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fermentation and to utilize them as dietary supplements. Among these subtypes, MK-7 has definitely been most profoundly studied for this purpose as compared to the other common subtypes such as MK-4 [7]. Both of these subtypes are used in the US as dietary food supplements but MK-7 has superior effects in human metabolism [8, 9].

Health benefits of MK-7 for humans have been studied for several decades [10]. There are two major areas where MK-7 has shown promising effects; in reducing the risk of cardiovascular diseases [11, 12] and osteoporosis [13]. Therefore, such extraordinary benefits associated with MK-7 has expanded the demand for its industrial production through microbial fermentation [5].

When it comes to MK-7 fermentation, there are only a few strains of the *Bacillus* genus including *B. subtilis natto* [14], *B. licheniformis* [15] and *Bacillus amylolyquifaciens* [16] that have been studied for this purpose. All of these bacteria are aerobes and have a potent tendency to form pellicles and biofilms [5]. Solid state fermentation (SSF) strategies have been studied for MK-7 production [16, 17], but they are basically problematic to scale up, although they are generally associated with less operating costs [18]. On the other hand, liquid state fermentation (LSF) strategies without agitation or aeration preserve pellicle and biofilm formations that are beneficial to MK-7 biosynthesis, since MK-7 secretion is hypothesized to be effected by the precursors engaged in the formation of the extracellular matrices in the pellicle and biofilm formations [19, 20]; but creates operational issues including heat and mass transfer problems [21]. Thus, the ideal case is to create a suitable condition for biofilm formations to form and at the same time being able to robustly agitate and aerate the system. This is efficiently possible with the use of biofilm reactors [22].

In general, biofilm reactors harness the same extraordinary features that microbial cells gain by switching from planktonic cell form to mature biofilm form through passive immobilization [23]. Gaining more resistance to desiccation, grazing, and antimicrobial agents in the biofilm structure as well as boosted expression of certain metabolites through gene expression changes that occur due to immobilization, are among such features [24]. Production of many value-added products have been enhanced by the use of biofilm reactors and formation of biofilms [25–28].

In the past studies, different carbon sources have been investigated, namely glucose, mannose, sucrose, etc [26]. However, glycerol-based media have been recommended due to the fact that glycerol is not only metabolized by B. subtilis as a cheap carbon source but also may improve MK-7 biosynthesis and secretion through cell membrane and media alterations [14]. Therefore, the aim of this study was to form biofilm reactors and evaluate and optimize the key growth factors effecting MK-7 production in *Bacillus subtilis natto*.

Materials and methods

Microorganisms and media

As previously described [5], *Bacillus subtilis natto* (NF1) were isolated from commercially available natto using tryptic soy agar plates and were grown at 40 °C for 24 h in Tryptic Soy Broth (TSB) (Difco, Detroit, MI) supplemented with 0.8% yeast extract (TSBYE) (Difco) as a base medium at static fermentation for inoculum preparation. TSB medium including 10% (w/v) glucose (Tate & Lyle, Decatur, IL) and 0.8% yeast extract (Biospringer, Milwaukee, WI) (TSBGYE) was used for biofilm formation. Main fermentation media consisted of 100 g of soytone (Difco), 35 g of yeast extract (Difco), 45 g of glycerol (EMD Chemicals, Gibbstown, NJ) and 0.6 g of K_2HPO_4 (VWR, West Chester, PA) per liter of deionized water, as suggested by previous studies with some modifications [26]. The cultures were stored at 4 °C and sub-cultured monthly to maintain viability. For long-term storage, stock cultures were maintained at − 80 °C in a 20% glycerol solution.

Biofilm reactors

Sartorius Biostat B Plus twin system bioreactors (Allentown, PA) equipped with 2 L vessels were used. During the fermentation, sterile 4N sulfuric acid (EMD) and 4N sodium hydroxide (Amresco, Solon, OH) along with antifoam B emulsion (Sigma–Aldrich, Atlanta, GA) were automatically added to the bioreactors 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 in the Center for Crops Utilization Research at Iowa State University (Ames, IA) using a twin-screw co-rotating Brabender PL2000 extruder (model CTSE-V; C.W. Brabender Instruments, Inc., South Hackensack, NJ) as described by Ho et al. [27]. PCS tubes were cut in about 6.5 cm lengths and the grid-like fashion was formed on the agitator shafts (Fig. 1) as described in previous studies [27, 28].

Biofilm formation

To form the biofilms on the PCS grids, bioreactors were set up as described above 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. After sterile TSBGYE medium replaced aseptically the initial water, bioreactors were inoculated with 3% (v/v) 24-h grown suspended-cell culture at 25 °C. Then, TSBGYE medium

Fig. 1 PCS grid formation in bioreactors

was refreshed every 48 h for four times to allow biofilm formations on the PCS. Once the biofilm was in place, the fermentation broth was sampled and Gram-stained to verify a pure culture.

Experimental design

Response surface methodology (RSM) was used to investigate the effects of three growth variables each with three levels being temperature (35–45 °C), pH (6–8) and agitation (100–200 rpm) chosen in this study on MK-7 concentrations. A total of 15 consecutive batch fermentations were carried out and each run was 144 h long and samples were taken every 12 h before the medium was refreshed for the next set of conditions of the next batch. After each batch completion, medium was completely pumped out aseptically so that only biofilm formations on the PCS would remain and then quickly, fresh sterile medium was pumped into the fermenters to start the next batch. Maximum MK-7 concentration was treated as the sole response (Table 1). After optimization using the responses, repeated validation runs in biofilm reactors were performed by under suggested optimum growth parameters. In addition, repeated suspended-cell fermentations as a control were also performed and compared with the biofilm reactors. Furthermore, repeated bioreactors implemented with PCS tubes the same as biofilm reactors were carried out; but in this case, the biofilm formation steps were not carried out to provide comparison to illustrate the effects of the biofilm formation steps.

MK-7 measurement

MK-7 (Menaquinone-7) was extracted from 3 mL of fermentation broth using 2:1, v/v *n*-hexane:2-propanol mixture [26]. In this study, *n*-hexane:2-propanol (2:1, v/v) with 1:4 (liquid:organic, v/v) was used for MK-7 extraction. The mixture was vigorously shaken with a vortex mixer for

Table 1 Response surface methodology design including variable temperature (°C), pH, agitation (rpm) and 1 vvm aeration in predicting MK-7 concentrations (mg/L) in biofilm reactors

Run	Growth factors			MK-7 Concentration (observed) (mg/L)	MK-7 Concentration (predicted) (mg/L)
	Temperature (°C)	pH	Agitation (rpm)		
1	40	7.5	150	11.92	10.36
2	45	8.0	150	9.14	8.59
3	45	6.0	150	3.69	4.11
4	35	7.5	200	12.65	12.52
5	45	7.5	100	7.88	8.195
6	40	6.0	200	10.96	9.82
7	40	6.0	100	6.82	6.80
8	40	8.0	200	9.19	9.58
9	35	6.0	150	11.75	12.50
10	40	7.5	150	10.03	10.36
11	45	7.5	200	10.97	11.21
12	40	8.0	100	6.85	6.56
13	35	7.5	100	10.16	9.51
14	35	8.0	150	7.09	7.55
15	40	7.5	150	8.87	10.36

3 min and then the organic phase was separated and evaporated under forced air flow. Then, dried pellets were dissolved in methanol in a Biosonic ultra-sonication water bath (Cuyahoga Falls, OH) 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). High performance liquid chromatography (HPLC) (Waters, Milford, MA) equipped with a 2489 UV/Visible detector and a Supelcosil C18 column (15 cm × 4.6 mm, 5 µm, Supelco Analytical, Bellefonte, PA) 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 used for calibration and analysis. The MK-7 calibration curve obtained from 99.9% pure MK-7 (Chroma Dex Corporation, Irvine, CA) was linear between 0.1 and 30 mg/L ($R^2 = 0.999$).

Glycerol measurement

Glycerol is the primary carbon source in fermentations in this study. In addition, glycerol is believed to affect MK-7 biosynthesis through altering cell membrane composition in *B. subtilis* and medium viscosity [14]. Therefore, it is essential to investigate and monitor the consumption of glycerol concentration throughout the runs. In this fashion, fermentation broth was centrifuged at 9000*g* for 5 min (Microfuge 20 Series, Beckman Coulter Inc., Brea, CA) 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) 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 ($R^2 > 0.999$).

Statistical analysis

Using Minitab 17.0 Analysis of Variance (ANOVA) (Minitab Inc., State College, PA) with statistical model and regression analysis with Box–Cox transformation optimal λ, the effects of temperature (°C), pH and agitation (rpm) along with second order and two-way interaction effects were obtained. Minitab is able to design RSM tables and analyze the sole or several responses (in this case MK-7) that are measured in the experiments through ANOVA or analysis of covariance (ANCOVA) to determine statistically significant differences in the responses and through optimization determine the optimal conditions (in this case temperature, pH and agitation) for extremum (in this case maximum) response(s). Since it was hypothesized that higher levels of interactions are not significantly effective, ANOVA was selected instead of ANCOVA. A confidence level of 95% was implemented throughout the analysis procedures to distinguish significant parameters [30, 31].

Results and discussions

Temperature, pH and agitation were chosen as the key factors to optimize *B. subtilis natto* growth in glycerol-based medium for MK-7 production in biofilm reactors. Table 1 shows the factorial design used and the observed MK-7 values in the analysis and predicted values from the model. As mentioned above, these responses were the maximum MK-7 concentrations observed in each run in the design, which were almost entirely obtained towards the end of the runs in the sixth day of fermentation. The maximum observed value in the entire runs was 12.65 mg/L at 35 °C, pH 7.5 and 200 rpm (Table 1). Following ANOVA of these observed values rendered a statistical model to explain the effect of each variable on MK-7 concentrations.

Temperature

In this study, it was concluded that temperature affects MK-7 concentrations in biofilm reactors in a different way as compared to the results reported in previous studies, despite the fact that similar *B. subtilis natto* strains were used in both studies. In the respective studies, 40 and 45 °C were deemed best temperature for MK-7 secretion [21, 32] but in this study, 35 °C was found to be the optimum (Fig. 2). Although similar medium compositions and bacterial strain were utilized in these studies, it must be taken into consideration that fermentation in shake flasks and biofilm reactors are distinctive enough to cause such differences. In this case, temperature effects were statistically most significant as compared to agitation and pH (p value < 0.000). The RSM design temperature range was 35–45 °C and ANOVA analyses indicated a constant increase in MK-7 secretion as temperature decreased (Fig. 3b, e); rendering 35 °C to be optimum (Fig. 2). However, further tests in lower temperatures (25, 28, and 30 °C) indicated that 35 °C is in fact the optimum temperature in this case (Fig. 4). Temperatures less than 30 °C are not really expected to work anyway since glycerol as the primary carbon source is elaborative to metabolize for *Bacillus* strains [33] and low temperatures certainly do not help. Especially going down from 28 to 25 °C hindered the metabolism as glycerol consumptions

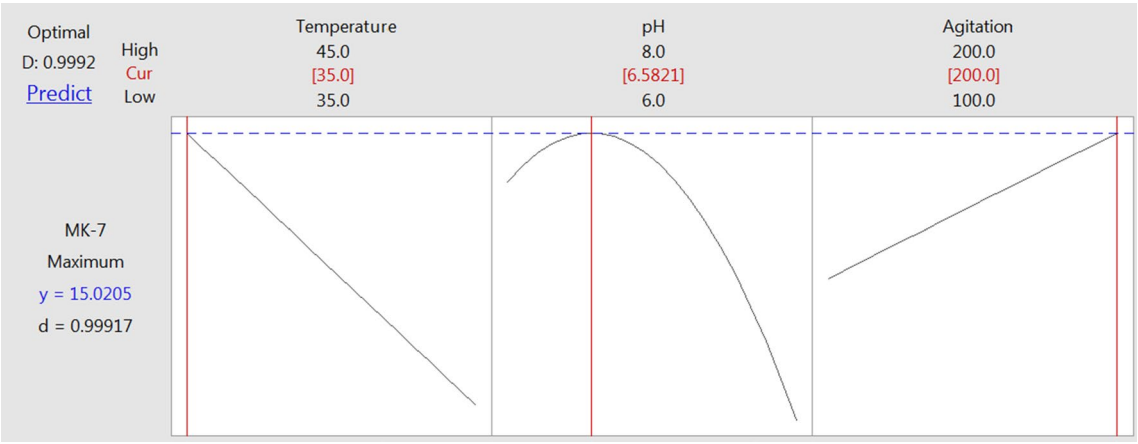

Fig. 2 Optimal conditions and the optimal MK-7 concentration predicted by the model optimizer in biofilm reactors with 1 vvm aeration

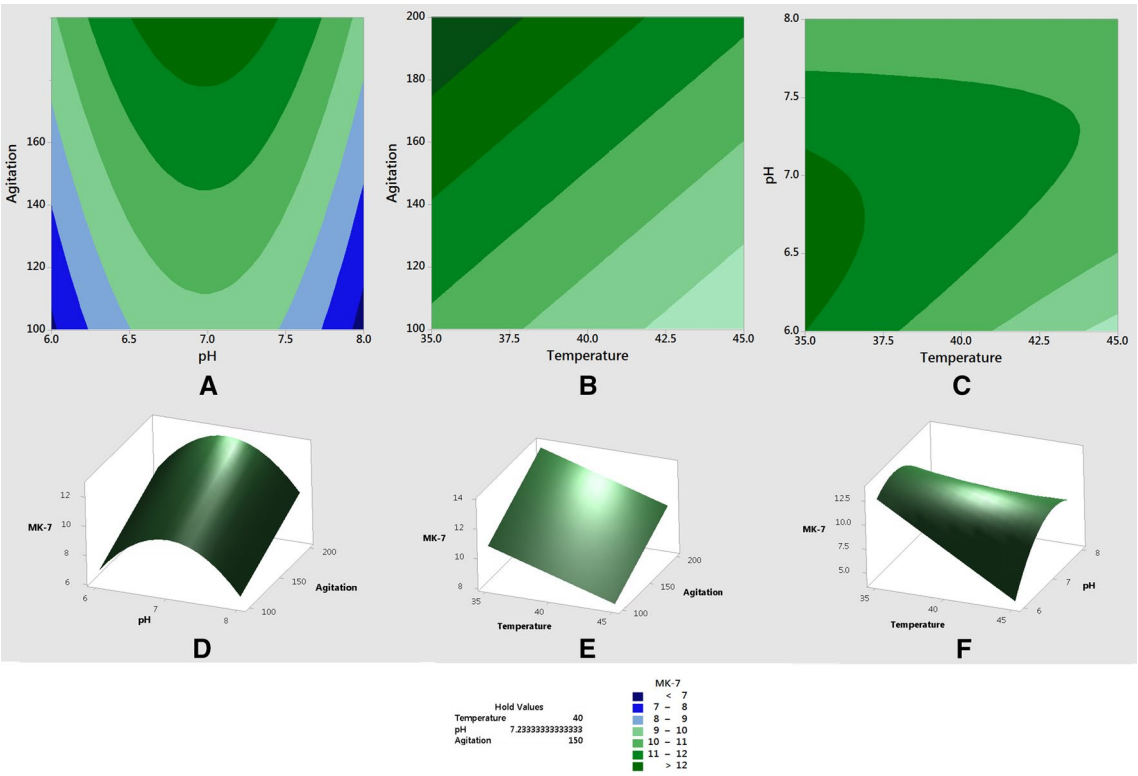

Fig. 3 Contour and surface plots generated by the model for MK-7 concentration (mg/L) behaviors with response to different temperature (°C), pH and agitation (rpm) in biofilm reactors with 1 vvm aeration

were declined by around 80% which explains the insignificant MK-7 concentrations in those temperatures (Fig. 4).

pH

In previous studies in the literature, pH was never controlled as it was believed that significant pH changes that occur during the fermentation is important for MK-7 secretion [14, 34]. However, in biofilm reactors, pH controlling seems to be beneficial to MK-7 secretion despite the fact that without pH control, a similar pH profile is observed throughout the fermentation as compared to Sato and Berenjians' findings (Fig. 5). pH itself was found statistically significant by second order (p value < 0.002) (Table 2) and RSM plots confirmed a significance of pH–temperature interaction (Fig. 3c, f) that was originally predicted by ANOVA

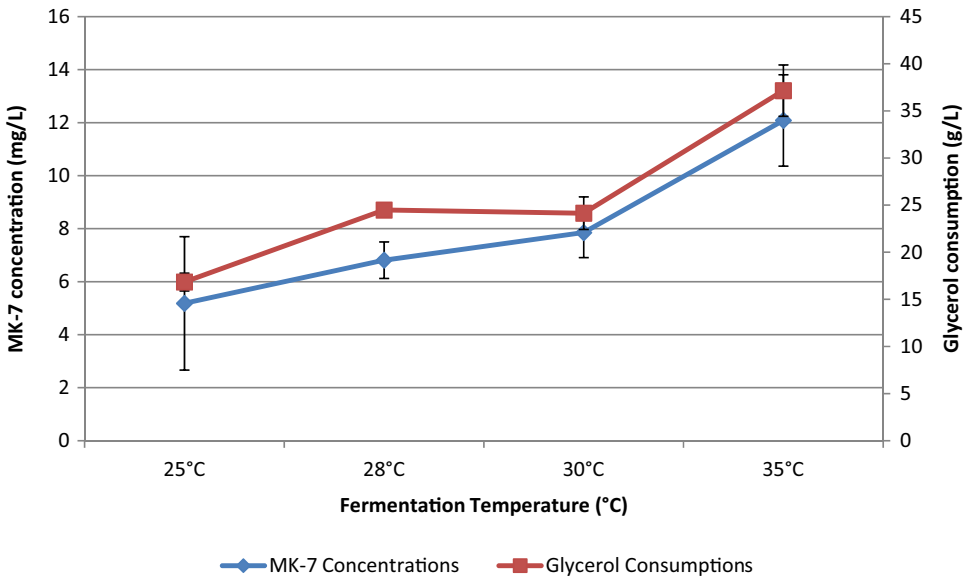

Fig. 4 MK-7 concentrations (mg/L) (repeated) and glycerol consumptions (g/L) (repeated) observed under pH 6.58 and agitation 200 rpm and temperatures equal and below 35 °C in biofilm reactors with 1 vvm aeration

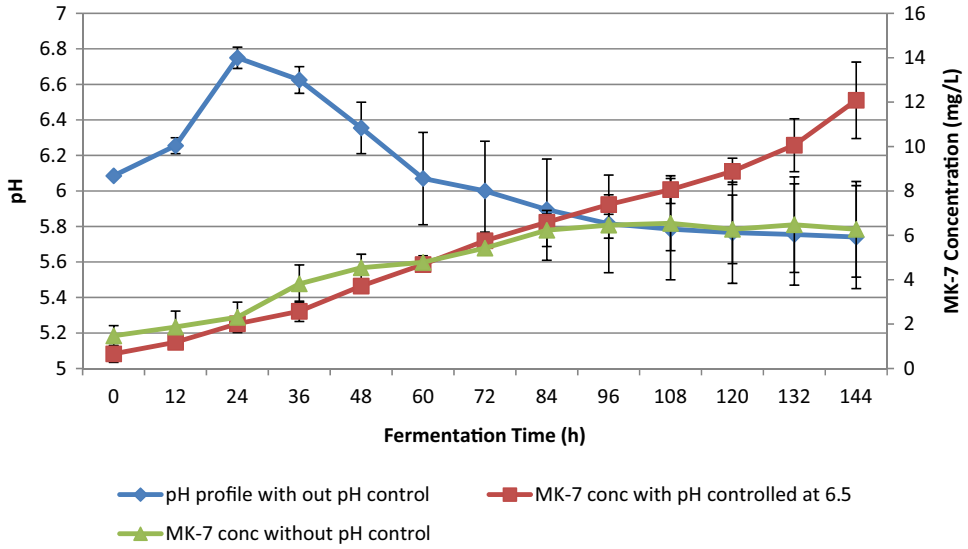

Fig. 5 MK-7 concentrations (mg/L) (repeated) with pH controlled at 6.58 and without pH control both in biofilm reactors under optimal temperature (35 °C), agitation (200 rpm) and 1 vvm aeration

Table 2 ANOVA output for MK-7 concentrations (mg/L) versus temperature (°C), pH, agitation (rpm) and 1 vvm aeration in biofilm reactors

Source	DF	Seq SS	Contribution (%)	Adj SS	Adj MS	*F* value	*p* value
Regression	5	73.31	89.98	73.31	14.66	16.17	0.000
Temperature	1	12.42	15.25	28.51	28.51	31.43	0.000
pH	1	1.08	1.32	4.40	4.40	4.85	0.055
Agitation	1	18.18	22.31	18.18	18.18	20.04	0.002
pH × pH	1	16.60	20.38	16.60	16.60	18.31	0.002
Temperature × pH	1	25.03	30.72	25.03	25.03	27.60	0.001
Error	9	8.16	10.02	8.16	0.91		
Lack-of-fit	7	3.42	4.20	3.42	0.49	0.21	0.952
Pure	2	2.00	474.20	4.74	2.37		
Total	14	81.475	100.00				

(Table 2). Thus, pH and temperature two-way interaction was statistically significant and incorporated into the finalized model (p value < 0.001) and an optimum pH of 6.58 was determined by RSM (Fig. 2). Furthermore, MK-7 concentrations decreased when pH was not controlled as compared to the pH-controlled fermentation at the optimum pH (6.58) (Fig. 5). This observation is yet another proof of how significantly scaling up production can effect MK-7 secretion, in this case from shake-flask fermentations to 1.5 L benchtop bioreactors. Similar to low temperatures, extremely high pH conditions seem to have similar effects; maintaining pH conditions above 8 completely inhibits metabolism although under extreme nutrient deficiencies pH may naturally reach pH 9 in the fifth or sixth day (data not shown).

Agitation

Generally for aerobic fermentations in biofilm reactors, oxygen transfer is a challenge [22]. Therefore, higher agitation and aeration rates are usually believed to be favorable in many cases. Similarly, higher agitation rates were also found to be favorable in this study for MK-7 fermentation (Fig. 3a, d). While aeration rates were kept at a constant 1vvm throughout all the experiments in this study and it is practically the highest feasible in the utilized bioreactors in the long-term, agitation rates were kept below 200 rpm. Higher agitation rates were possible, but not recommended since they may cause overstress on the PCS sticks on the propellers that may lead to biofilm detachment. Naturally, agitation was a significant factor (p value < 0.002) and the highest evaluated agitation rate (200 rpm) was recommended by the optimizer software as a constant MK-7 concentration enhancement was observed as agitation rates increased (Figs. 2, 3b, e).

Optimization of evaluated growth factors

Among these variables, all three were found significantly effective on MK-7 concentration (Table 2). Using ANOVA, the effective terms of the model were obtained as shown in Table 2. Despite the fact that the first order pH term was less significantly affecting the response (p value > 0.055), it was kept in the model to maintain hierarchy. The other terms, temperature and agitation were more significantly effective (p value < 0.000) (Table 2). The regressed model is displayed in Eq. 1.

$$\begin{aligned}\left[\text{MK}-7\ \text{Concentration}\left(\frac{\text{mg}}{\text{L}}\right)\right] &= [8.6]-[3.669]\\ &\times\left[\text{Temperature}\ (^{\circ}\text{C})\right]+[22.6]\times\left[\text{pH}\right]+[0.03015]\\ &\times\left[\text{Agitation}\ (\text{rpm})\right]-[2.971]\times\left[\text{pH}\right]\times\left[\text{pH}\right]+[0.4717]\\ &\times\left[\text{Temperature}\ (^{\circ}\text{C})\right]\times\left[\text{pH}\right]\end{aligned} \quad (1)$$

As also shown in Fig. 6, regression of the predicted values versus observed values renders a slope of 1.0 and $R^2 = 0.90$ with no significant abnormalities. Temperature of 35 °C, pH 6.5 and agitation of 200 rpm were predicted by the model to be optimum. Optimizing conditions targeting maximum MK-7 concentration, a maximum MK-7 concentration of 15.02 mg/L was predicted (Fig. 2). Thus, validating this prediction, a maximum concentration of 12.09 ± 1.72 was achieved, which is within a 75% accuracy range (Fig. 7). This accuracy is close to the prediction accuracy that the statistical model suggests (70.8%). However, 12.09 ± 1.72 mg/L concentration in the biofilm reactors is still 58% higher than the ones in suspended-cell reactors under the same optimum conditions (7.67 ± 2.15 mg/L) (Fig. 7), which clearly shows the contribution of biofilm reactors. In addition, increasing agitation rate from 200 to 250 RPM does not seem to meaningfully make a difference in MK-7 concentration or

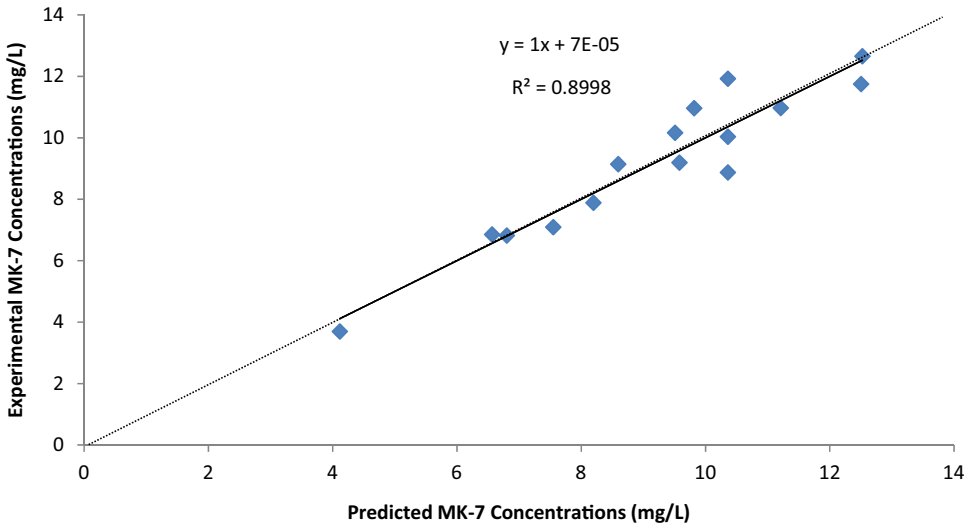

Fig. 6 Experimental MK-7 concentrations (mg/L) in biofilm reactors at pH 6.58, 200 rpm, 35 °C and 1 vvm plotted versus predicted values generated by the model

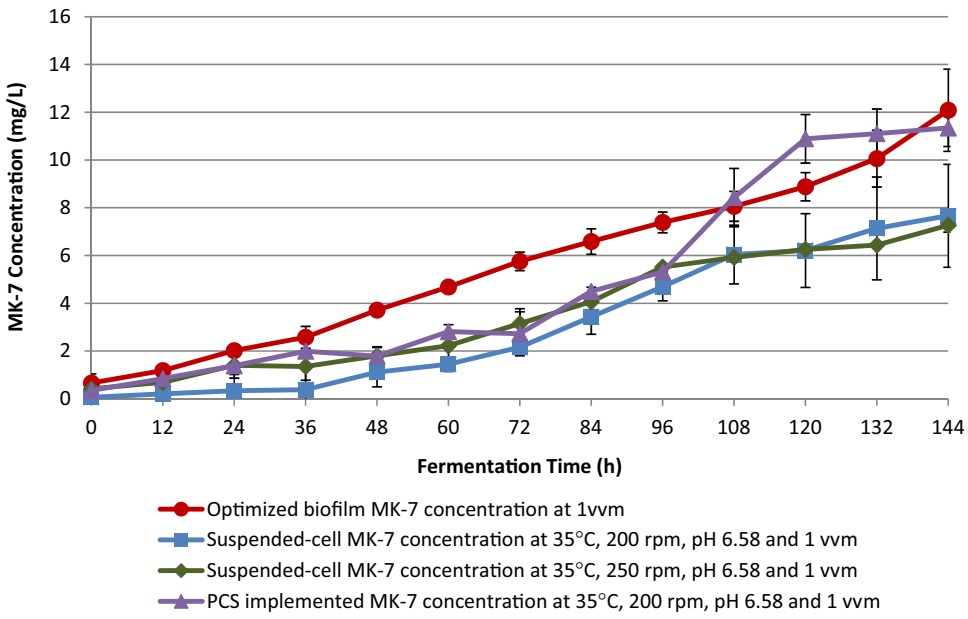

Fig. 7 MK-7 profile for fermentation in biofilm reactors, suspended-cell bioreactors and PCS implemented bioreactors (repeated)

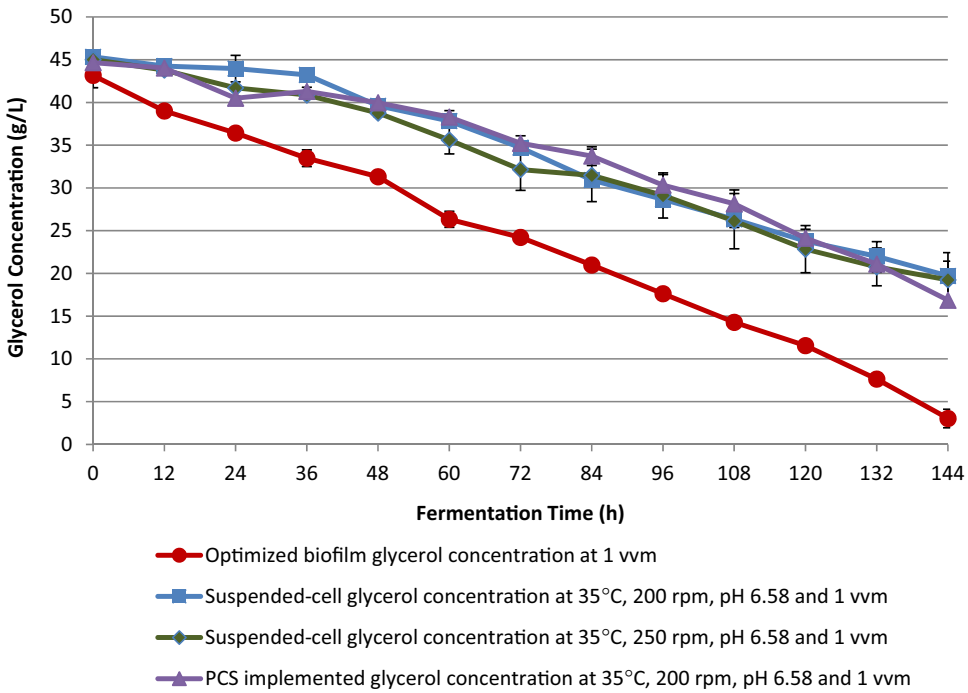

Fig. 8 Glycerol profile for fermentation in biofilm reactors, in suspended-cell bioreactors and PCS-implemented bioreactors (repeated)

even glycerol metabolism (Fig. 8). In addition, running PCS implemented bioreactors resulted in glycerol consumption patterns similar to suspended-cell reactors, although MK-7 profile seemed different. As Fig. 8 depicts, there is a clear gap between the glycerol consumption pattern in biofilm reactors and all the controls (suspended-cell at 200 or 250 rpm and PCS implemented), where glycerol is almost depleted in biofilm reactors but barely goes below 20 g/L in the controls (Fig. 8). This proves that biofilm formations on the PCS significantly boost the metabolism that also leads to enhanced MK-7 biosynthesis. As Fig. 7 indicates, again there is a meaningful gap between MK-7 biosynthesis profile in biofilm reactors compared to suspended-cell reactors. However, PCS implementation created a pattern in MK-7 biosynthesis similar to suspended-cell reactors until 96 h but then there was a sudden increase that led to concentrations similar to the ones in biofilm reactors by the end of the sixth day (Fig. 7). This is possibly due to the fact that biofilm formations are slowly forming on the PCS and kick in after 4 days of fermentation in the PCS-implemented bioreactors

and hence the sudden jump in concentrations. This only confirms how efficient biofilm formations are in enhancing metabolism in *B. subtilis* and consequently MK-7 biosynthesis. Therefore, this can be a critical step towards improved industrial scale production of MK-7 using biofilm reactors.

Conclusions

In this study, biofilm reactors were successfully constructed and used to produce MK-7. Using RSM with a general factorial design, temperature, agitation and pH were optimized to achieve the highest MK-7 biosynthesis in biofilm reactors. Maximum MK-7 concentration was achieved under the optimum conditions which was significantly higher than its counterpart in suspended-cell reactors. This finding promises a potential of using biofilm reactor in MK-7 production and addressing current issues. In addition, the results give a perspective towards future studies to optimize media components and investigate different media to even further enhance the concentrations.

Acknowledgements This study was funded in-part by the Pennsylvania Agricultural Experiment Station. The authors thank the Statistical Consulting Center at The Pennsylvania State University for their support in providing useful consultation for data processing.

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