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Reduced Biofilm Formation in Menaquinone-7 Production Process by Optimizing the Composition of the Cultivation Medium

Dinali Ranmadugala[1], Alireza Ebrahiminezhad[2,3,*], Merilyn Manley-Harris[1], Younes Ghasemi[3,4], Aydin Berenjian[1,*]

[1]Faculty of Science & Engineering, University of Waikato, Hamilton, New Zealand.

[2]Department of Medical Biotechnology, School of Medicine, and Noncommunicable Diseases Research Centre, Fasa University of Medical Sciences, Fasa, Iran.

[3]Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

[4]Department of Pharmaceutical Biotechnology, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran.

Abstract

Nutrient components in the culture medium can affect not only the growth and metabolic activity of bacteria, but also bacterial attachment to solid surfaces. Based on the fact that response surface methodology (RSM) has been successfully applied for the optimization of Menaquinone-7 (MK-7) production, the objective of this research was to investigate the feasibility of reducing biofilm formation with added nutrient components to the optimum medium previously described for MK-7 production without compromising MK-7 production. Monovalent and divalent salts and urea, which were suspected to have an impact on bacterial biofilms, were screened using a full factorial design. Calcium chloride and urea were found to significantly influence the biofilm biomass and MK-7 production (P<0.05). Central composite face design (CCF) was used for the optimization. The minimum biofilm biomass of 0.56 g and MK-7 concentration of 17.98 mg/L was achieved at the optimum conditions. This is the first report on biofilm reduction in the MK-7 production process through optimization of nutrient components.

Keywords: Biofilm formation, *B. subtilis*, Fermentation, MK-7, Response surface methodology.

1. Introduction

Vitamin K has many health benefits beyond its role in blood coagulation, such as improvement of bone health (1, 2) and the reduction of cardiovascular diseases (3). Menaquinones (vitamin K2), especially MK-7, is suggested to be more effective in these roles than phylloquinone (vitamin K1) (3, 4). However, the concentration of MK-7 in food products are very low, Therefore, MK-7 is mainly produced by *B. subtilis* fermentation on an industrial scale (1).

Nutrients have a marked effect on biofilm formation as well as MK-7 production by *B. subtilis*. Berenjian et al. (1) have previously developed an efficient media for high MK-7 production. However, it is well documented that the presence of complex nutrients in the liquid fermentation media influence bacterial attachment (5) and as a consequence biofilm formation posed a major problem in the MK-7 production process (6, 7). Reduction in biofilm formation would reduce costly periodic cleaning and improve mass transfer. Therefore, biofilm formation is an area worthy of serious at-

Corresponding Author: Aydin Berenjian, Faculty of Science & Engineering, University of Waikato, Hamilton, New Zealand.
Email: aydin.berenjian@waikato.ac.nz

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tention in the MK-7 production process. Common strategies for biofilm control are the use of biocides and disinfectants (8). However, in industrial production of MK-7, it is of the utmost importance that the removal strategy for biofilm should not affect bacterial cell viability. Furthermore, previous studies have demonstrated a linear dependency of MK-7 production on biofilm formation (6). Therefore, strategies to optimize the overall productivity of MK-7 production process with reduced biofilm formation and maximum MK-7 production, while maintaining the biological activity of bacteria, can be achieved either by reducing biofilm attachment (7) or enhancing biofilm detachment (9).

In addition to bacterial surface attachment, nutrient conditions in the medium can also greatly influence detachment (10). In this regard, supplementation of the optimum medium for MK-7 production with nutrient components that would promote biofilm detachment might achieve low biofilm biomass and optimum overall productivity in the MK-7 production process. Experimental evidence from the biofilm research field shows that biofilm detachment resides in the structural integrity of the biofilm (11). Structural integrity of biofilms in turn results from a combination of forces including long range van der Waals forces and short range forces such as electrostatic and hydrophobic interactions (9). Disruption of these forces can therefore bring about biofilm detachment. In recent years, the use of monovalent and divalent cations has presented as options for biofilm removal by changing the structure of the biofilm (9, 10,12). Apart from the salts, there are indications that nitrogen source influences the amount and size of exopolysaccharides (13), which are a major component of biofilm matrix. Urea for example, is a well-known organic nitrogen as well as a carbon source, which is said to speed up the growth of bacteria in many instances (14). While rapid growth rate itself can bring about biofilm biomass loss (11), experimental evidence show that urea can also act as a chaotropic agent that would disrupt the network of hydrogen bonding interactions responsible for biofilm cohesion (9, 12). Therefore, the aim of the present study was to optimize the cultivation medium composition to achieve the lowest possible biofilm biomass with the highest possible MK-7 production.

2. Materials and methods

2.1. Materials

Yeast extract, peptone from soy meal, calcium chloride, urea, absolute ethanol for analysis, 2-propanol, and *n*-hexane were obtained from Merck (USA). K_2HPO_4 was purchased from Scharlab S.L (Spain). Glycerol was purchased from Ajax Finechem (New Zealand). Sodium chloride was obtained from a domestic supplier. Lipase enzyme was obtained from Novozymes (Denmark). Pure MK-7 standard (97.6%) was purchased from ChramoDex (USA) for calibration and HPLC analysis. *Bacillus subtilis* (ATCC 6633) was obtained from the New Zealand reference culture collection (New Zealand).

2.2. Microorganism and inoculum preparation

B. subtilis (ATCC 6633) cells were cultured on nutrient agar plates at 37 °C for 2 days. Plates were scraped after 2 days and a spore suspension was prepared by suspending the cells in 0.9% (w/v) sodium chloride solution. The spore suspension was kept in a water bath at 80 °C for 30 min to kill the residual vegetative cells. Cell debris was removed by centrifuging at 3,000 rpm for 10 min.

2.3. Analytical methods

The optical density was measured at 600 nm with a UV spectrophotometer (UV-1700 PharmaSpec, Shimadzu, Japan). The pH was measured using a laboratory standard pH meter directly from the cultivation medium.

2.4. Extraction and analysis of MK-7

Extraction of MK-7 from the fermentation media (3 mL) was carried out using a mixture of propan-2-ol and n-hexane (2:1 v/v). The enzymatic hydrolysis of triglycerides through treatment with 1% lipase followed by ethanol-water (4 mL and 2 mL) treatment were carried out before extracting with propan-2-ol and *n*-hexane.

The MK-7 concentration was measured by high-performance liquid chromatography (HPLC). The HPLC system consisted of a HP 2440 (Waters Co., UK) pump with a photo diode array UV de-

tector and a C_{18} reversed-phase analytical column; Gemini 250 mm x 4.6 mm, i.d (Phenomenex). Mobile phase solvent was propan-2-ol and *n*-hexane (2:1, v/v). Flow rate was 0.5 ml/min and the injection loop volume was 20 µl. Detection was carried out with an excitation wavelength of 248 nm. The concentration was calculated by peak area, using a standard curve from a serially diluted standard solution of 0.2 mg/mL of MK-7 (Chromadex) (15).

2.5. Biofilm biomass assay

Pellicles were harvested and dried in an oven for five h at 40 °C to constant weight before measuring the dry weight. The dry weight was taken using a 5-digit balance.

2.6. Experimental design for screening medium components that affect biofilm formation and MK-7 production

A full factorial design was used to study the individual and interactive effects of the potent factors on biofilm formation and MK-7 production. Sodium chloride, calcium chloride, and urea were selected as potent factors. In all the experiments, the growth medium consisted of 5% w/v yeast extract, 18.9% w/v soy peptone, 5% w/v glycerol, and 0.06% w/v K_2HPO_4, which is the optimum medium previously described for MK-7 production (1).

B. subtilis were grown up to a turbidity of 0.5 McFarland standard ($OD_{600\ nm}$=0.1). The McFarland suspension was diluted 1:20 and treated with different concentrations of salts (NaCl and $CaCl_2$) and urea according to the full factorial design. Cultures were grown at 37 °C at 120 rpm for 60 h before screening for MK-7 production and biofilm biomass.

2.7. Optimization of the MK-7 production process

In the second phase, a full factorial central composite design (CCD) was applied for further optimization of the significant effective factors chosen from the screening step. Statistical analysis was performed using MODDE software version 9 (Umetrics, Sweden).

3. Results and discussion

3.1. Screening the effect of main factors on biofilm formation and MK-7 production applying a full factorial design

In order to study the effect of salts and urea on biofilm formation and MK-7 production, *B. subtilis* (ATCC6633) were grown in the optimum medium for MK-7 production previously described by Berenjian, et al. (1) with added salts and urea. When selecting parameters that affect biofilm formation, the main consideration was the parameters that would reduce/ remove biofilm formation without showing any detrimental effect on the bacterial growth.

The initial screening process was carried out using Design of Experiments (DOE) methodology with 3 different variable factors, namely NaCl, $CaCl_2$, and urea. The three distinct variables were exploited to investigate the most important factors for reducing biofilm biomass and increasing MK-7 production. Experimental range and levels of independent variables used for the full factorial design are presented in Table 1. Concentrations of these variables that were used in experimental design, and the two responses of biofilm formation and MK-7 production are presented in Table 2. Statistical analysis is provided in Table 3 to demonstrate the main effective factors on biofilm formation and MK-7 production. The linear regression coefficient (R^2) was 0.984 and 0.963 for MK-7 production and biofilm biomass, respectively. The R^2 adj

Table 1. Nutrient levels for microbial production of MK-7 and biofilm biomass Full-Factorial design.

Variable number	Variable name	Coded value		
		Low level (-1)	Central level (0)	High level (+1)
X1	NaCl	0	0.5	1
X2	$CaCl_2$	0	0.5	1
X3	Urea	0	2.5	5

Table 2. The effect of selective variables on MK-7 production and biofilm biomass of *B. subtilis*.

Run	Variable factors [a]			MK-7 production (mg/L)	Biofilm biomass (g)
	NaCl% (w/v)	CaCl2% (w/v)	Urea % (w/v)		
1	0 (-1)	0 (-1)	0 (-1)	22.94	0.76637
2	1 (+1)	0 (-1)	0 (-1)	21.91	0.74428
3	0 (-1)	1 (+1)	0 (-1)	15.81	0.44331
4	1 (+1)	1 (+1)	0 (-1)	12.42	0.42380
5	0 (-1)	0 (-1)	5 (+1)	13.09	0.57875
6	1 (+1)	0 (-1)	5 (+1)	11.17	0.54652
7	0 (-1)	1 (+1)	5 (+1)	12.38	0.38612
8	1 (+1)	1 (+1)	5 (+1)	6.78	0.43780
9	0.5 (0)	0.5 (0)	2.5 (0)	16.36	0.51557
10	0.5 (0)	0.5 (0)	2.5 (0)	14.32	0.48102
11	0.5 (0)	0.5 (0)	2.5 (0)	15.12	0.49195

[a]values are expressed in (%w/v).

values for MK-7 production and biofilm biomass were 0.960 and 0.907, respectively. The data show the validity of the two models, which is visually displayed by the coefficient plots (Figure 1).

3.1.1. Effect of salts on biofilm biomass and MK-7 production

The monovalent salt (NaCl) and the divalent salt ($CaCl_2$) were evaluated to find out their influence on MK-7 production and biofilm formation. As illustrated in Table 3 and Figure 1, the presence of NaCl had no significant impact on biofilm formation by *B. subtilis*; although earlier reports suggested that sodium chloride (NaCl) promotes biofilm detachment (9, 12) as well as reducing the bacterial gel strength of extracellular polymeric substances (16).

However, the presence of $CaCl_2$ significantly reduced the biofilm biomass ($P<0.05$). According to the coefficient plots, $CaCl_2$ showed the highest influence on biofilm biomass. These findings were similar to the previous studies where calcium chloride is said to reduce the biofilm by disrupting biofilm crosslinking forces such as electrostatic interactions and destroying structural biofilm components (9, 12). There are other reports showing that calcium can increase bacterial adhesion in a concentration dependent manner (17). However, Das et al. (18) reported that Ca^{2+} could increase biofilm biomass only in the presence of eDNA (18), and the response could vary depending on the bacterial strain (19) and their

Table 3. Statistical analysis of full factorial design matrix for MK-7 production and biofilm biomass.

Term	MK-7 (mg/L) [a]			Biofilm biomass (g) [b]		
	Coefficient	Standard error	*P* value	coefficient	Standard error	*P* value
constant	14.7546	0.278049	7.54908e-007	0.528681	0.0114889	1.33391e-006
X_1	-1.49464	0.326041	0.0101511	-0.00276873	0.0134719	0.847203
X_1	-2.71334	0.326041	0.00113902	-0.118111	0.0134719	0.00093314
X3	-3.70802	0.326041	0.000340891	-.0535712	0.0134719	0.0164491
X_1X_2	-0.755142	0.326041	0.0814852	0.0108113	0.0134719	0.467234
X_1X_3	-0.387568	0.326041	0.300302	0.00763124	0.0134719	0.601356
X_2X_3	1.43933	0.326041	0.0115595	0.0427737	0.0134719	0.0336953

[a]R^2=0.984, significance code : $P<0.05$ R^2 adj 0.960.

[b]R^2=0.963, significance code : $P<0.05$ R^2 adj 0.907.

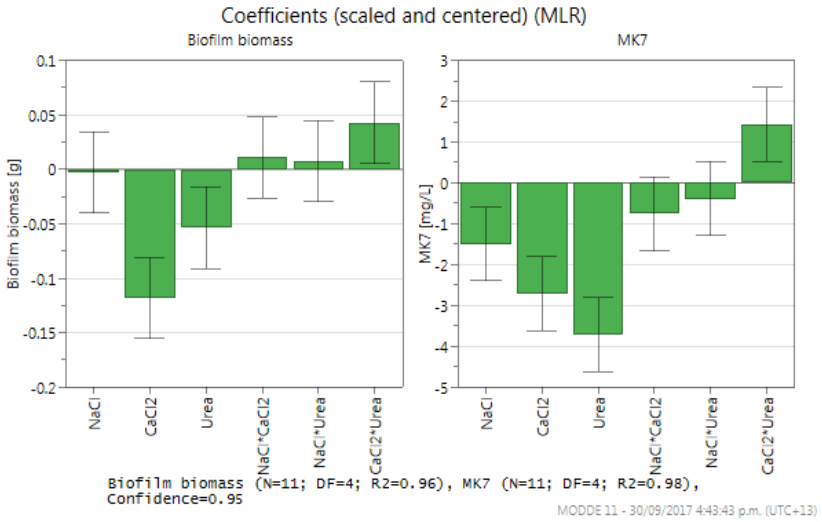

Figure 1. Coefficients (scaled and centered).

growth stage (20). The presence of NaCl and $CaCl_2$ both, however, showed a negative effect on MK-7 production (P<0.05).

3.1.2. Effect of urea on biofilm biomass and MK-7 production

According to the screening results, the presence of urea significantly reduced biofilm biomass (P<0.05) (Figure 1 and Table 3). This agrees with the findings of Chen and Stewart (12), who demonstrated that treatment with 2M Urea reduced biofilm viscosity by 46% by disrupting the hydrogen bonding network between water molecules (9, 12). The presence of urea showed a negative effect on MK-7 production. However, urea together with $CaCl_2$ showed a positive effect on MK-7 production. Based on these results, $CaCl_2$ and urea were used as variables for the optimization of MK-7 production process.

3.2. Optimization of MK-7 production process

The statistical analysis of this research was performed using RSM by a CCF design. Individual factors and their interaction effects on MK-7 production and biofilm formation were determined and statistical models were created to obtain the optimum conditions to minimize biofilm formation and maximize MK-7 production. Quadratic models are proposed. The summary of the ANOVA results of the model fitting are shown in Table 6. For both the model equations, the F-value of 43.4574 and 42.8068 imply that the models are significant. Values of P less than 0.05 indicate that model terms are significant. In this case, all the model terms i.e X_1, X_2, X_1^2, X_2^2, and X_1X_2 are significant for MK-7 and biofilm biomass. The empirical relationships between the two responses (MK-7 production and biofilm biomass) and the variables ($CaCl_2$ and urea concentration) were built and are shown by the regression equa-

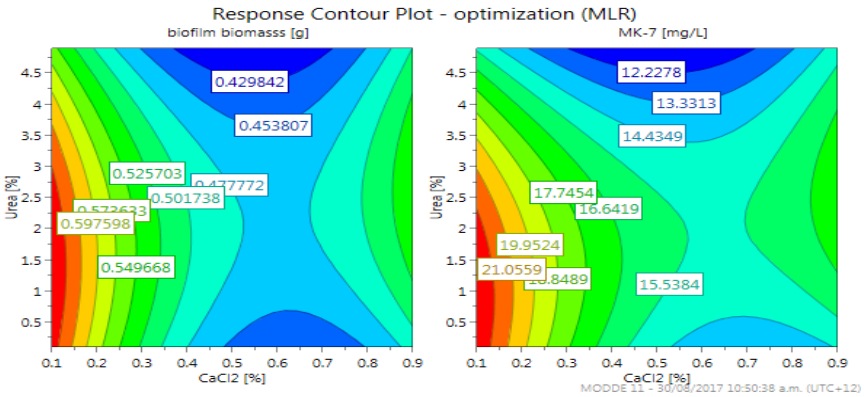

Figure 2. Response contour plots for the biofilm biomass and MK-7 production.

Table 4. Experimental conditions for the central composite face design and responses for MK-7 and biofilm biomass (for both original and scaled factors).

	CaCl2 (% w/v)	Urea (% w/v)	MK-7 observed (mg/L)	MK-7 predicted (mg/L)	Biofilm biomass observed (g)	Biofilm biomass predicted (g)
1	0.1 (-1)	0.1(-1)	23.4072	23.058	0.64438	0.656392
2	0.9 (+1)	0.1 (-1)	14.6551	14.5367	0.47595	0.488007
3	0.1(-1)	4.9 (+1)	14.9736	15.3643	0.55708	0.557863
4	0.9 (+1)	4.9 (+1)	14.3781	14.9996	0.50126	0.502088
5	0.1 (-1)	2.5 (0)	21.917	21.8755	0.67023	0.657435
6	0.9 (+1)	2.5 (0)	17.9356	17.4325	0.55824	0.545355
7	0.5 (0)	0.1 (-1)	14.2737	14.7413	0.47372	0.449651
8	0.5 (0)	4.9 (+1)	12.1382	11.126	0.40904	0.407428
9	0.5 (0)	2.5 (0)	15.5531	15.598	0.47512	0.478847
10	0.5 (0)	2.5 (0)	14.941	15.598	0.46414	0.478847
11	0.5 (0)	2.5 (0)	15.7554	15.598	0.4716	0.478847

tions (1) and (2).

$$Y_1 = 15.598 - 2.2215\,X_1 - 1.80768\,X2 + 4.05598\,X1^2 - 2.66436\,X_2^2 + 2.03915\,X1X2 \quad \text{(Eq. 1)}$$

$$Y_2 = 0.478847 - 0.05604X1 - 0.0211117\,X2 + 0.122548\,X1^2 - 0.0503071\,X2^2 + 0.0281525X1X2 \quad \text{(Eq. 2)}$$

Y1 and Y2 are the predicted values of MK-7 and biofilm biomass, respectively, and X_1, X_2 represents $CaCl_2$ and urea concentration, respectively. Figure 2 shows the response contour plots based on equations (1) and (2) to visualize the influence of the effective variables on biofilm formation and MK-7 production. Correspondingly, the models (equations. (1) and (2)) assumed that the highest MK-7 production (17.53 mg/L) and lowest biofilm biomass (0.51 g) could be achieved using 0.32% $CaCl_2$ (w/v) and 0.10% urea (w/v), respectively. As can be seen on Figure 2, biofilm biomass decreased with the increase in $CaCl_2$ and urea concentration. Increasing the percentage of urea in the medium up to 2.25% (w/v) resulted in higher MK-7 production. However, further increase in urea concentration reduced the MK-7 production. Therefore, MK-7 can be improved by adding an optimal amount of urea, which act as a source of nitrogen in microbial metabolic pathway. According to the results, $CaCl_2$ and urea were found crucial factors to influence both biofilm formation and MK-7 production. Biofilm biomass can be remarkably reduced by adding $CaCl_2$ and urea. These results are in agreement with the ob-

Table 5. Statistical analysis of the Central composite face (CCF) design experiments.

Term	MK-7 production (mg/L)[a]			Biofilm biomass (g)[b]		
	coefficient	Standard error	*P* value	coefficient	Standard error	*P* value
constant	15.598	0.372155	1.45857e-007	0.478847	0.00884155	4.05861e-008
X_1	-2.2215	0.296169	0.00066593	-0.05604	0.0070363	0.000503318
X_2	-1.80768	0.296169	0.00170982	-0.0211117	0.0070363	0.0300858
$X_1$2	4.05598	0.455794	0.000298285	0.122548	0.00108286	9.41854e-005
$X_2$2	-2.66436	0.455794	0.00207403	-0.0503071	0.018286	0.0056036
X_1 X_2	2.03915	0.362732	0.00246588	0.0281525	0.00861768	0.0222765

X_1 Calcium chloride , X_2 Urea.

[a]R^2=0.978, significance code : P<0.05 R^2 adj 0.955.

[b]R^2=0.977, significance code : P<0.05 R2[a] adj 0.954.

Table 6. Analysis of variance for the quadratic model

Source of variation	Biofilm biomass (g)						MK-7 production (mg/L)					
	DF	SS	MS	SD	F	P	DF	SS	MS	SD	F	P
Total corrected	10	0.0650657	0.00650657	0.0806633	42.8068	0.000	10	116.989	11.6989	3.42036	43.4574	0.000
Regression	5	0.0635804	0.0127161	0.112766				114.357	22.8715	4.78241		
Residual	5	0.00148529	0.000297057	0.0172354				2.63148	0.526296	0.725463		
Residual	5	0.00148529	0.000297057	0.0172354				2.63148	0.526296	0.725463		
Residual	5	0.00148529	0.000297057	0.0172354				2.63148	0.526296	0.725463		

servations reported by Chen and Steward, where urea and $CaCl_2$ showed a negative effect on biofilm biomass (9).

3.3. Validation of the model and the effect of $CaCl_2$ and urea on MK-7 production and biofilm formation

In order to evaluate the predicted optimal conditions from the models, a verification test was conducted using the two independent variables, $CaCl_2$ and urea concentrations. The experimental conditions used for validation experiment are given in Table 7.

Results of the validation test are given in Table 8. The MK-7 production and biofilm biomass were similar to the predicted values from the model with only 2% and 8% variations, respectively. The outstanding correlation between predicted and measured values verified the model accuracy for these experiments.

Table 7. Validation experiment conditions.

Experiment	% $CaCl_2$ (w/v)	% urea (w/v)
	0.317283	0.100917

3.4. Evaluation of MK-7 production and biofilm formation in the optimum media

Based on the results, fermentation experiments were performed for 108 h to monitor the production of biofilm biomass, MK-7, bacterial growth, and pH in the optimum medium. As can be concluded from Figure 3, a rapid growth of *B. subtilis* was seen in the presence of $CaCl_2$ (0.3%) and urea (0.1%) during the first 48 h. Cell density reached a maximum of 45.76 (OD600 nm) at 48 h and started to decline thereafter. Experimental evidence show that bacterial detachment depends on microbial growth rate, where high microbial growth rates would lead to instability in biofilm accumulation and would trigger biomass loss (11). Therefore, the reduced biofilm biomass accumulation seen in the optimum media in comparison to the controlled conditions after 60 h of fermentation, can also be partly explained with this phenomenon. MK-7 production increased rapidly after the cell density reached its maximum at 48 h and peaked to 30.83 mg/L after 84 h of fermentation. The results are in agreement with previous reports (21-23) where the increase in MK-7 concentration during the latter period was said to be caused by sporulation (23). A typical HPLC chromatogram of MK-7 produced by the *B. subtilis* strain (ATCC 6633) in the optimum medium is shown in Figure 4. The pH of the medium decreased from 7.1 to 6.3 during the first 12 h and gradually increased up to 9.12 thereafter with the increase in cell growth. The increase in pH may be due to decomposition of proteins by *B. subtilis*, which is proteolytic in

Table 8. Verification test.

	Verification test	Predicted values	% variation
Biofilm biomass (g)	0.55	0.51	8%
MK-7 production (mg/L)	17.98	17.53	2%

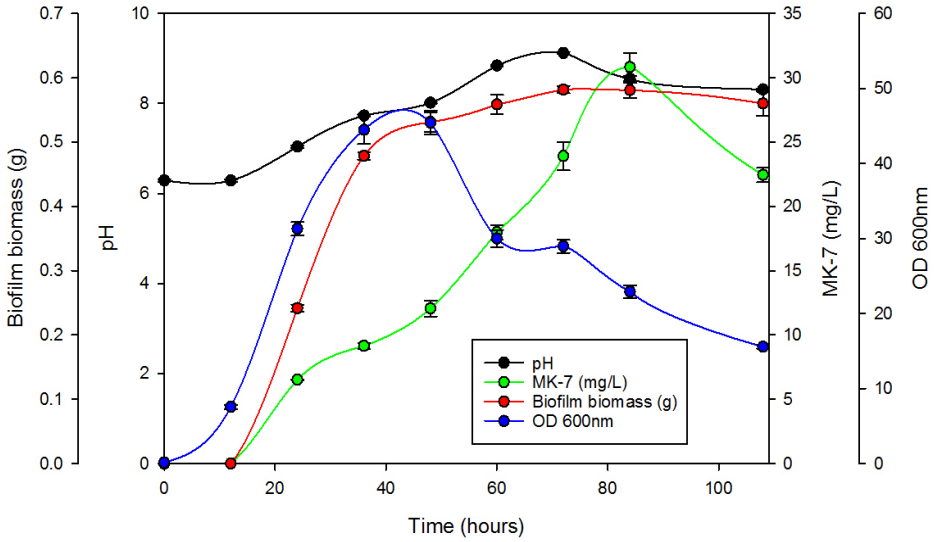

Figure 3. Changes in biofilm formation, MK-7 production, bacterial growth, and pH during the time course of fermentation for optimized media consisting of 5% (w/v) yeast extract, 18.9% (w/v) soy peptone, 5% (w/v) glycerol, 0.06% (w/v) K_2HPO_4, 0.32% (w/v) $CaCl_2$, and 0.1% (w/v) urea.

nature and leads to the release of ammonia (23). A slight decrease in pH was seen after the cell growth was at the maximum level, a possible explanation for which, is the conversion of organic substances to volatile fatty acids and poorly-soluble gases; both weakening the biofilm structure (24).

4. Conclusion

Calcium chloride and urea were found to be the key nutrient influencing both biofilm formation and MK-7 production. The optimum condition to minimize biofilm formation while maximizing MK-7 production was achieved by using a response surface methodology approach. The lowest biofilm biomass and highest MK-7 production was achieved when the medium was supplemented with 0.32% (w/v) $CaCl_2$ and 0.10% (w/v) urea. Importantly, the approach presented here can be applied to biofilm control in any type of bioreactor to reach the optimal desirable properties for different applications.

Conflict of Interest

None declared.

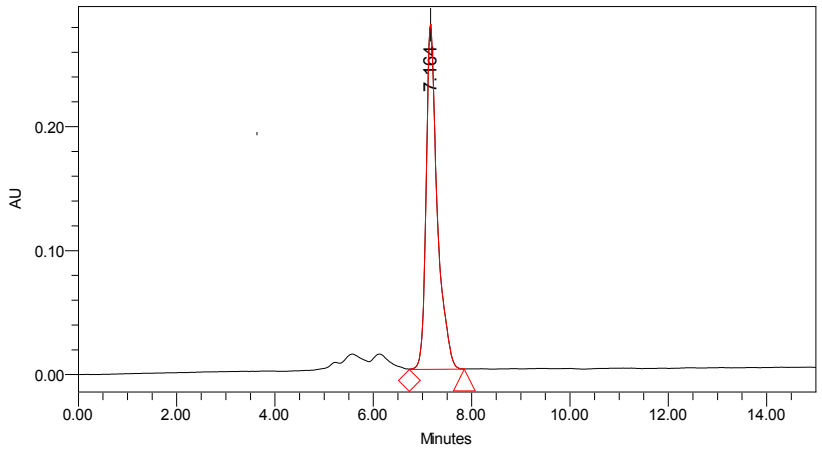

Figure 4. HPLC Chromatogram of MK-7 in the culture of *B. subtilis* in the optimum medium designed in this study. MK-7 elutes at 7.164 min. HPLC conditions are described in the section of materials and methods.

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