Biocatalysis and Agricultural Biotechnology 19 (2019) 101172

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Development of a Menaquinone-7 enriched product through the solid-state fermentation of *Bacillus licheniformis*

Neha Lal, Mostafa Seifan, Donya Novin, Aydin Berenjian*

School of Engineering, Faculty of Science and Engineering, The University of Waikato, Hamilton, 3216, New Zealand

ARTICLE INFO

Keywords:
Solid-state fermentation
MK-7
Bacillus licheniformis
Bone fractures
Animals

ABSTRACT

Bone fracture is a significantly prevalent issue in both large and small animals, in particular cats, dogs, and horses. Recent studies have shown that adequate consumption of the vitamin Menaquinone-7 (MK-7) has the ability to reduce the incidence of bone fractures, by increasing the bone mineral density. Therefore, the development of an MK-7 enriched animal feed poses as a promising concept that is likely to significantly benefit the health and wellbeing of animals, and improve their quality of life. The aim of this investigation was to build on the different aspects of previously conducted studies, and apply the relevant findings and experimental procedures to enhance the production of MK-7 through the solid-state fermentation of *Bacillus licheniformis*, for the unique application of an MK-7 enriched animal feed, in order to limit the incidence of bone fractures in animals. An optimisation study was performed using composite face-centered experimental design. The most ideal combination of the experimental variables for the biosynthesis of MK-7 was determined to be 10% (w/w) tryptone, 20% (w/w) glucose, and 2% (w/w) K_2HPO_4, when the soybean substrate is inoculated with 5% (v/w) of *B. licheniformis* and incubated at a temperature of 32 °C, for a fermentation period of 72 hours. The optimisation process was then validated and the highest concentration of MK-7 (12.507 mg/kg) that was obtained was fairly similar to the value predicted by the model. The results and observations from this research are a valuable step towards achieving a better understanding of the ability of feed grade bacterial cultures to enhance the production of MK-7 through solid-state fermentation for the development of functional animal feed in the future.

1. Introduction

Vitamin K is the collective name for a group of fat-soluble compounds that have a common 2-methyl-1, 4-napthaquinone nucleus, but differ in the structure of a side chain at the 3-position (Berenjian et al. 2011, 2015; Mahanama et al., 2012b). Vitamin K naturally occurs in two forms phylloquinone (vitamin K1) and a series of menaquinones (vitamin K2), both of which play an important role in hemostasis (Berenjian et al., 2015). MK-7 is a class of menaquinone that can be produced by the fermentation of bacteria, such as *Escherichia coli, Flavobacterium*, and *Bacillus subtilis* natto (Berenjian et al., 2014). MK-7 has recently gained widespread interest in the Biotechnology field, as recent studies have shown that besides its role in blood coagulation, adequate MK-7 consumption has a protective effect against osteoporosis and cardiovascular diseases (Berenjian et al. 2011, 2012, 2015; Mahanma et al., 2011).

In the body, vitamin K functions as a cofactor in the γ-glutamyl carboxylation of certain glutamic acid (Gla) residues of vitamin K-dependent proteins, which is required for their activation (Mahanama et al., 2012b; Berenjian et al., 2015). Osteocalcin is a vitamin K-dependent protein that is produced by osteoblasts and plays an important role in bone formation and in maintaining healthy levels of bone mineral density (Berenjian et al., 2015). The γ-carboxylation of osteocalcin is required for its activation and contributes to calcium and hydroxyapatite binding, allowing for osteocalcin deposition in mineralised bone matrix (Patti et al., 2013; Berenjian et al., 2015). In comparison, non-carboxylated osteocalcin has a low affinity for hydroxyapatite and is more easily released into the circulation, where it may increase the risk of arterial calcification (Patti et al., 2013; Berenjian et al., 2015).

Studies have also shown that vitamin K plays an important role in the prevention of arterial calcification, through the activation of the vitamin K-dependent matrix Gla protein that is expressed in vascular tissue, which binds to calcium ions and acts as an inhibitor of vascular mineralisation (Berenjian et al., 2015). Various clinical trials and studies have compared the effects of phylloquinone and menaquinone on the prevention of osteoporosis and arterial calcification. It has been found that menaquinone is more effective than phylloquinone, and

* Corresponding author. School of Engineering, Faculty of Science and Engineering, The University of Waikato, Hamilton, New Zealand.
E-mail address: aydin.berenjian@waikato.ac.nz (A. Berenjian).

https://doi.org/10.1016/j.bcab.2019.101172
Received 29 March 2019; Received in revised form 29 April 2019; Accepted 21 May 2019
Available online 22 May 2019
1878-8181/

confers a greater health benefit, as a high dietary intake of menaquinone is associated with a significant improvement in bone strength and the reduction of coronary artery calcification (Berenjian et al., 2015). The ability of vitamin K2 to reduce the incidence of bone fractures by increasing the bone mineral density (Davani-Davari et al., 2019), suggests that it may be an effective dietary supplement for animals (Mahanama et al., 2011a). Bone fractures are a serious issue in both large and small animals, in particular cats, dogs, and horses. The severity of the sustained fracture varies depending on the type of animal and the nature of the fracture, as does the treatment method (Mohnacky, 2019, Willows Veterinary Center and Referral Service, 2019). The cost associated with veterinary care is also very substantial, and it is likely to increase proportionately with the severity of the fracture, and the type of treatment required (Wag, 2019). Generally, fractures are considerably easier to heal and the chances of complete recovery are far greater in small animals, in comparison to large animals, due to a greater range of different treatment options (American College of Veterinary Surgeons, 2019; Willows Veterinary Center and Referral Service, 2019). There is also the potential for the poor recovery of certain animals, depending on the severity of the sustained fracture, type of treatment method, and other age and health-related factors (Wag, 2019, Willows Veterinary Center and Referral Service, 2019). Thus, the development of an MK-7 enriched animal feed poses as a promising concept that is likely to significantly benefit the health and wellbeing of animals, and improve their quality of life. Numerous studies have been conducted to enhance the yield of MK-7 through fermentation. Majority of the initial research that has been conducted in this area has focused on liquid-state fermentation; however, more recently solid-state fermentation has received more extensive attention. Solid-state fermentation could be a more viable and economical alternative to liquid-state fermentation, as it may require less pre-processing energy, produce less wastewater, result in superior productivity, and have improved product recovery (Mahanama et al. 2011a, 2011b). In addition, solid-state fermentation is of particular interest in processes where the crude fermented product may be used directly as a food supplement (Mahanama et al., 2011a).

An issue that has been commonly encountered between most of the previously conducted investigations is the low yield of MK-7 in fermentation processes. The MK-7 yield obtained in solid-state fermentation processes is dependent on a variety of physiochemical and biochemical parameters such as the particle size, initial substrate moisture content, medium components, pH, substrate pre-treatment, relative humidity, incubation temperature, fermentation period, inoculum size, supplementation of trace elements and additional nutrients, as well as the choice of microorganism and other fermentation conditions (Berenjian et al., 2015; Puri et al., 2015). The individual effects of these parameters as well as their interactive effects determines the amount of MK-7 obtained through fermentation, and because it is often difficult to consider and control all of these variables during fermentation, the MK-7 yield obtained in most studies is low (each study tends to consider only a selection of the different variables affecting MK-7 production). Thus, it is evident that MK-7 biosynthesis and the yield of MK-7 in solid-state fermentation processes is dependent on a wide variety of factors, which must be optimised to improve the amount of MK-7 obtained. Therefore, numerous studies have been conducted to compare the effects of various factors such as, different substrates and nutrients, dynamic versus static fermentation conditions, as well as genetic manipulation on the fermentation process (Berenjian et al., 2011; Mahanama et al., 2011a; Song et al., 2014; Puri et al., 2015). Also, majority of the previous investigations have focused on enhancing the production of MK-7 using the bacterium *B. subtilis* (more specifically *Bacillus subtilis* natto), to develop strategies that will allow for the bulk production of a low cost dietary supplement or functional food rich in MK-7, for human consumption (Berenjian et al., 2015; Southee et al., 2016). Thus, research into other areas, such as the use of different bacterial strains to enhance the production of MK-7 through solid-state fermentation for other (non-human related) applications has not been as widely considered.

B. licheniformis (CAS: 57-13-6) has been identified as the ideal bacterial strain for MK-7 biosynthesis, as it is characterised as a probiotic microorganism (Cutting, 2011; Nithya et al., 2012; Prieto et al., 2014) that has been safely implemented in animal feed to improve animal health. Although the use of *B. licheniformis* for probiotic applications has been extensively researched, its potential for use in MK-7 production studies has not been as widely considered. The study conducted by Goodman et al. (1976) demonstrates the ability of the bacterium *B. licheniformis* to produce MK-7, but for an entirely different purpose that does not relate to the development of a functional food product or animal feed rich in MK-7. Hence, although *B. licheniformis* and other *Bacillus* strains, such as *Bacillus amyloliquefaciens*, have been shown to produce MK-7, *Bacillus subtilis* natto is the most dominant strain, which is preferentially used in MK-7 studies (Mahdinia et al. 2019a, 2019b). Thus, there is limited available literature that considers the use of *B. licheniformis* in studies involving MK-7 biosynthesis. Therefore, the aim of this investigation was to evaluate the possibility of MK-7 production through the solid-state fermentation of *B. licheniformis*, for the unique application of an MK-7 enriched animal feed that may have the potential to limit the incidence of bone fractures in animals.

2. Materials and methods

2.1. Chemicals

Tryptone, dipotassium phosphate (K_2HPO_4), LB Broth, n-hexane, and 2-propanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glucose was purchased from Merck Millipore (Germany) and nutrient agar plates were sourced from Fort Richard Laboratories (Auckland, New Zealand). MK-7 with a purity of 99.5% was purchased from ChromaDex (ChromaDex Co., USA) to construct a calibration curve. Lipase was acquired from Sigma-Aldrich (St. Louis, MO, USA) and soybean granules were purchased from a local grocery store.

2.2. Microorganism and inoculum preparation

Freeze-dried *B. licheniformis* (CAS: 57-13-6) was revived in an autoclaved LB broth medium, by incubating the culture overnight at 37 °C, under dynamic conditions (150 rpm). The culture was then transferred to nutrient agar plates (500 μL) and incubated for another 24 h at 37 °C (Seifan et al., 2017). The bacterial cells were then scraped and suspended in a 0.9% saline solution. The suspension was then incubated in a water bath for 30 min at 80 °C and centrifuged at 3000 rpm for 10 min to remove the cell debris (Seifan et al., 2016). The pure spore suspension that was obtained following this process was refrigerated and used as required for solid-state fermentation experiments.

2.3. Substrate preparation

In this investigation, soybeans have been chosen as the ideal substrate for solid-state fermentation, as it is a common component of animal feed, which has also been shown to enhance the MK-7 yield when fermented with *B. subtilis* natto (Berenjian et al., 2015, Riverina Australia, 2017). For each experiment, the soybeans were ground for 1 min in small batches of 100 g using a grinder, such that the soybeans resembled a fine-grained mixture. This resulted in an assortment of soybean particles of different sizes, which has been suggested to improve the MK-7 yield, as a combination of small and large particle sizes may result in optimised nutrient availability and oxygen transfer (Mahanama et al., 2011a). The grinding time was kept constant between all batches of soybeans for all experiments, so as to maintain consistency and reduce any experimental variation or error.

2.4. Extraction and measurement of MK-7

MK-7 was extracted from the media through the addition of n-hexane:2-propanol at the ratios of (2:1, v/v) and 1:4 (liquid:organic, v/v). The mixture was vortexed for two minutes and then centrifuged at 3000 rpm for 10 min, in order to separate the organic phase from the aqueous phase. The separated organic layer was then removed and evaporated to recover the extracted MK-7 (Berenjian et al., 2011; Ranmadugala et al., 2018). The Thermo Scientific Dionex high-performance liquid chromatography (HPLC) system equipped with a photodiode array UV detector (UVD-340U) was used for MK-7 analysis. The samples were analyzed by a Phenomenex C18 Gemini column (5 μm, 150 × 4.6 mm, Phenomenex, USA) at 40 °C. The flow rate of the mobile phase (methanol) was 1 mL min^{-1}, and the wavelength was set at 248 nm (Berenjian et al., 2014).

2.5. Experimental design and statistical analysis

For the optimization process, the selection of the carbon, nitrogen, and salt sources were based on previous studies (Berenjian et al. 2012, 2013; Mahdinia et al. 2018a, 2018b). To maximize the production of MK-7, the experimental design was created using a design of experiment package (MODDE pro, Umetrics v12.1, Umeå, Sweden). A total of five variables were analysed at three coded levels (−1, 0, +1), and consisted of three nutrients (tryptone, glucose, and K_2HPO_4), inoculation loading, and an operating condition (incubation temperature). Central composite face-centered (CCF) design was employed to optimise the level of effective variables. Analysis of variance (ANOVA) was carried out with the data obtained to assess any significant lack of fit with the experimental results. The experimental data was then fitted to a second-order polynomial regression model to predict the biosynthesis of MK-7 (Eq. (1)).

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

where Y is the concentration of MK-7, b_0 represents a constant coefficient, b_i, b_{ii}, and b_{ij} are the coefficients of the linear, quadratic, and synergic effects respectively, and X_i and X_j represent the coded values of the significant factors.

3. Results and discussion

3.1. Optimisation of MK-7 production through RSM

In this investigation, an optimisation study was performed to maximise the production of MK-7 through the solid-state fermentation of *B. licheniformis*. The effect of different concentrations of influential components on the biosynthesis of MK-7, such as nitrogen, carbon, and salt sources, along with operating conditions including inoculum loading and temperature, have been investigated. The amount of each constituent nutrient was prepared according to the experimental combination of the central composite face-centered (CCF) design matrix, as tabulated in Table 1. The individual and interactive effects of the fermentation parameters (temperature, inoculum size, and nutrients) are discussed below.

3.2. Effect of fermentation temperature and inoculum size

The relationship between the fermentation temperature and inoculum size, and the remaining experimental variables were considered to determine their individual and combined effects on the MK-7 yield.

Three levels of the fermentation temperature were considered in this investigation, namely 30 °C, 35 °C, and 40 °C. Fig. 1 shows the relationship between the fermentation temperature and each of the remaining experimental variables, and all four plots (Figure a–d) suggest that an intermediate fermentation temperature of 35 °C, favours a greater MK-7 yield. This is emphasised by the decrease in the MK-7 concentration that is observed when the incubation temperature increases to 40 °C and decreases to 30 °C, with the lowest MK-7 concentration occurring at both temperature extremes, for all four combinations of variables. This suggests that the optimum growth temperature of *B. licheniformis* is approximately 35 °C. This observation is validated by the conclusions drawn from the study conducted by Kabir et al. (2017), which demonstrates that optimum growth for most *Bacillus* species occurs at a temperature of 35.5 ± 2 °C. *B. subtilis* mainly *B. subtilis* natto is known as the main model microorganism for MK-7 production. According to previous studies, the concentration of MK-7 produced by *B. subtilis* has been found to be diverse under different conditions, particularly at different temperatures. Although in some studies the temperature range 30–45 °C has been tested, temperatures between 35 °C and 40 °C has been considered as the optimum (Sato et al., 2001; Berenjian et al. 2011, 2014; Song et al., 2014; Mahdinia et al., 2018c).

Three inoculum volumes were investigated in this study, specifically 1% (v/w), 3% (v/w), and 5% (v/w). Fig. 1b and 2 a, b, and c display the correlation between the inoculum size and each of the remaining experimental variables, and all four plots indicate that a greater inoculum size of approximately 5% (v/w) is preferred, as it results in greater MK-7 productivity. This is clearly demonstrated by all four plots, which show an increase in the MK-7 concentration with an increase in the inoculum size from 1% (v/w) to 5% (v/w), and is consistent for all four combinations of variables. Thus, a greater inoculum size results in a higher MK-7 concentration. This observation complies with what would theoretically be expected, as a greater inoculum size (initial quantity of bacteria) is likely to result in a greater final bacterial population, which consequently results in a greater concentration of MK-7 that is achieved through fermentation.

However, this observation is in contrast to the conclusions drawn from previous studies, such as the studies conducted by Mahanama et al. (2011b, 2012a), which state that a greater inoculum size has an adverse effect on the MK-7 production. It has been suggested that a greater inoculum loading may deplete the concentration of essential nutrients that are required for microbial metabolite production, and may reduce the amount of available oxygen during fermentation, which has a detrimental effect on the MK-7 productivity (Mahanama et al., 2011b). It must be noted that the studies conducted by Mahanama et al. (2011b, 2012a) considered the effect of the inoculum loading for a different bacterial strain (*B. subtilis*), thus it is possible for the different bacterial species to have different growth and metabolic requirements, and consequently show different responses to the initial inoculum size. This conclusion is supported by the findings from other optimisation studies involving *B. licheniformis*, such as the investigations conducted by Nilegaonkar et al. (1992) and Perego et al. (2003), which have illustrated that an increase in the inoculum size has a positive effect on the product yield. Thus, it can be concluded that different *Bacillus* species show contrasting responses to the initial inoculum size, with respect to product formation.

3.3. Effect of nutrients (carbon, nitrogen and salt source)

The effect of three different nutrients was considered in this study, glucose (carbon source), tryptone (nitrogen source), and K_2HPO_4 (salt source), in order to determine the individual and interactive effects of these nutrients on MK-7 biosynthesis.

Glucose was selected as the carbon source in this study, and three levels of the glucose concentration were considered, 20% (w/w), 35% (w/w), and 50% (w/w). Figs. 1a, 2a, 2d and 3a demonstrate the relationship between the glucose concentration and each of the remaining experimental variables, and all four plots suggest that the lowest quantity of glucose (20% (w/w)) promotes a greater MK-7 yield. This trend is consistent across all four response surface plots, which show that the greatest MK-7 concentration is obtained at a glucose

Table 1
Coded level of the variables examined in the optimisation study using a CCF design.

Run	Temperature (°C)	Inoculum size (% v/w)	Tryptone (g/kg)	Glucose (g/kg)	K_2HPO_4 (g/kg)	MK-7 mg/kg (observed)	MK-7 mg/kg (predicted)
N1	−1	−1	−1	−1	1	9.620	9.275
N2	1	−1	−1	−1	−1	4.362	4.239
N3	−1	1	−1	−1	−1	10.422	10.154
N4	1	1	−1	−1	1	7.077	7.053
N5	−1	−1	1	−1	−1	4.318	4.496
N6	1	−1	1	−1	1	6.250	6.672
N7	−1	1	1	−1	1	6.059	6.336
N8	1	1	1	−1	−1	6.197	6.696
N9	−1	−1	−1	1	−1	3.290	2.840
N10	1	−1	−1	1	1	4.651	4.445
N11	−1	1	−1	1	1	5.273	4.922
N12	1	1	−1	1	−1	4.832	4.703
N13	−1	−1	1	1	1	2.773	2.868
N14	1	−1	1	1	−1	4.671	4.988
N15	−1	1	1	1	−1	4.056	4.228
N16	1	1	1	1	1	4.573	4.989
N17	−1	0	0	0	2	3.084	3.778
N18	1	0	0	0	2	4.784	3.611
N19	0	−1	0	0	2	5.290	5.402
N20	0	1	0	0	2	7.149	6.559
N21	0	0	−1	0	2	5.885	7.782
N22	0	0	1	0	2	9.363	6.987
N23	0	0	0	−1	0	6.793	6.177
N24	0	0	0	1	0	3.422	3.559
N25	0	0	0	0	−1	4.807	4.612
N26	0	0	0	0	1	5.423	5.139
N27	0	0	0	0	0	4.171	5.312
N28	0	0	0	0	0	4.974	5.312
N29	0	0	0	0	0	4.875	5.312

All variables were tested at three different levels: temperature (30, 35, and 40 °C), Inoculum size (1, 3, and 5% v/w), tryptone (10, 15, and 20 g/kg), glucose (20, 35, and 50 g/kg) and K_2HPO_4 (1, 2, and 3 g/kg).

concentration of 20% (w/w), for all four combinations of variables. It can also be observed that the MK-7 productivity rapidly declines as the glucose quantity increases from 20% (w/w) to 50% (w/w), with the lowest MK-7 yield occurring at a glucose concentration of 50% (w/w).

Tryptone was chosen as the nitrogen source in this investigation, and three levels of the tryptone concentration were considered, 10% (w/w), 15% (w/w), and 20% (w/w). Figs. 1d, 2c, 3a and 3b demonstrate the association between the quantity of tryptone and each of the remaining experimental variables, and all four plots invariably illustrate that the lowest tryptone concentration (10% (w/w)) favours greater MK-7 productivity.

The concentration of both the carbon (glucose) and nitrogen (tryptone) sources showed a similar effect on the concentration of MK-7 that was obtained, as the lowest concentration of each nutrient resulted in the greatest MK-7 yield. This is comparable to the observations from the study conducted by Berenjian et al. (2011) and Mahdinia et al. (2019a), which suggest that lower nutrient concentrations are preferred for both carbon and nitrogen sources, as a high nutrient concentration is attributed to an increase in the osmotic pressure and a decrease in the water activity, both of which have an inhibitory effect on cell growth

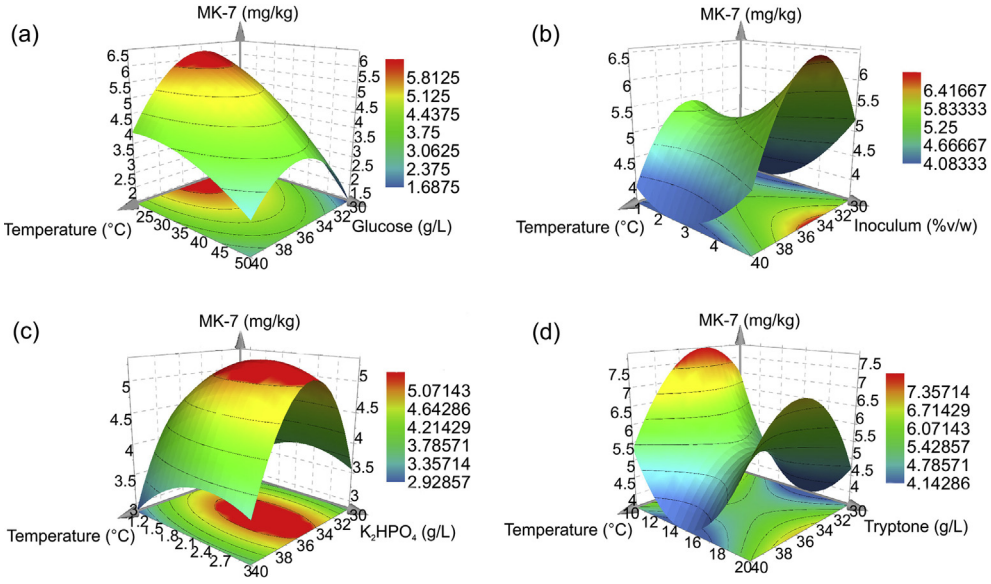

Fig. 1. Response surface plot for the MK-7 concentration obtained as a function of (a) temperature and glucose, (b) temperature and inoculum size, (c) temperature and K_2HPO_4, (d) temperature and tryptone.

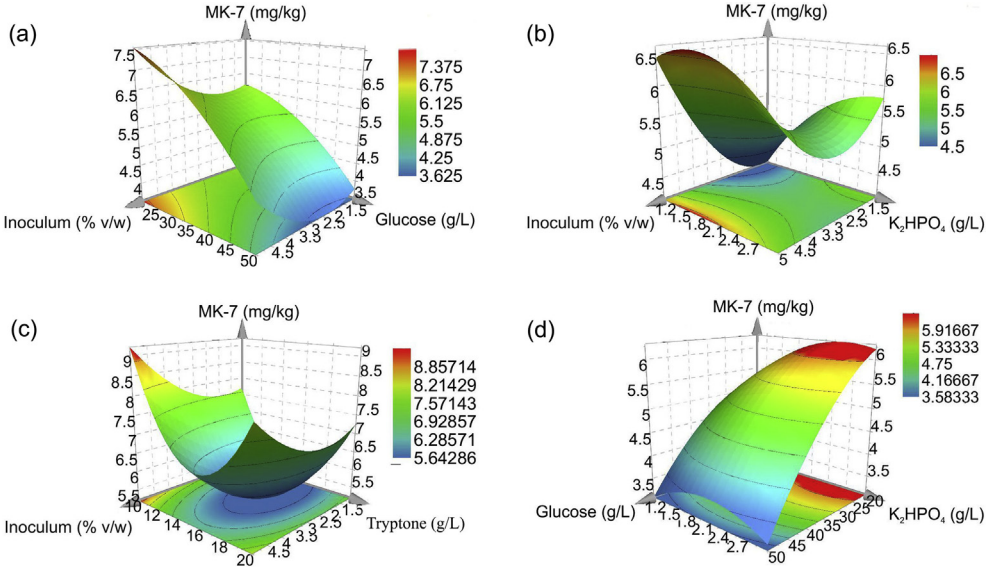

Fig. 2. Response surface plots for the MK-7 concentration obtained as a function of (a) inoculum size and glucose, (b) inoculum size and K_2HPO_4, (c) inoculum size and tryptone, (d) glucose and K_2HPO_4.

and metabolism. This is further validated by the findings from the investigation conducted by Perego et al. (2003), which also demonstrated that a decrease in product formation was associated with an increase in the concentration of nutrients.

K_2HPO_4 was selected as the salt source for this investigation, as previous studies have demonstrated that K_2HPO_4 has a positive effect on the MK-7 production, as it is a phosphate source that is involved in the synthesis of key primary and secondary microbial metabolites during fermentation (Berenjian et al., 2011). Three concentrations of K_2HPO_4 were considered, 1% (w/w), 2% (w/w), and 3% (w/w). Figs. 1c, 2b, 2d and 3b illustrate the correlation between the amount of K_2HPO_4 and each of the remaining experimental variables, and it is implied by all four response surface plots that an intermediate quantity of K_2HPO_4 (2% (w/w)) promotes a greater MK-7 yield. This is emphasised by the decrease in the MK-7 concentration that is observed when the amount of K_2HPO_4 increases to 3% (w/w) and decreases to 1% (w/w), and the lowest MK-7 concentration is obtained at both concentration extremes, for all four combinations of variables. A possible explanation for this observation may be that a K_2HPO_4 concentration less than 2% (w/w) is not sufficient for the production of microbial metabolites, while a K_2HPO_4 concentration greater than 2% (w/w) results in a high osmotic pressure and decreased water activity, which has a detrimental effect on MK-7 biosynthesis. The observations from the present study are supported by the findings from the previously conducted investigation (Berenjian et al., 2011), which has also determined that an intermediate K_2HPO_4 concentration is the most ideal, as it promotes greater MK-7 productivity.

3.4. Experimental verification

A monitoring study was performed to validate the developed experimental model and investigate the variation in the MK-7 concentration over the fermentation period (72 h). To achieve this, the obtained experimental results were fitted to a quadratic model (Eq. (2)) and the regression equations ware solved within the experimental region. The fermentation medium was prepared with the optimum concentrations/levels of variables (glucose 20 g/L, tryptone 10 g/L, K_2HPO_4 2 g/L, inoculum loading 5 %v/w, and temperature 32 °C).

$$Y = 2.840 + 0.044X_1 + 0.309X_2 - 0.212X_3 - 0.700X_4 + 0.141X_5 - 0.864X_1^2 + 0.357X_2^2 + 1.108X_3^2 - 0.237X_4^2 - 0.233X_5^2 - 0.102X_1X_2 + 0.407X_1X_3 + 0.330X_1X_4 + 0.028X_1X_5 - 0.094X_2X_3 - 0.062X_2X_4 - 0.307X_2X_5 + 0.223X_3X_4 - 0.110X_3X_5 - 0.110X_4X_5 \quad (2)$$

where Y is the predicted MK-7 concentration, and X_1, X_2, X_3, and X_4 represent the coded values for temperature, inoculum size, tryptone, glucose, and K_2HPO_4, respectively. The statistical analysis of the model was performed by analysis of variance (ANOVA) and the results are shown in Table 2. The multiple correlation coefficient value (R^2) was 84.7% with an insignificant lack of fit (*p*-value 0.075). This indicates a good correlation between the obtained experimental data and the predicted values by the model (Bezerra et al., 2008).

Fig. 4 illustrates that the MK-7 concentration gradually increases over the fermentation period, and a maximum concentration (12.507 mg/kg) is achieved at 72 h. It can be observed that the MK-7 concentration increases linearly between 0 and 36 h. A 48% increase in the MK-7 concentration occurred between 12 and 24 h, which was

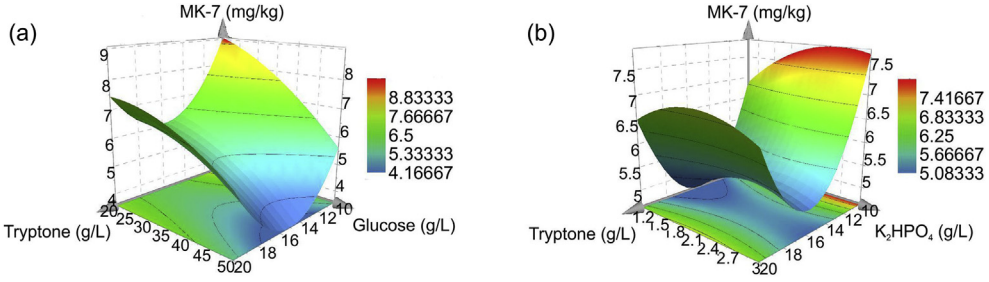

Fig. 3. Response surface plots for the MK-7 concentration obtained as a function of (a) tryptone and glucose, (b) tryptone and K_2HPO_4.

Table 2
Analysis of variance (ANOVA) to determine the suitability of the generated quadratic model in fitting the experimental data.

Source	DF	SS	MS	SD	F-value	*p*-value
Total	29	963.639	33.229	–	–	–
Constant	1	865.673	865.673	–	–	–
Residual	8	15.0226	1.878	1.370	–	–
Lack of Fit	6	14.6392	2.440	1.562	12.727	0.075
Pure error	2	0.383409	0.192	0.438	–	–

DF: degree of freedom, SS: sum of squares, MS: mean sum of squares, SD: standard deviation, $R^2 = 0.847$, $R^2_{adj.} = 0.463$.

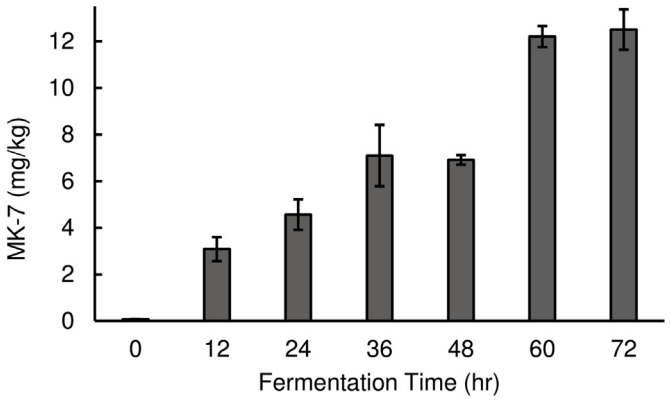

Fig. 4. The variation in the MK-7 concentration over the fermentation period (72 h).

followed by a 56% rise in the MK-7 concentration as the fermentation time increased from 24 to 36 h. The MK-7 concentration then showed a downward trend between 36 and 48 h, which corresponded to a 2.6% decrease in the concentration. A possible reason for this could be the vast difference in the MK-7 concentration that was obtained from the two duplicate samples at 36 h; the large error bars indicate this inconsistency. There is a considerable increase in the MK-7 concentration (76%) between 48 and 60 h, reaching a concentration of 12.204 mg/kg at 60 h. The MK-7 concentration then shows a negligible increase of 2.5% between 60 h and the end of the fermentation period (72 h).

The trends in the changes of the MK-7 concentration over the fermentation period may be attributed to the microbial growth profile. The slight increase in the MK-7 concentration that has been observed between 0 and 12 h is likely to correspond to the lag phase of microbial growth, as the newly inoculated microbial cells are adjusting to their new environment and are preparing to divide. The four-fold increase in the concentration that occurs between 12 and 60 h, is likely to correspond to the exponential phase of microbial growth, as all cells are undergoing rapid growth and metabolism, hence a large quantity of the product is produced. This observation confirms that MK-7 is a primary microbial metabolite as it is a vitamin, which is largely synthesised during the exponential phase of microbial growth. The very little variation in the MK-7 concentration that has been observed between 60 h and the end of the fermentation period (72 h), is likely to correspond to the stationary phase of microbial growth, where the rate of cell growth is balanced by the rate of cell death, and occurs as a result of nutrient depletion and the accumulation of toxins in the fermentation media.

Fermentation was not conducted for a period greater than 72 h for several reasons. This study suggests that bacterial growth is likely to transition from the stationary phase of growth to the death phase after a period of 72 h. Thus, the size of the bacterial population will decline (the rate of cell death will be greater than the rate of cell growth) as the fermentation time proceeds beyond 72 h, and since MK-7 biosynthesis depends on the number of live bacterial cells, the amount of MK-7 that is produced will also decrease. The studies conducted by Berenjian et al. (2011, 2014) also demonstrate that no significant increase in MK-7 production occurs after 72 h. Consequently, in the interest of time, energy and resources, fermentation was only conducted for 72 h. It has also been suggested that longer fermentation periods may lead to the accumulation of free amino acids in the fermentation media, which has an adverse effect on MK-7 production (Berenjian et al., 2015). Therefore, for the reasons outlined above a fermentation period of 72 h was determined to be the most ideal.

A similar variation in the MK-7 concentration has been observed in other similar investigations involving the bacterium *B. subtilis*. In particular, it was demonstrated that the MK-7 concentration increases very slightly over the first day (24 h) of the fermentation period, after which it shows a notable increase, reaching a maximum concentration at around 3 days (72 h), following this the MK-7 concentration remains fairly constant (and increases only slightly) for the remainder of the fermentation period (Berenjian et al., 2011). It was also noted that the MK-7 production shows a similar trend to the cell growth profile, which supports the conclusions drawn from the present investigation.

The MK-7 yield obtained in different solid-state fermentation studies involving different solid substrates and bacterial strains (from the *Bacillus* species) varies between 0.53 and 140 mg/kg (Berenjian et al., 2015). Of particular relevance to this investigation is the study conducted by Mahanama et al. (2011a), which considered the solid-state fermentation of *B. subtilis* natto on soy granules under similar fermentation conditions (Berenjian et al., 2015). The maximum MK-7 yield obtained from this study was 28.26 ± 0.11 mg/kg, which is considerably greater than the maximum concentration achieved in the present study (12.507 mg/kg). This suggests that the amount of MK-7 obtained is greater through the solid-state fermentation of *B. subtilis*, compared to *B. licheniformis*. This observation is supported by several studies that have determined *B. subtilis* natto to be the highest MK-7 producing bacterial strain, as the greatest MK-7 concentrations have been obtained from studies involving the solid-state fermentation of *B. subtilis* natto (Berenjian et al., 2015).

3.5. Recommendations

This investigation attempted to build on the different aspects of previous studies that have been conducted using *B. subtilis*, and the relevant findings and experimental procedures from these studies have been applied to enhance the production of MK-7 through the solid-state fermentation of *B. licheniformis*, for a novel application. Thus, it has generally been assumed that many of the experimental methods and observations from previous investigations (involving *B. subtilis*) are also relevant and applicable to *B. licheniformis*. However, it must be appreciated that although both of these bacterial strains are from the same genus (*Bacillus*), they are from different species, thus it is highly likely that the specific growth and metabolic requirements of each species are different. Therefore, it may be worthwhile to conduct further research into the specific growth and metabolic requirements for *B. licheniformis*. A screening study could also be conducted to determine the most ideal carbon, nitrogen and salt sources that have the ability to enhance the MK-7 production through the solid-state fermentation of this bacterium.

This study focuses on the use of soybeans as a substrate for the solid-state fermentation of *B. licheniformis*, as soybeans are a common component of animal feed, and have been shown to enhance the MK-7 concentration obtained in previously conducted studies involving *B. subtilis*. However, it may be promising to investigate the ability of other substrates to enhance the MK-7 production, such as the various types of cereals and grains that are commonly incorporated into animal feed. It has been determined that soybeans are mainly a component of animal feed that is manufactured for large animals, such as horses and livestock, thus it would be valuable to investigate a more versatile substrate that can also be incorporated into the diet of small animals, such as cats and dogs.

It may also be worthwhile to investigate the effect of other

experimental variables, such as the incubation time, initial substrate moisture level, particle size, rate of aeration, and type of fermentation vessel, on the MK-7 production as well. In particular, it has been found that many of the previously conducted studies, such as the studies conducted by Mahanama et al. (2011a, 2011b, 2012a) have used Petri dishes or specimen jars as the fermentation vessel. This may offer an advantage over the use of tubes, as it is likely to reduce the substrate bed thickness, which has been shown to favour a high yield of MK-7, as it reduces the formation of temperature, oxygen, and nutrient gradients (Mahanama et al. 2011a, 2011b). It may also be ideal to mix the samples more often during the fermentation/incubation period (that is at regular intervals), as it may improve the access of microorganisms to nutrients and other media components. Mixing was only carried out when the samples were wet with water during fermentation, thus investigating the effect of mixing (that is the frequency of mixing) on the MK-7 production may also be advantageous. Overall, although this investigation has been successful with respect to its ability to satisfy the various experimental aims, there is still plenty of scope for further research and improvement.

4. Conclusions

The overall aim of this investigation was to expand on the different aspects of previous studies that have been conducted using *B. subtilis*, in order to evaluate and develop an MK-7 enriched animal feed through the solid-state fermentation of *B. licheniformis*. The experimental results demonstrate the ability of the bacterium *B. licheniformis* to produce MK-7 through the solid-state fermentation of soybeans. This was a significant observation, as it validated the findings from other investigations that have demonstrated the ability of *B. licheniformis* to produce MK-7, and has suggested that the bacterium is suitable for the development of a fermented MK-7 enriched animal feed. The results and observations obtained from this research investigation are a valuable step towards achieving a better understanding of the ability of different bacterial strains to enhance the production of MK-7 through solid-state fermentation for other (non-human related) applications. Therefore, it is anticipated that the findings of this experiment have laid the foundations for subsequent investigations that aim to explore other areas of this topic to develop a functional animal feed, which may have the potential to limit the incidence of bone fractures in animals.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

This study was financially supported by the University of Waikato, New Zealand.

References

American College of Veterinary Surgeons, 2019. Fractures in Horses-Emergency First Aid and Stabilization. Retrieved. https://www.acvs.org/large-animal/fractures-horses, Accessed date: 25 February 2019.

Berenjian, A., Chan, N.L.C., Mahanama, R., Talbot, A., Regtop, H., Kavanagh, J., Dehghani, F., 2013. Effect of biofilm formation by *Bacillus subtilis* natto on menaquinone-7 biosynthesis. Mol. Biotechnol. 54 (2), 371–378.

Berenjian, A., Mahanama, R., Kavanagh, J., Dehghani, F., 2015. Vitamin K series: current status and future prospects. Crit. Rev. Biotechnol. 35 (2), 199–208.

Berenjian, A., Mahanama, R., Talbot, A., Biffin, R., Regtop, H., Valtchev, P., Kavanagh, J., Dehghani, F., 2011. Efficient media for high menaquinone-7 production: response surface methodology approach. N. Biotech. 28 (6), 665–672.

Berenjian, A., Mahanama, R., Talbot, A., Regtop, H., Kavanagh, J., Dehghani, F., 2012. Advances in menaquinone-7 production by *bacillus subtilis* natto: fed-batch glycerol addition. Am. J. Biochem. Biotechnol. 8 (2), 105–110.

Berenjian, A., Mahanama, R., Talbot, A., Regtop, H., Kavanagh, J., Dehghani, F., 2014. Designing of an intensification process for biosynthesis and recovery of menaquinone-7. Appl. Biochem. Biotechnol. 172 (3), 1347–1357.

Bezerra, M.A., Santelli, R.E., Oliveira, E.P., Villar, L.S., Escaleira, L.A., 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta 76 (5), 965–977.

Cutting, S.M., 2011. Bacillus probiotics. Food Microbiol. 28 (2), 214–220.

Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S.J., Berenjian, A., Ghasemi, Y., 2019. Prebiotics: definition, types, sources, mechanisms, and clinical applications. Foods 8 (3).

Goodman, S.R., Marrs, B.L., Narconis, R.J., Olson, R.E., 1976. Isolation and description of a menaquinone mutant from *Bacillus licheniformis*. J. Bacteriol. 125 (1), 282–289.

Kabir, M.S., Hsieh, Y.-H., Simpson, S., Kerdahi, K., Sulaiman, I.M., 2017. Evaluation of Two Standard and Two Chromogenic Selective Media for Optimal Growth and Enumeration of Isolates of 16 Unique Bacillus Species, vol. 80. pp. 952–962.

Mahanama, R., Berenjian, A., Dehghani, F., Kavanagh, J., 2012a. Modeling the effect of bed height and particle size for vitamin K2 production in a static bed fermenter. Eng. Lett. 20 (1), 16.

Mahanama, R., Berenjian, A., Dehghani, F., Kavanagh, J.M., 2011a. Solid-substrate Fermentation of Menaquinone 7 with *Bacillus subtilis*: Comparison of Continuous Rotation with Stationary Bed Fermentation at Different Initial Moisture Levels. Chemeca. Engineering a Better World, Sydney Hilton Hotel, NSW, Australia, pp. 315.

Mahanama, R., Berenjian, A., Regtop, H., Talbot, A., Dehghani, F., Kavanagh, J.M., 2012b. Modeling Menaquinone 7 production in tray type solid state fermenter. ANZIAM J. 53, 354–372.

Mahanama, R., Berenjian, A., Talbot, A., Biffin, R., Regtop, H., Dehghani, F., Kavanagh, J., 2011b. Effects of inoculation loading and substrate bed thickness on the production of menaquinone 7 via solid state fermentation. Cardiovasc. Dis. 2 **2**, 19–22.

Mahanma, R., Berenjian, A., Valtchev, P., Talbot, A., Biffin, R., Regtop, H., Dehghani, F., Kavanagh, J.M., 2011. Enhanced production of menaquinone 7 via solid substrate fermentation from *Bacillus subtilis*. Int. J. Food Eng. 7 (5).

Mahdinia, E., Demirci, A., Berenjian, A., 2018a. Effects of medium components in a glycerol-based medium on vitamin K (menaquinone-7) production by *Bacillus subtilis* natto in biofilm reactors. Bioproc. Biosyst. Eng. 1–10.

Mahdinia, E., Demirci, A., Berenjian, A., 2018b. Implementation of fed-batch strategies for vitamin K (menaquinone-7) production by *Bacillus subtilis* natto in biofilm reactors. Appl. Microbiol. Biotechnol. 102 (21), 9147–9157.

Mahdinia, E., Demirci, A., Berenjian, A., 2018c. Optimization of *Bacillus subtilis* natto growth parameters in glycerol-based medium for vitamin K (Menaquinone-7) production in biofilm reactors. Bioproc. Biosyst. Eng. 41 (2), 195–204.

Mahdinia, E., Demirci, A., Berenjian, A., 2019a. Effects of medium components in a glycerol-based medium on vitamin K (menaquinone-7) production by *Bacillus subtilis* natto in biofilm reactors. Bioproc. Biosyst. Eng. 42 (2), 223–232.

Mahdinia, E., Mamouri, S.J., Puri, V.M., Demirci, A., Berenjian, A., 2019b. Modeling of vitamin K (Menaquinoe-7) fermentation by *Bacillus subtilis* natto in biofilm reactors. Biocatal. Agric. Biotechnol. 17, 196–202.

Mohnacky, 2019. Animal Hospitals of Carlsbad. In: Fracture Repair, Retrieved 25 February, 2019, from. https://mohnackycarlsbad.com/orthopedics/fracture-repair/.

Nilegaonkar, S., Bhosale, S.B., Kshirsagar, D.C., Kapadi, A.H., 1992. Production of 2,3-butanediol from glucose by *Bacillus licheniformis*. World J. Microbiol. Biotechnol. 8 (4), 378–381.

Nithya, V., Muthukumar, S.P., Halami, P.M., 2012. Safety assessment of *Bacillus licheniformis* Me1 isolated from milk for probiotic application. Int. J. Toxicol. 31 (3), 228–237.

Patti, A., Gennari, L., Merlotti, D., Dotta, F., Nuti, R., 2013. Endocrine actions of osteocalcin. Int. J. Endocrinol. 2013, 10.

Perego, P., Converti, A., Del Borghi, M., 2003. Effects of temperature, inoculum size and starch hydrolyzate concentration on butanediol production by *Bacillus licheniformis*. Bioresour. Technol. 89, 125–131.

Prieto, M., O'Sullivan, L., Tan, S., McLoughlin, P., Hughes, H., Gutierrez, M., Lane, J., Hickey, R., Lawlor, P., Gardiner, G., 2014. In vitro assessment of marine *Bacillus* for use as livestock probiotics. Mar. Drugs 12 (5), 2422–2445.

Puri, A., Iqubal, M., Zafar, R., Panda, B.P., 2015. Influence of physical, chemical and inducer treatments on menaquinone-7 biosynthesis by *Bacillus subtilis* MTCC 2756. Songklanakarin J. Sci. Technol. 37 (3), 283–289.

Ranmadugala, D., Grainger, M., Manley-Harris, M., Berenjian, A., 2018. Determination of menaquinone-7 by a simplified reversed phase-HPLC method. Curr. Pharmaceut. Biotechnol. 19 (8), 664–673.

Riverina Australia, 2017. Protein Meals and Ingredients. Retrieved 25 February, 2019, from. http://www.riverina.com.au/products/soybean-meal/.

Sato, T., Yamada, Y., Ohtani, Y., Mitsui, N., Murasawa, H., Araki, S., 2001. Efficient production of menaquinone (vitamin K2) by a menadione-resistant mutant of *Bacillus subtilis*. J. Ind. Microbiol. Biotechnol. 26 (3), 115–120.

Seifan, M., Samani, A.K., Berenjian, A. J. A. m., biotechnology, 2016. Induced Calcium Carbonate Precipitation Using Bacillus Species, vol. 100. pp. 9895–9906 23.

Seifan, M., Samani, A.K., Berenjian, A. J. A. m., biotechnology, 2017. New Insights into the Role of pH and Aeration in the Bacterial Production of Calcium Carbonate ($CaCO_3$), vol. 101. pp. 3131–3142 8.

Song, J., Liu, H., Wang, L., Dai, J., Liu, Y., Liu, H., Zhao, G., Wang, P., Zheng, Z., 2014. Enhanced production of vitamin K2 from *Bacillus subtilis* (natto) by mutation and optimization of the fermentation medium. Braz. Arch. Biol. Technol. 57 (4), 606–612.

Southee, R., Haroon, S., Ebrahiminezad, A., Ghasemi, Y., Berenjian, A., 2016. Novel functional fermented dairy product rich in menaquinone-7. Biocatal. Agric. Biotechnol. 7, 31–35.

Wag, 2019. Surgical Fracture Repair in Dogs. Retrieved February, 2019, from. https://wagwalking.com/treatment/surgical-fracture-repair.

Willows Veterinary Center, Referral Service, 2019. Fracture Treatment-An Overview. Retrieved 25 February, 2019, from. https://www.willows.uk.net/specialist-services/pet-health-information/orthopaedics/fracture-treatment.