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EVALUATION OF BIOCONVERSION CONDITIONS ON REUTERIN PRODUCTION USING RESPONSE SURFACE METHODOLOGY AND *LISTERIA MONOCYTOGENES* AS TARGET BACTERIA

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ABSTRACT

The objective of this study was to evaluate the effect of bioconversion conditions on reuterin production by *Lactobacillus reuteri* using response surface methodology (RSM). A central composite design consisted of three independent factors at 5 levels: concentration of *Lactococcus reuteri* (log CFU/ml), glycerol concentration (mM), and incubation time (h) was set up and consisted of 18 experimental runs. The dependant factor was the relative reuterin concentration expressed through its antibacterial activity (Units reuterin per ml, U/ml) against *L. monocytogenes* using microbroth dilution assay. A polynomial equation for prediction of reuterin production (U/ml) produced by *L. reuteri* was created based on obtained data. Results showed that incubation time, bacterial and glycerol concentrations had linear positive effects on reuterin production. The quadratic effect of incubation time had negative effect on reuterin production in which at a fixed bacterial concentration of *L. reuteri*, a longer incubation time with low concentration of glycerol will cause the reduction in relative reuterin concentration. It was found that approximately 450 U/ml can be produced by *L. reuteri* with glycerol concentration ranging from 320 to 340 mM, *L. reuteri* concentration of 9.4 log CFU/ml and 2 h of incubation. Results demonstrated that applying suitable conditions for glycerol bioconversion into reuterin by *L. reuteri*, a high relative reuterin concentration could be obtained and used for food preservation against *L. monocytogenes*.

Keywords: Antibacterial activity, Reuterin, *Lactobacillus reuteri*, *Listeria monocytogenes*, response surface methodology

INTRODUCTION

Lactobacillus reuteri is a member of gastrointestinal bacteria of healthy human and animals. It has been demonstrated as a potential probiotic bacterium in biotransformation and health stimulation (van Niel *et al.*, 2012) and in immunomodulation (Mechoud *et al.*, 2012). Further, *L. reuteri* is well-known as a producer of 3-hydroxypropionaldehyde (3-HPA) or reuterin during anaerobic metabolism of glycerol (Axelsson *et al.*, 1989; Chung *et al.*, 1989). Glycerol is converted to reuterin by a coenzyme B₁₂-dependent dehydratase (Talarico and Dobrogosz, 1990). Reuterin can be further reduced by NADH to form 1,3-propanediol which is catalyzed by 1,3-propanediol: NAD oxidoreductase (Talarico *et al.*, 1990). Reuterin can inhibit the growth of yeast, protozoa, and many bacteria, both Gram-negative and Gram-positive (Axelsson *et al.*, 1989; Chung *et al.*, 1989).

Reuterin is water soluble compound, and it is active in a wide range of pH. It is also resistance to proteolytic and lipolytic enzymes (El-Ziney *et al.*, 2000; Vollenweider and Lacroix 2004). Therefore, potential of using reuterin for preservation of food products are of great interest (El-Ziney and Debevere 1998; Arqués *et al.*, 2004; Montiel *et al.*, 2014). Reuterin could inhibit the growth of pathogenic bacteria such as *Listeria monocytogenes*, and *Escherichia coli* O157: H7 in milk and cottage cheese (El-Ziney and Debevere 1998), *L. monocytogenes* in cold-smoked salmon (Montiel *et al.*, 2014). A synergistic effect of reuterin plus nisin against *L. monocytogenes* and *Staphylococcus aureus* was obtained in refrigerated milk at the end of storage of 12 days (Arqués *et al.*, 2004).

It has been found that *L. monocytogenes* was more resistant to reuterin than some other bacteria. For example, reuterin at the same activity (8 U/ml) was more effective in eliminating *Staphylococcus aureus* than *L. monocytogenes* at 37 °C in milk (Arqués *et al.*, 2004). Similarly, it has been observed that *E. coli* strains were sensitive to reuterin than *Listeria* strains including *L. monocytogenes* (El-Ziney *et al.*, 2000). Thus, it is important to find suitable conditions so that *L.*

reuteri can produce high reuterin concentration to eliminate *L. monocytogenes* in food products.

It has been demonstrated that several factors such as the glycerol and biomass concentrations, incubation time, temperature and pH can influence on reuterin production produced by *L. reuteri* (Lüthi-Peng *et al.*, 2002a,b; Tobajas *et al.*, 2007). However, most of studies focused on evaluation of varying effect of a single factor while other factors were fixed on reuterin activity and therefore, the possible interactive effects among different factors on reuterin production by *L. reuteri* could not be established.

Response surface methodology (RSM) is known as a combination of mathematical and statistical techniques for optimization of a process where dependent factors are affected by several independent factors. This method can be used to determine the possible interactive effects among different independent factors on the dependent factors in an experimental design (Adjalle *et al.*, 2011; Khan *et al.*, 2014).

Thus, the aim of this study was to evaluate the interactive effects of bioconversion condition on reuterin activity against *L. monocytogenes* using RSM. A central composite consisting of three independent factors (glycerol concentration, *L. reuteri* concentration and incubation time) at 5 levels was designed. The design formed 18 experimental bioconversion conditions. Reuterin production or relative reuterin concentration expressed through its activity (Units per ml, U/ml) against *L. monocytogenes* was determined using microbroth dilution method.

MATERIALS AND METHODS

Bacterial and growth conditions

Lactobacillus reuteri ATCC 53608 was used in this study. The stock culture of *L. reuteri* in MRS broth containing 10 % glycerol was preserved as aliquot of 1 ml in cryovial tubes at -80 °C. Before each experiment, one ml of stock culture was inoculated into test tube containing 9 ml of MRS broth, incubated at 37°C for

24 h to obtain pre-inoculum. Then, the pre-inoculum (1.5 ml) was inoculated in Erlenmeyer containing 30 ml of MRS broth and incubated at 37 °C for 24 h to obtain inoculum.

L. monocytogenes 2812 stock cultures were stored at -80 °C in Tryptic Soya Broth medium containing glycerol (10% v/v). Before each experiment, stock cultures were grown through two consecutive 24 h growth cycles in Tryptic Soya Broth at 37 °C. The cultivated cultures were centrifuged at 5000 g for 15 minutes and the obtained pellets were washed twice in sterile saline water (0.85% w/v) to obtain working cultures containing approximately 10^9 CFU/mL. The working cultures were diluted in sterile saline water (0.85 %, w/v) to have final concentration of 10^6 CFU/ml. This bacterial solution was then used for microbroth dilution assay to evaluate relative reuterin activity (U/ml).

Biomass production of *L. reuteri*

Preliminary experiment on the effect of fermentation time on reuterin production and on its antibacterial activity showed that biomass of *L. reuteri* collected at the end of exponential phase (24 h of fermentation) gave better antimicrobial activity against *L. monocytogenes*. Therefore, biomass of *L. reuteri* was collected at 24 h of fermentation.

The inoculum of *L. reuteri* was inoculated (5%, v/v) into Erlenmeyers containing 200 ml of MRS broth. The Erlenmeyers were incubated in an incubator at 37 °C under static conditions for 24 h. The fermented broth was collected in centrifugal tubes and centrifuged at 5,000 x g for 10 min at 4°C. The supernatant was discarded and biomass was washed twice with 50 mM sodium phosphate buffer (pH 7.5) to eliminate residual components of culture medium (MRS broth). The washing step was conducted by suspended biomass in 50 mM sodium phosphate buffer, then centrifuged at 5000 x g for 10 minutes at 4°C and the supernatant was discarded after each washing step. Finally, biomass was suspended in 50 mM sodium phosphate buffer (pH 7.5). The cell concentration of *L. reuteri* (CFU/ml) in suspended biomass was enumerated by 10-fold serial dilution and plating on petri dishes containing MRS agar. Further, the suspended biomass was diluted by 1, 1.2, 1.4, 1.6, 1.8 and 2 folds and the optical density (OD) values of diluted

solutions were recorded at 595 nm. Based on cell concentration determined by plating counting method and the OD of different diluted solutions of suspended biomass, the linear correlation between CFU/ml and OD values was established. This correlation helped to determine the approximately bacterial concentration of *L. reuteri* in subsequent experiments based on OD value of the cultures.

Experimental design for reuterin production

A central composite consisting of 3 independent factors (glycerol concentration, bacterial concentration and incubation time) at 5 levels (Table 1) was designed to find the suitable conditions for reuterin production against *L. monocytogenes*. This included 18 experimental bioconversion conditions (Table 2).

Table 1 Level of independent factors in the central composite design

Independent factors	Level				
	-2	-1	0	1	2
Glycerol concentration (X_1 , mM)	166	200	250	300	334
Bacterial concentration (X_2 , log CFU/ml)	8.0	8.3	8.7	9.1	9.40
Incubation time (X_3 , h)	0.3	1	2	3	3.7

Bacterial suspension of *L. reuteri* at different concentrations was added into falcon tubes, and glycerol was added to obtain final concentration of bacteria and glycerol as required depending on each experimental condition (Table 2). The final volume of each falcon tube was 10 ml. The mixture in falcon tubes was mixed well and falcon tubes were anaerobically incubated at 37 °C for different periods (h) as required in the experimental design. Then, tubes were centrifuged at 5 000 x g for 10 minutes and supernatant was collected. The supernatant was filtered through a 0.45 µm filter. The supernatant was kept at 4 °C and used for evaluation of its activity against *L. monocytogenes* using microbroth dilution assay.

Table 2 Experimental runs and relative reuterin concentration

Experimental condition	Glycerol concentration (mM)	Bacterial concentration (log CFU/ml)	Incubation time (h)	Observed relative reuterin concentration (U/ml)	Predicted relative reuterin concentration (U/ml)
1	200	8.3	1.0	64.0	57.5
2	200	8.3	3.0	96.0	128.2
3	200	9.1	1.0	256.0	237.8
4	200	9.1	3.0	256.0	308.5
5	300	8.3	1.0	96.0	96.5
6	300	8.3	3.0	96.0	167.2
7	300	9.1	1.0	256.0	276.8
8	300	9.1	3.0	384.0	347.5
9	166	8.7	2.0	192.0	204.9
10	334	8.7	2.0	256.0	270.5
11	250	8.0	2.0	128.0	86.7
12	250	9.4	2.0	384.0	388.7
13	250	8.7	0.3	64.0	78.8
14	250	8.7	3.7	256.0	197.7
15	250	8.7	2.0	256.0	237.7
16	250	8.7	2.0	256.0	237.7
17	250	8.7	2.0	256.0	237.7
18	250	8.7	2.0	256.0	237.7

Evaluation of reuterin production (U/ml) using microbroth dilution assay

Reuterin production was evaluated by relative reuterin concentration which is expressed through its antibacterial activity (U/ml) using dilution assay (Chung *et al.*, 1989). In this study, *L. monocytogenes* was used as target bacterial strain and the microbroth dilution assay with some modification was applied for evaluation of reuterin production (Turgis *et al.*, 2012). The supernatants obtained from experimental conditions were diluted by 8, 12, 16, 24, 48, 96, 128 and 192 times using sterile sodium phosphate buffer (50 mM, pH 7.5). First, 125 µl of sterile Mueller-Hinton Broth (MHB) were filled into wells of a microplate. Then, 50 µl of reuterin solution at different concentrations were added into wells of eight columns of the microplate. Finally, 25 µl of working culture of *L. monocytogenes* (10^6 CFU/ml) were inoculated into the wells of six rows of the microplate. The last two rows containing MHB, different concentrations of reuterin and without *L. monocytogenes* served as blank. The columns containing MHB with *L. monocytogenes* and without reuterin served as positive control. The final volume of the wells in the microplate was 200 microliters. Thus, the final reuterin concentration in the tested wells was diluted by 4 fold. This factor plus the dilution level mentioned above (8, 12, 16, 24, 48, 96, 128 and 192) were considered in calculation the reuterin activity against *L. monocytogenes*. The microplate was incubated for 24 h at 37 °C, then, the optical absorbance (OD) of the microplate was measured at 595 nm using an Ultra Microplate Reader (Biotek

instruments, Winooski, VT, USA). Relative reuterin concentration (Unit reuterin per ml or U/ml) was calculated as the reciprocal of the highest dilution of the sample that can inhibit the growth of *L. monocytogenes* (Chung *et al.*, 1989).

Statistical analysis

Data obtained from experimental design were used for analysis of variance (ANOVA), and regression analysis using STATISTICA 12 (STATSOFT Inc., Tulsa, US). A general second-order polynomial equation was built to predict reuterin production (equation 1).

$$Y = B_0 + \sum_{i=1}^3 B_i X_i + \sum_{i=1}^3 B_{ii} X_i^2 + \sum_{i=1}^2 \sum_{j=i+1}^3 B_{ij} X_i X_j \quad (\text{equation 1})$$

where Y is the predicted response; B_0 , intercept; X_i and X_j are values of various levels of the independent variables; B_i is the values of linear coefficients; and B_{ii} is the values of quadratic coefficients and B_{ij} is the values of the interaction between 2 independent factors (Adjallé *et al.*, 2011). Response surface plots were created to show the effects between the independent variables (glycerol concentration (X_1), bacterial concentration (X_2) and incubation time (X_3)) on the reuterin production (U/ml).

RESULTS

Analysis of variance for reuterin production and regression coefficients of factors for the model

The relative reuterin concentrations (U/ml) of 18 experimental bioconversion conditions are presented in Table 2. It can be observed that depending on conditions of three independent factors (glycerol, bacterial concentrations and incubation time), the observed relative reuterin concentration could vary from minimum of 64 to maximum of 384 U/ml. The higher relative reuterin concentration (U/ml) of a sample means that even at higher factor of dilution of this sample, it can still have the capacity to eliminate *L. monocytogenes* in tested system.

Analysis of variance (ANOVA) was conducted and the model had a regression coefficient factor (R^2) of 0.89, indicating that 89% of the variation in the responses could be explained by the combination of the responses. Further, there was no lack of fit of the model. Thus, the model can be used for prediction of reuterin production based on incubation time, glycerol, and bacterial concentration.

The reduced ANOVA table (after eliminating the factors which are not important in the model) is presented in Table 3. It can be observed that the linear effect of bacterial concentration was a very important factor on reuterin production ($P = 0.000001$). The linear and quadratic effects of incubation time were also important to affect reuterin production ($P = 0.004$). The linear effects of glycerol ($P = 0.07$) was important on reuterin production but less than those of incubation time and bacterial concentration (Table 3).

Table 3 Analysis of variance for reuterin production

Source	Sum of square	df	P value
Glycerol concentration (L)*	5244.9	1	0.07
Bacterial concentration (L)	110631.7	1	0.000001
Incubation time (L)	17075.4	1	0.004
Incubation time (Q)	16846.3	1	0.004
Total sum of square	167876.5		

* L and Q represent for linear and quadratic effect. There is no lack of fit of the model.

Table 4 Regression coefficients of the predictive model

Factors	Regression Coefficient	P value
Constant	-2032.00	0.000001
Glycerol concentration (L)*	0.39	0.07
Bacterial concentration (L)	225.39	0.000001
Incubation time (L)	175.94	0.0009
Incubation time (Q)	-35.15	0.004

* L means Linear effect, Q means Quadratic effect.

The final regression coefficients of the factors for the model are presented in Table 4 and were used to create a predictive model (equation 2):

$$Y = -2032.00 + 0.39X_1 + 225.39X_2 + 175.94X_3 - 35.15X_3^2 \quad (\text{equation 2})$$

where Y = reuterin activity (U/ml); X_1 is glycerol concentration (mM); X_2 is *L. reuteri* concentration (log CFU/ml); and X_3 is incubation time (h).

It can be observed that all linear effect of glycerol, biomass and incubation time have positive effects on the reuterin production while the quadratic effect of incubation time have a negative impact on the activity (Table 3). Based on the model, the predicted values of relative reuterin concentration (U/ml) of experimental runs are also presented in the Table 2.

Response surface plots of the interactive effects between different independent factors on reuterin activity

Response surface plots of the relation between different independent factors on the reuterin production was created and expressed in Figure 1 to Figure 3.

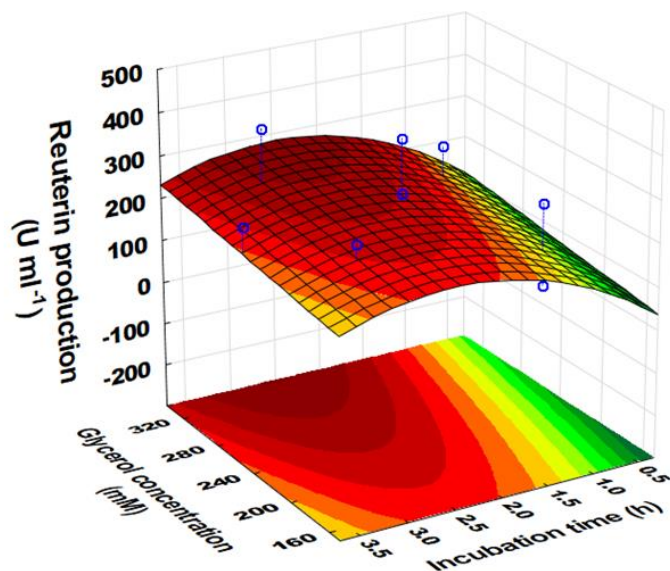


Figure 1 Effect of glycerol concentration and incubation time on reuterin production (bacterial concentration was fixed at 8.7 log CFU/ml)

Figure 1 presents the relation between glycerol concentration (X_1 , mM) and incubation time (X_3 , h) on reuterin production when the concentration of *L. reuteri* (X_2) was kept constant at 8.7 log CFU/ml. It can be observed that increase in both incubation time (from 0.5 to about 2.5 h) and glycerol concentration (140-340 mM) can cause an increase in relative reuterin concentration; however, further increase in incubation time and at low glycerol concentration, a decrease in relative reuterin concentration can be occurred. For example, when the incubation time is around 1 h, and glycerol concentration is from 140 to approximately 200 mM, a relative reuterin concentration of equal or less than 140 U/ml can be produced by 8.7 log CFU/ml of *L. reuteri* (Figure 1). When glycerol concentration is from 280 to 340 mM and the incubation time is from 2 to 3 h, almost 300 U/ml can be produced by the same concentration of *L. reuteri* (8.7 log CFU/ml). However, when the incubation time is more than 3 h and glycerol concentration is less than 240 mM, relative reuterin concentration of less than 200 U/ml can be obtained (Figure 1). Thus with a fixed bacterial concentration of *L. reuteri*, a longer incubation time with low concentration of glycerol will cause the reduction in reuterin production.

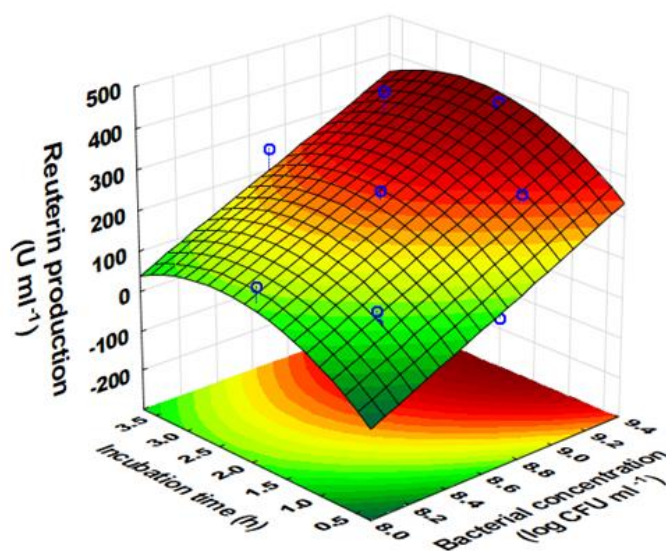


Figure 2 Effect of bacterial concentration and incubation time on reuterin production (glycerol concentration was fixed at 250mM)

Figure 2 presents the relation between bacterial concentration (X_2 , log CFU/ml) and incubation time (X_3 , h) on reuterin production when glycerol concentration was kept constant at 250 mM. In general, it can be observed that the increase in bacterial concentration (from 8 to 9.4 log CFU/ml) and the increase in incubation time (from 0.5 to 2.5 h) cause a significant increase in relative reuterin concentration; however, further increase in incubation time (more than 2.5 h) of solution containing 250 mM glycerol with irrespective of bacterial concentrations

cause a decrease in reuterin production (Figure 2). It can be seen that high relative reuterin concentration (approximately 400 U/ml) can be obtained using 9.4 log CFU/ml of *L. reuteri*, 250 mM glycerol and 2.5 h of incubation. With the same conditions but with the incubation time of 3.5 h, only 350 U/ml against *L. monocytogenes* can be obtained.

Figure 3 presents the interactive effects between glycerol concentration (X_1 , mM) and bacterial concentration (X_2 , log CFU/ml) on reuterin production when the incubation time was kept constant at 2 h. It can be observed that increasing both *L. reuteri* and glycerol concentrations cause an increase in reuterin production. It is of interest to observe that since the incubation time is fixed, a lower concentration of glycerol require a higher concentration of bacteria or a higher concentration of glycerol require a lower concentration of bacteria to produce similar reuterin concentration (similar antilisterial activity). For example, when glycerol concentration is around 200 mM, and bacterial concentration is around 8.8 log CFU/ml, a relative reuterin concentration of approximately 200 U/ml can be obtained. Similarly, when glycerol concentration is around 280 mM, and bacterial concentration is around 8.6 log CFU/ml, a relative reuterin concentration of approximately 200 U/ml can also be obtained. With incubation time of 2 h, a higher relative reuterin concentration of approximately 450 U/ml can be obtained by using the bacterial concentration of approx. 9.4 log and a glycerol concentration of 320 to 340 mM (Figure 3). Figure 3 also showed that further increasing glycerol and bacterial concentration at fixed incubation time of 2 h may obtain higher relative reuterin concentration.

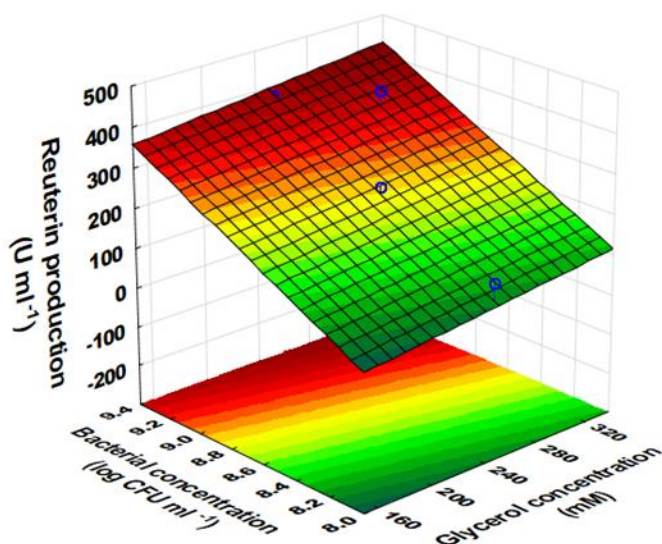


Figure 3 Effect of glycerol concentration and *L. reuteri* concentration on reuterin production (incubation time was fixed at 2 h)

DISCUSSION

Reuterin (3-hydroxypropionaldehyde, 3HPA) is a low-molecule-weight, nonproteinaceous antimicrobial substance containing aldehyde group. Reuterin is not a bacteriocin (Vollenweider and Lacroix 2004) which is different with other proteinaceous bacteriocins which are produced by many other strains of lactic acid bacteria (LAB) Turgis et al., 2013). In addition to *L. reuteri*, some other bacterial genera such as *Klebsiella*, *Enterobacter*, *Citrobacter*, *Clostridium*, and *Bacillus* can also produce reuterin (Vollenweider and Lacroix 2004).

It has been observed that *E. coli* strains were sensitive to reuterin than *Listeria* strains including *L. monocytogenes* (El-Ziney et al., 2000). El-Ziney and Debevere (1998) found that adding reuterin at 100 and 150 Units g⁻¹ cheese caused a reduction of *E. coli* by 3 and 6 log CFU, respectively, while a reduction of 2 and 5 log was observed for *L. monocytogenes* at day 7 of storage. The results showed that *L. monocytogenes* was more resistant to reuterin than that of *E. coli*. Thus, to apply reuterin as an antimicrobial agent against foodborne pathogens such as *L. monocytogenes*, a high reuterin concentration is required. Therefore, current study focused on effects of different combined conditions (Table 2) on bioconversion glycerol into reuterin by *L. reuteri* and the relative reuterin concentration was evaluated through its activity against *L. monocytogenes*.

It was found that incubation time, bacterial and glycerol concentration had positive effect on reuterin production. It showed that utilization of 320-340 mM glycerol, 9.4 log CFU/ml of *L. reuteri* and incubation time of 2 h, a high relative reuterin concentration of approximately 450 U/ml can be obtained (Figure 3). However, at a fixed concentration of bacterial and glycerol concentrations, longer incubation time caused a decrease in reuterin production. The decrease in relative reuterin concentration or its activity after long incubation time was also observed by other studies (Lüthi-Peng et al., 2002b). The decrease in its activity could be

due to reuterin was reduced to form 1,3-propanediol (Slininger et al., 1983; Talarico et al., 1990; Vollenweider and Lacroix, 2004). Further, it is also known that reuterin at high concentration can also be toxic for *L. reuteri*, the producer (Lüthi-Peng et al., 2002b; Vollenweider and Lacroix, 2004).

Concerning the mechanism of action of reuterin, it was proposed that the aldehyde group of reuterin is mainly responsible for antimicrobial activity (Schaefer et al., 2010; Vollenweider et al., 2010). Schaefer et al., (2010) demonstrated that reuterin induce oxidative stress in target organisms. The authors confirmed that the aldehyde form interacting with thiol groups of small molecules and proteins of target organisms and cause growth inhibition. When cysteine was added into growth media of *E. coli* or *Clostridium difficile* before exposing to reuterin, the antibacterial effects of reuterin against these bacteria was suppressed (Schaefer et al., 2010). In other study, Vollenweider et al., (2010) demonstrated that reuterin induces a depletion of reduced glutathione (GSH) in *E. coli* and then attack on protein-linked SH- groups, changing protein functions which finally result in cell death. Further, adding exogenous GSH caused an increase in intracellular GSH concentrations up to 9-fold and prevented the alteration of protein thiols, and resulted in rescuing the bacteria (Vollenweider et al., 2010). These properties explain for the reason why reuterin can act against a broad spectrum of bacteria, yeast, protozoa and it is a candidate for food preservation.

CONCLUSIONS

This study evaluated the bioconversion conditions including incubation time, glycerol and concentration of *L. reuteri* on reuterin production using response surface methodology and the relative reuterin concentration was expressed through its activity against *L. monocytogenes*. A polynomial predictive equation for relative reuterin concentration produced by *L. reuteri* was established. Incubation time, glycerol and *L. reuteri* concentrations had positive effects on reuterin production. At a fixed concentration of bacteria, longer incubation time and low concentration of glycerol caused a decrease in reuterin production. It was found that with glycerol concentration of 320-340 mM, 9.4 log CFU/ml of *L. reuteri* and 2 h of incubation, a high relative reuterin concentration of approximately 450 U/ml against *L. monocytogenes* can be obtained. Future study on application of reuterin alone or in combination with other antibacterial agents such as essential oils, organic acid salts to control *L. monocytogenes* or other foodborne pathogens in food products is necessary.

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