Modeling Menaquinone 7 production in tray type solid state fermenter

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Abstract

The fermented Japanese food *Natto* contains menaquinone 7 which is known to reduce the incidence of bone fractures and cardio vascular diseases. Natto is traditionally produced by the solid state fermentation of soy beans by *Bacillus subtilis* natto. A mathematical model is developed for describing the production of menaquinone 7 in a static solid substrate bed supported on a tray fermenter using parameters obtained from literature for similar micro-organisms. Two model parameters were fitted to experimental data obtained to predict menaquinone 7 production. The postulated model presented in the form of a sensitivity analysis is likely to yield valuable insights on the dynamic behaviour of bacterial kinetics, including the formation of products such as menaquinone 7 as the first step towards scaling up.

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Contents

1 Introduction

Menaquinone 7 (MK7) is part of the family known as vitamin K_2, and is necessary for the synthesis of blood coagulation factors, the activation of proteins involved in the building of bones and inhibition of vascular calcification [1, 2]. Solid state fermentation (SSF) of MK7 in static bed reactors has only been the subject of limited research [3, 4, 5, 6, 7, 25], where the relevant phenomena are yet to be fully understood; hence mathematical models play an important

role in fermenter design, optimization and potential scale up. This study proposes a model for an overall performance of a bioreactor. This mathematical model consists of transport phenomena and microbial kinetics to estimate the effects of environmental conditions on MK7 production. Shallow trays have a maximum bed thickness of 1 cm to avoid the O_2 transfer resistance. The set up used for collecting experimental data was designed in house and the model predicted the effect of process parameters on the biomass growth and production of MK7.

1.1 Model development

The model is a set of four balanced equations. The lumped parameter model describes the production of MK7 via *Bacillus subtilis* in tray type fermenters.

1.1.1 Basic kinetic equations

Mathematical models of SSF bioreactors are commonly based on logistic equations [8]. These equations rely on a simplification of mathematical modeling by employing a single equation for estimating the whole growth rate profile including the lag phase and cessation of growth in the last stage of fermentation. This simplified differential equation,

$$\frac{dX}{dt} = \mu X\left(1 - \frac{X}{X_m}\right), \tag{1}$$

eradicates the need for an additional equation for substrate measurement and estimating the parameters of SSF [9]. Here, X is microbial biomass, t is time, μ is the specific growth rate constant, and X_m is the maximum possible microbial biomass.

1.1.2 The effect of environmental conditions on growth

The two most important environmental variables that have significant impact on bioreactor operation are temperature and the water activity for the fermentation bed. The constant condition approach determines the effect of these environmental variables in the model,

$$\mu_T = \frac{A \exp\left[\frac{-E_{a1}}{R(T+273)}\right]}{1 + B \exp\left[\frac{-E_{a2}}{R(T+273)}\right]}, \tag{2}$$

where A and B are dimensionless, E_{a1} and E_{a2} fitting parameters, R is the universal gas constant, T is temperature, and μ_T is the effect of temperature on specific growth rate parameter. The assumption of an isothermal system was used to develop the model for determining the effect of temperature. A similar concept was also applied by von Meien and Mitchell [11] for predicting the effect of water activity on bacterial growth which can be depicted as

$$\frac{\mu_W}{\mu_{opt}} = \exp\left[D_1(a^3)_{ws} + D_2(a^2)_{ws} + D_3(a)_{ws} + D_4\right], \tag{3}$$

where D_1 to D_4 are fitting parameters, a_{ws} is water activity of the solid substrate phase, μ_W is the effect of water activity on the specific growth rate parameter, and μ_{opt} is the optimum specific growth rate parameter. In subsequent equations we use the true specific growth rate μ_G [12], which is the geometric mean of the temperature and water activity specific growth rate parameters:

$$\mu_G = \sqrt{\mu_W \mu_T}\,. \tag{4}$$

To date, most models of SSF bioreactors used this approach to describe the effect of environmental conditions on the parameters of the growth equation [10, 12, 13, 14] although the form of the empirical equation that is used varies. In any case, use of these equations within the kinetic submodel of a bioreactor model has the implicit assumption that the growth of the microorganism depends only on the current values of the environmental variables.

1.1.3 Death kinetics

In SSF bioreactors, the environmental conditions, especially the temperature, can attain values that are sufficiently adverse to cause death [15]. The modeling of death kinetics in SSF systems has received relatively little attention due to a problematic definition of death via measuring it experimentally through total and viable counts [15].

If death is simply defined as a permanent loss of the ability to grow, then autolysis is not a necessary consequence of death. Therefore death will not necessarily lead to a reduction in the amount of biomass, so it is not a simple matter to quantify death experimentally. As a result, current models of death used in SSF bioreactor models are very simple. For example, Sangsurasak and Mitchell [16] assumed first order death kinetics and segregated the microbial biomass into living and dead sub-populations:

$$\frac{dX_V}{dt} = \mu_G X_V \left(1 - \frac{X_V + X_D}{X_m} - k_D X_V\right), \tag{5}$$

$$\frac{dX_D}{dt} = k_D X_V\,, \tag{6}$$

where X_V is viable cells, X_D is dead cells, μ_G is true specific growth rate, and k_D is specific death rate coefficient.

It is not a simple matter to determine the true specific growth rate and the first order death constant (k_D) for use in these equations. We employed the equation proposed by Szewczyk and Myszka [17] by fitting two Arrhenius-type terms to a plot of observed specific growth rate versus temperature [15, 16],

$$\mu_{obs} = \mu_G - k_D = \mu_G - k_{D_0} \exp\left(-\frac{E_{a_D}}{RT}\right), \tag{7}$$

where k_D and k_{D_0} are frequency factors, E_{a_D} is activation energy of death kinetic, respectively; and μ_{obs} is used as μ in Equation (1).

1.1.4 Product formation

MK7 production is assumed to be of the form,

$$r_P = \frac{dP}{dt} = Y_{PX}\frac{dX}{dt} + m_p X, \tag{8}$$

where r_P is overall rate of product formation, Y_{PX} is growth associated product formation rate (fitted parameter), and m_P is nongrowth associated product formation rate (fitted parameter).

1.1.5 CO_2 production and O_2 consumption

The consumption of O_2 and CO_2 production are estimated by

$$\frac{dCO_2}{dT} = \mu\left(\frac{X}{Y_{X/CO_2}}\right) + m_{CO_2}X, \tag{9}$$

$$\frac{dO_2}{dT} = \mu\left(\frac{X}{Y_{X/O_2}}\right) - m_{O_2}X, \tag{10}$$

where Y_{X/CO_2} is the yield of CO_2 from biomass, Y_{X/O_2} is the yield of biomass from O_2, and m_{O_2}/m_{CO_2} are maintenance coefficients. Equation (10) can be used to indirectly measure the evolution of heat which affects the temperature and hence the specific growth rate (μ) shown in Equation (2). Additionally, O_2 consumption and CO_2 evolution are of particular interest in model postulation, since they represent the most convenient way of estimating the growth parameters in a bioreactor, and are the focus of upcoming experiments. Equations were solved using Simulink `ode15s`.

2 Materials and methods

2.1 Microoganism

Strain *Bacillus subtilis var. natto* was isolated from commercially available *natto* after screening different types for highest MK7 producing strain as described by Berenjian et al. [18].

2.2 Inoculum

Spores of *Bacillus subtilis* incubated on a liquid culture were suspended in 0.9% NaCl solution to obtain the standard spore solution of 10.8 ± 0.04 log CFU gm^{-1}). Solid state fermentation was carried out in square type Petri dishes (100 mm × 100 mm × 15 mm, *Greiner*, Germany) with a spore loading of 8.4 ± 0.04 log CFU gm^{-1}.

2.3 Medium

An equal mixture of corn and soy was autoclaved in the absence of water at 121°C for 20 minutes before inoculation. The water content after inoculation was adjusted to 70%.

2.4 Substrate preparation and fermentation

Substrates used in this experiment were nixtamalized corn grits and soy protein granules. These substrates were employed in a mixture of equal corn and soy without any supplementation with other carbon and nitrogen sources. The SSF procedure used has been described previously [6]. Briefly, the initial moisture content was adjusted at 50% by addition of sterilized

water to the autoclaved substrate that was kept in a fridge (4°C) overnight to allow complete swelling of the granules. Fermentation was carried out at 37°C inside an unaerated chamber (*Thermoline Scientific*, Australia) where relative humidity was maintained at 90–95% to minimize water evaporation from the substrate bed. The relative humidity, temperature and dew point were measured throughout the incubation period using a data logger (*Lascar Electronics*, UK). The production of MK7 was measured on days three, five and seven during the fermentation. Individual sample trays were prepared for each day to extract MK7 to minimize error in sampling and measurement.

2.5 MK7 extraction and determination

MK7 was extracted from 3 gm of homogenized wet substrate using 12 mL 2-propanol: n-hexane (v:v 1:2) and determined using high performance liquid chromatography (HP 1050, *Hewlett-Packard*, USA) using the method described in detail previously [18]. The LC-MS system (LCMS-2010EV, *Shimadzu*, Kyoto) was used to confirm the structure of MK7 [18].

2.6 Parameters and assumptions

The critical parameters for *Bacillus subtilis* do not exist in the literature. Therefore, the parameters listed in Table 1 for other microorganisms were used to estimate the constants for the equations for *Bacillus subtilis* fermentation. Microbiological research relies on the use of model organisms that act as representatives of their species or subspecies, these are frequently well-characterized laboratory strains. However, it has often become apparent that the model strain initially chosen may not represent important features of the species [26].

- Heat evolution is assumed to be 460 kJ $mol^{-1}O_2$ (14.375 kJ $gm^{-1}O_2$) [21].
- Enthalpy of evaporation of H_2O assumed to be 2 MJ kg^{-1} of H_2O [22].

Table 1: Coefficient values and model microorganisms (where appropriate, values have been converted from the units used by the cited source).

coeff.	value*	model strain	Ref	Eqn
A	$2.694 \times 10^{11}\,\mathrm{h}^{-1}$	*Aspergillus niger*	[10]	(2)
B_1	3×10^{47}	*Aspergillus niger*	[10]	(2)
A_{a1}	$70225\,\mathrm{J\,mol}^{-1}$	*Aspergillus niger*	[10]	(2)
E_{a2}	$283356\,\mathrm{J\,mol}^{-1}$	*Aspergillus niger*	[10]	(2)
D_1	618.92	*Aspergillus niger*	[19]	(3)
D_2	−1863.53	*Aspergillus niger*	[19]	(3)
D_3	1865.1	*Aspergillus niger*	[19]	(3)
D_4	−620.67	*Aspergillus niger*	[19]	(3)
Y_{X/CO_2}	$0.76394\,\mathrm{kg\,C\,O_2\,kg\,X}^{-1}$	*Rhizopus sp.*	[12]	(9)
m_c	$0.031\,\mathrm{kg\,C\,O_2\,kg\,X}^{-1}\,\mathrm{h}^{-1}$	*Rhizopus sp.*	[12]	(9)
Y_{X/O_2}	$0.9510\,\mathrm{kg\,X\,kg\,O_2}^{-1}$	*Gibberella fujikori*	[20]	(10)
m_{O_2}	$0.013\,\mathrm{kg\,O_2\,kg\,X}^{-1}\,\mathrm{h}^{-1}$	*Gibberella fujikori*	[20]	(10)

- Oxygen transfer resistance is negligible.
- Unlimited substrate availability (nitrogen and carbon sources).
- Temperature and water activity (a_w) remain constant throughout cultivation.
- Model parameters remain constant throughout cultivation.
- Shikimate pathway for MK7 production was neglected.

3 Results and discussion

SSF modeling is challenging due to the heterogeneous nature of fermentation and lack of available data for microorganisms of interest. Additionally possible

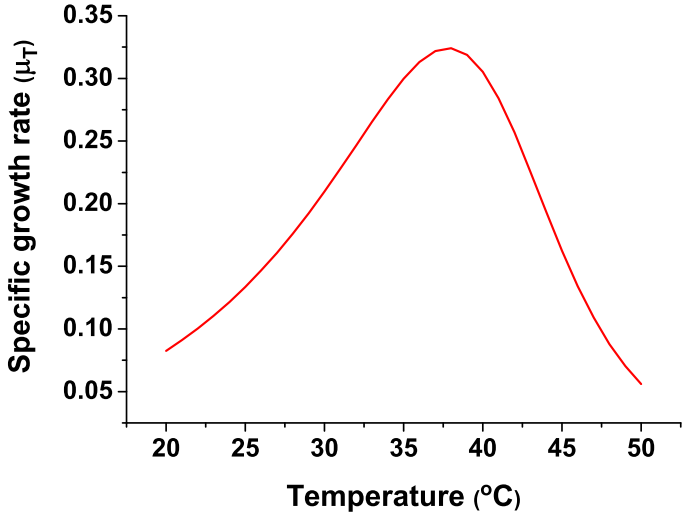

Figure 1: Dependence of specific growth rate upon temperature.

deviations are expected since the dissimilarity of fitting parameters obtained by different model microorganisms. The kinetic submodel is described including the dependency on key environmental variables, because these variables typically cannot be simply controlled at their optimum values in a SSF bioreactor [8].

The effects of temperature and water activity on growth are described in Figures 1 and 2, by expressing the parameters in the kinetic equation as functions of the local conditions. These functions were calculated assuming "isothermal" and "isohydric" conditions, which were maintained throughout the growth cycle, whereas in real SSF processes the temperature and the water activity change during the process. It is possible that expressions for the effects of temperature and water activity that were obtained from isothermal and isohydric assumptions cannot describe the true effect on growth of the time varying conditions that are encountered by the organism in SSF processes at large scales [23].

According to Equation (2) the effect of the specific growth rate parameter μ

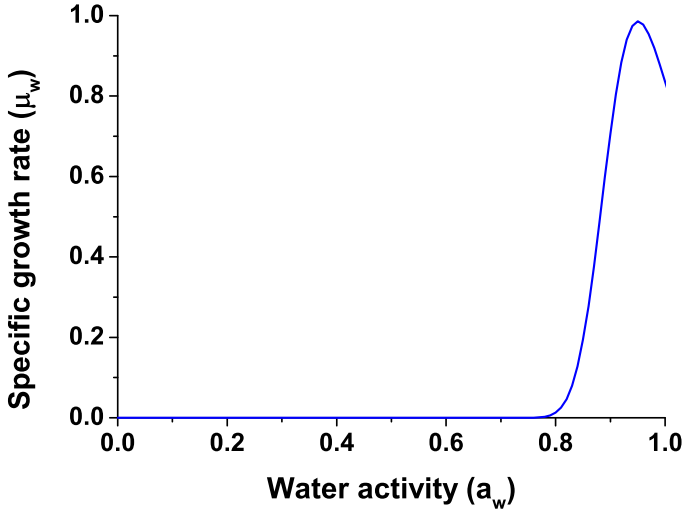

Figure 2: Dependence of specific growth rate upon water activity.

on temperature is described using the double-Arrhenius equation of Saucedo–Castaneda et al. [10]. The symbol μ_T denotes that the equation describes specifically the effect of temperature on the specific growth rate parameter. The dependence of the specific growth rate parameter μ_W on water activity was described by Equation (3) of von Meien and Mitchell [11], and the geometric mean of the individual specific growth rates were calculated for their combined effect in Equation (4).

The variation of MK7 production during the cultivation at 37°C and a_w of 0.95 is presented in Figure 3, in which the solid line represents the estimation from the model by fitting the experimental data and adjusting growth associated product formation (Y_{PX}) and nongrowth associated product formation rate (m_P). There was a good agreement between experimental data and results predicted by the model. The data in Figure 4 shows that by increasing the temperature from 35°C to 45°C the yield of MK7 decreased by 40%. The predicted value for MK7 production by the model was 140 mg kg^{-1} within the temperature range 35°C to 37°C, which is similar to value acquired

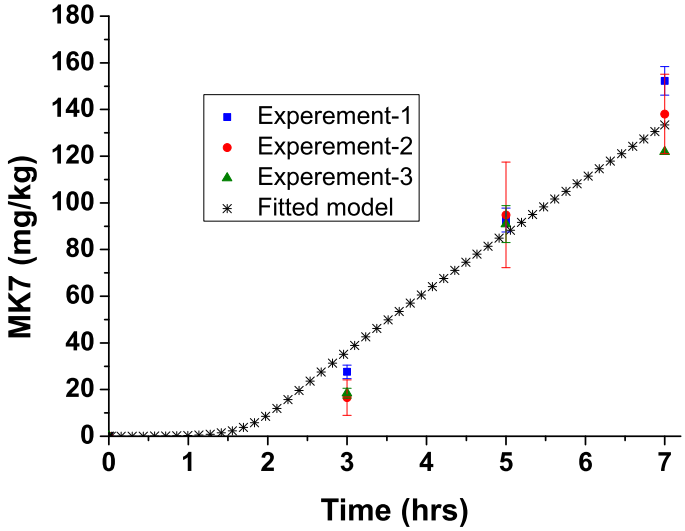

Figure 3: Model estimation versus experimental values.

by Mahanama et al. [5, 6, 7] using experimental data and response surface methodology.

The effect of water activity on the production of MK7 at 37°C is shown in Figure 5. The results of our study demonstrate that the high moisture content is critical for the production of MK7 and its production ceased when the moisture content dropped below 80%. This data shows that bacteria growth in SSF is different from fungi that can grow even at lower moisture content such as 50% humidity [24]. The low moisture level dries the culture and decreases the growth rate, subsequently dropping the MK7 yield. The production of MK7 was enhanced for all solid substrates with the initial moisture level of 70% when using static fermentations [6]. According to Lonsane et al. [25] only limited water is used in SSF, but water exhibits profound effects on the physicochemical properties of solids, which in turn affects process productivities. Different models were used to investigate the effect of water activity and moisture on fermentation and MK7 production. Our results shows the significant impact of water activity on MK7 production.

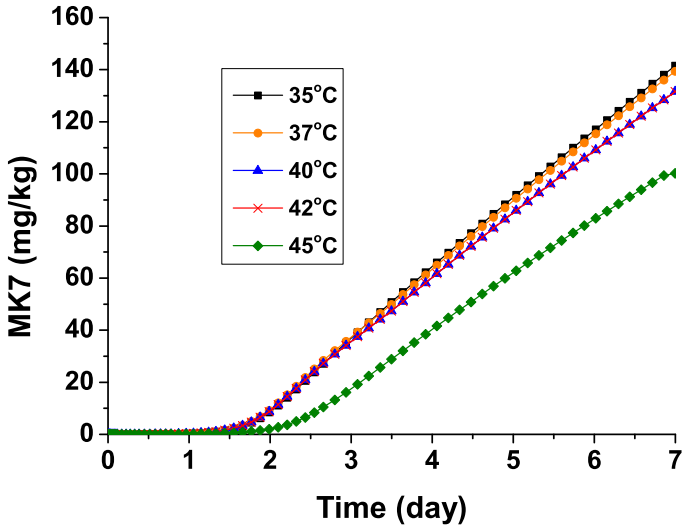

Figure 4: The effect of temperature on MK7 production

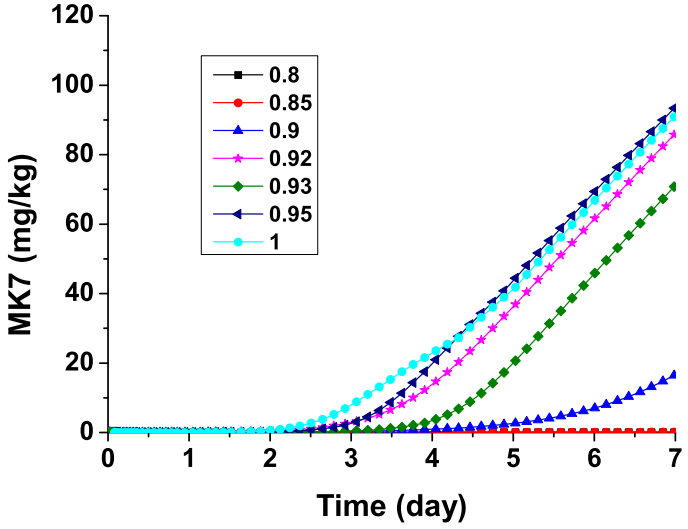

Figure 5: The effect of water activity on MK7 production

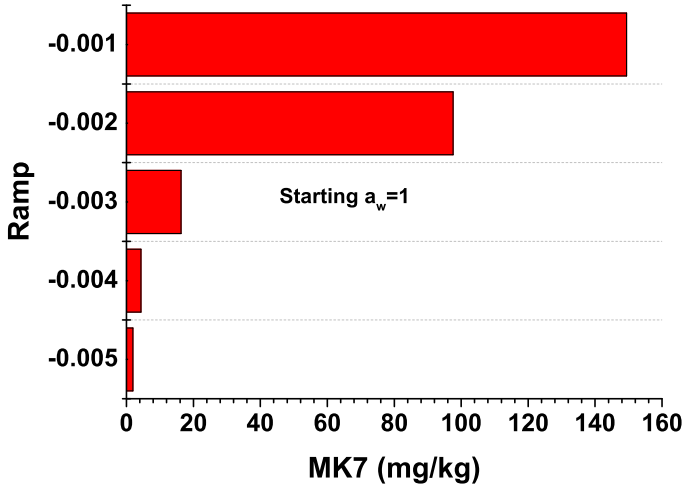

Figure 6: Predicted MK7 concentrations in reducing water activities

As shown in Figure 6, the MK7 recovery at the end of fermentation was decreased with a reducing ramp that was associated with a faster moisture evaporation rate. The model predicted the MK7 concentration of 150 $\mathrm{mg\,kg^{-1}}$ at the lowest level of moisture content reduction (that is, −0.001). The postulated MK7 concentration was dropped below 12 $\mathrm{mg\,kg^{-1}}$ when the ramp increased fivefold. At the end of the fermentation period the moisture contents for ramps −0.001 and −0.005 were 83% and 16%, respectively. These results are in good agreement with our previous data in which the sudden drop of moisture content within the range of 10% to 20% dramatically decreased MK7 production to 80% [6].

4 Conclusions

The mathematical model developed in this study is based on parameters from *Gibberella fujikori*, *Aspergillus niger* and *Rhizopus sp.* microoganisms. The model could be matched to measurements, and qualitatively predict the effect

on drying in MK7 production via a solid sate static bed fermenter. Water activity had a significant impact on MK7 production; however, temperature had a negligible effect within the range examined. The evaporation of water from the substrate bed had an adverse effect on MK7 production due to the drying and shrinkage of the bed. The proposed model has the potential to be used for developing a tray type bioreactor with a thicker bed for the production of MK7. Further research is required in a broader range to determine the effects of process parameters on the growth rate of microorganisms and MK7 production.

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