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Improvement of Folate Biosynthesis by Lactic Acid Bacteria Using Response Surface Methodology

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Summary

Lactic acid bacteria (*Lactococcus lactis* NZ9000, *Lactococcus lactis* MG1363, *Lactobacillus plantarum* I-UL4 and *Lactobacillus johnsonii* DSM 20553) have been screened for their ability to produce folate intracellularly and/or extracellularly. *L. plantarum* I-UL4 was shown to be superior producer of folate compared to other strains. Statistically based experimental designs were used to optimize the medium formulation for the growth of *L. plantarum* I-UL4 and folate biosynthesis. The optimal values of important factors were determined by response surface methodology (RSM). The effects of carbon sources, nitrogen sources and *para*-aminobenzoic acid (PABA) concentrations on folate biosynthesis were determined prior to RSM study. The biosynthesis of folate by *L. plantarum* I-UL4 increased from 36.36 to 60.39 μg/L using the optimized medium formulation compared to the selective Man de Rogosa Sharpe (MRS) medium. Conditions for the optimal growth of *L. plantarum* I-UL4 and folate biosynthesis as suggested by RSM were as follows: lactose 20 g/L, meat extract 16.57 g/L and PABA 10 μM.

Key words: folate, lactic acid bacteria, *Lactobacillus plantarum* I-UL4, response surface methodology

Introduction

Folate plays an important role in human life as one of the most important essential components for the synthesis of purine, guanine, adenine, pyrimidine and thymine. Sufficient daily doses of folate may prevent diseases such as colon cancer, growth retardation, anaemia and neural tube defects in newborn. The human body needs 200 to 400 μg of folate per day. However, pregnant women are advised to take double doses (*1*). Folate is mostly found in green, leafy vegetables, legumes, beans, citrus, and dairy products, but spinach and beans have been found to contain the highest amount of folate (*2*). Folic acid (also known as vitamin B9 or folacin), folate (the naturally occurring form), pteroyl-L-glutamic acid and pteroyl-L-glutamate are forms of the water-soluble vitamin B9. Folic acid is not biologically active but after the conversion of tetrahydrofolate and other derivatives into dihydrofolic acid in the liver, it becomes biologically important. Folic acid found in fortified foods such as vitamin supplements is the synthetic form of water-soluble vitamin B9 (*3*). Based on the bioavailability, folic acid is more stable than the natural form of folate because folic acid is more readily and quickly absorbed into the human body.

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On the other hand, a good selection of microbial species such as lactic acid bacteria (LAB) and cultural conditions could enhance the level of folate in the fermented milk and dairy products (*4*). It has been reported that the folate levels in most of the products are low (*5,6*). Therefore, several researchers have focused on the biosynthesis of folate using LAB strains (*7*). Large differences in folate biosynthesis for different LAB strains and different growth conditions have been reported in literature. *Lactococcus lactis*, as claimed by Sybesma *et al.* (*8*), has a great potential to produce high amount of folate and its metabolism was further explored to maximize folate biosynthesis. Other researchers have also reported that many strains of bifidobacterium were able to produce folate (*5*). Among the *Lactobacillus* species, only *L. plantarum* can produce folate, whereas other species were shown to consume folate during growth (*7*).

The introduction of response surface methodology (RSM) by Box and Wilson (*9*) gives an alternative technique to analyze the optimized culture conditions, thus enabling the researcher to design the experiment, build the blocks, and evaluate the effects of different growth factors and responses. This approach allows the researchers to design experiments based on the RSM results and produce maximum yield of desired products (*10*). Furthermore, one of the major constraints involved in designing new cultivation media is the high number of experiments involved. RSM, a combination of good experimental design, regression modelling techniques and optimization, is a useful tool for process improvement. Therefore, RSM is a valuable tool used prior to industrial level production (*11*). Most of the researches on the optimization of medium formulation and cultural conditions for biosynthesis of products by LAB were focused on the conventional method rather than RSM approach (*5,7,12,13*).

The present work has been conducted to identify folate producers amongst our laboratory collection of LAB using microbiological assay. Folate in a biological extract is usually determined by a microbiological assay and this method is highly sensitive and ideal for routine assaying. The optimization of medium formulation for the growth and folate biosynthesis by the selected strains was then investigated through a conventional method and statistical approach of RSM.

Materials and Methods

Bacterial strains and maintenance

LAB strains were maintained in 5 % (by volume) glycerol at –80 °C. *L. lactis* NZ9000 and *L. lactis* MG1363 were kind gifts of Kees Leenhouts, the Netherlands. *L. plantarum* I-UL4 was isolated from local fermented food (*14,15*) and *L. johnsonii* DSM 20553 was purchased from DSMZ (German Collection of Microorganisms and Cell Cultures), Braunschweig, Germany.

Growth of LAB cultures

L. lactis NZ9000 and MG1363 were cultivated in folate-free M17 medium (*16*) supplemented with 0.5 % (*m/V*) glucose. *L. plantarum* I-UL4 and *L. johnsonii* DSM 20553 were cultivated in de Man-Rogosa-Sharpe (MRS) medium (*17*). All cultivations were carried out in a 250-mL Erlenmeyer flask containing 100 mL of medium at pH=7. The LAB strains were incubated at 30 °C at the agitation speed of 100 rpm for 24 h. The strains were cultivated in modified MRS medium containing (in g/L): glucose 10, peptone 10, yeast extract 5, meat extract 5, potassium hydrogen phosphate 2, sodium acetate 5, triammonium citrate 2, magnesium sulphate 0.2, manganese sulphate 0.2 and Tween 80 1 mL/L with the addition of *para*-aminobenzoic acid (PABA, 0.01 μM). All cultivations were seeded with 5 % (by volume) inoculum. Inocula were prepared by inoculating a colony of the strains grown on an M17 or MRS agar plate into 5 mL of M17 or MRS broth in a 10-mL test tube with continuous shaking (100 rpm) in a water bath at 30 °C for 12 h.

Central composite design

Three variables and five levels were used in this study. The three variables used were lactose, meat extract and PABA concentration. The experimental design for central composite design (CCD) is shown in Table 1.

Table 1. Coded and real values of variables selected for CCD

	Variable	Unit	Values of CCD variables				
			$-\alpha$	–1	0	+1	$+\alpha$
A	lactose	g/L	1.89	5	12.5	20	23.11
B	meat extract	g/L	1.89	5	12.5	20	23.11
C	PABA	μM	1.56	3	6.5	10	11.45

The results of the CCD were statistically evaluated and the data were analyzed by Design Expert v. 6.0.6 (Stat-Ease Inc., MN, USA). Each variable was investigated as the independent and dependent variable. Each independent variable indicates high level (+1) and low level (–1). An axial distance $\pm\alpha$ was chosen to be 1.4 to make the design rotatable, which means that the value is at the same distance from the design centre. The centre point, or typically known as dependent variable, was indicated as 0 and maintained at constant value, which provided an unbiased estimate of the process error variance. This centre point was set at middle point value, and six centres of this experiment were included to avoid the missing of a non-linear relationship (*18*). The response of folate concentration was subjected to quadratic regression model and expressed by the second-order polynomial:

$$Y=\beta_o+\Sigma\beta_j x_j+\Sigma\beta_{jj}x_{j2}+\Sigma\beta_{jk}x_j x_k \qquad /1/$$

where Y is the folate concentration, and β_j, β_{jj} and β_{jk} are linear, quadratic and interactive coefficient, respectively. The F-value was considered to be significant. The lack of fit should be non-significant and produce a good multiple correlation coefficient (R^2).

Analytical determination

Cell and substrate concentrations

A volume of 6 mL of the sample was withdrawn at 1-hour intervals for the analysis of folate, cell concentration and reducing sugar concentration. A volume of 5 mL of the sample was used for the analysis of intra- and extracellular folate and 1-mL sample was used for sub-

strate (*19*) and cell concentration analysis. The 1-mL samples were centrifuged (10 000×*g*, 10 min, 4 °C) to separate the cell pellet from the supernatant. The supernatants were collected for substrate determination and the absorbance was measured at 540 nm, while the cell pellets were used for cell concentration determination. The cell pellets were transferred to a pre-weighed dry filter paper and dried for 24 h at 100 °C. Dry cell mass (DCM) values were correlated with absorbance measurements at 600 nm to obtain a calibration standard of $A_{600\,nm}$ *vs.* DCM. $A_{600\,nm}$ readings were subsequently used to determine DCM.

Folate concentration

Folate concentration was quantified using a *Lactobacillus casei* microbiological assay. Cells and supernatants recovered from a cell culture were used to measure both intra- and extracellular folate concentrations as described previously (*20*). *L. casei* ATCC 7469 was used for folate bioassay and stored at –80 °C in MRS medium supplemented with 15 % (by volume) of glycerol. In the assay, *L. casei* ATCC 7469 was pre-grown in Folic Acid Casei Medium (Difco Laboratories, Surrey, UK) supplemented with folate 0.3 μg/L and the culture was incubated for 18 h at 37 °C. Volumes of 1.5 mL of aliquots of the culture were then stored in sterile tubes at –80 °C until use for folate determination. For measuring the intracellular and extracellular folate, a volume of 5 mL of cultivation broth was used and centrifuged (10 000×*g*, 10 min, at 20 °C) to separate the cell and the supernatant. The cells were washed with 0.1 M sodium acetate buffer (pH=4.8) and 1 % (by volume) of ascorbic acid and resuspended with the same buffer. The samples were then incubated at 100 °C for 5 min to release the folate from the cells. The supernatant was diluted 1:1 with 0.1 M sodium acetate buffer (pH=4.8) and 1 % (by volume) of ascorbic acid. The analysis of total folate concentration, including polyglutamyl folate, was conducted after enzymatic deconjugation of the folate samples with human plasma (Sigma-Aldrich, Malaysia) and incubated for 4 h at 37 °C. The microbiological assay was determined in 96-well microtiter plates. The wells were filled by adding 8 μL of working buffer containing 0.1 M potassium phosphate buffer with sodium acetate buffer (pH=4.8) and 1 % (by volume) of ascorbic acid to 122 μL of samples or 60 μL of reference sample, and the volume was increased by sterile distilled H_2O until it reached 150 μL and prior to filling with 150 μL of Folic Acid Casei Medium. The assay plate was covered and incubated at 37 °C for 18 h. For reference samples, folate was dissolved in the same buffer at a concentration ranging from 0 to 0.30 μg/L. The growth of LAB strains in the 96-well microtiter plates was determined by measuring the absorbance at 620 nm using the Zenyth Microplate Reader (Biochrom Ltd, Cambridge, UK).

Results and Discussion

Selection of folate producer

The results obtained in this study showed that the growth performance of folate producer was strain-specific. All the strains grew well and produced comparable intra- and extracellular folate. About 7.67 g/L of maximum cell concentration (X_{max}) was achieved in the cultivation of *L. plantarum* I-UL4 with a specific growth rate (μ) of 0.39 h^{-1} after 18 h of cultivation using MRS medium. The highest folate level was detected in *L. plantarum* I-UL4 (36.36 μg/L). *Lactobacillus* spp. was able to grow well and produce substantially high folate using chemically defined or complex media, compared to other strains (*7*). However, 27.63 μg/L of folate were obtained in the cultivation of *L. johnsonii* DSM 20553 with the maximum cell concentration of 7.73 g/L, which was the lowest, compared to the other strains.

Higher growth yield, $Y_{x/s}$ (1.69 g/g) and slightly lower folate production (30.61 μg/L) were obtained for *L. lactis* NZ9000 compared to *L. plantarum* I-UL4. These results suggest that the substrate was mainly consumed for the biomass build-up rather than the biosynthesis of folate. Furthermore, high cell concentration increased the viscosity of the culture, which could reduce the mixing efficiency and hence limit the substrate availability for folate biosynthesis (*21,22*).

The highest efficiency of cells to produce folate ($Y_{p/x}$) was observed in the cultivation of *L. lactis* MG1363 (6.31 μg/g). It was shown that this species has maximum efficiency in producing folate compared to other strains. From the results, it can be suggested that the biosynthesis of folate was non-growth associated process since good growth was unable to promote higher folate biosynthesis as observed in the cultivation of *L. lactis* NZ9000 and *L. johnsonii* DSM 20553. The highest productivity was observed in the cultivation of *L. plantarum* I-UL4 (3.71 μg/(L·h)). Hence, *L. plantarum* I-UL4 was selected as the superior folate producer and used for further optimization studies on medium composition *via* conventional and RSM approaches.

Effect of carbon sources on folate production

Preliminary optimization study on medium composition was conducted to determine important factors that could enhance the biosynthesis of folate. Three different carbon sources (lactose, maltose and glucose) were selected in this study and *L. plantarum* I-UL4 grew well on all of them. The highest biosynthesis of folate (36.19 μg/L) was obtained when lactose was used as the carbon source, compared to maltose and glucose. The strain also showed high efficiency when using lactose as a carbon source to produce folate (data not shown). The highest productivity of folate was obtained in the cultivation process using lactose (1.97 μg/(L·h)) compared to glucose (1.53 μg/(L·h)) and maltose (1.13 μg/(L·h)). Lactose is normally found in milk and it is the preferred carbon source for the growth of LAB (*23*).

Effect of nitrogen sources on folate production

The maximum cell concentration (X_{max}) was obtained when yeast extract was used as a nitrogen source (data not shown). Yeast extract, which consists of nitrogenous compounds and growth factors, stimulates cell growth (*24,25*). However, the best nitrogen source for the highest biosynthesis of folate (47.01 μg/L) was obtained when using meat extract. Slightly lower folate concentration was obtained when using yeast extract (42.83 μg/L) and pep-

tone (43.71 μg/L) as nitrogen sources (data not shown). Even though the cell efficiency of folate biosynthesis ($Y_{p/x}$) was the highest on peptone compared to meat extract and yeast extract, the highest productivity of folate (1.56 μg/(L·h)) was found on meat extract (data not shown). Therefore, meat extract is the most suitable nitrogen source for folate biosynthesis although it is quite an expensive source of nitrogen, especially for industrial application. Hence, a precise concentration of meat extract needed in a medium for higher folate biosynthesis should be further investigated.

Optimization of lactose concentration for folate production

Generally, a higher concentration of lactose in the medium exhibits an increased cell concentration. In this study, approx. 7.07 g/L of maximum cell concentration (X_{max}) was achieved using 20 g/L of lactose (Table 2). However, slightly lower folate concentration was obtained when more lactose was added to the medium. This indicated that the carbon flux in cells favoured more the build-up of cell biomass or biosynthesis of by-products rather than the folate biosynthesis. Excessive carbon source in the culture broth reduces biomass yield and growth efficiency due to metabolism overflow. Lactose concentration of 10 g/L was optimal for the strain to produce the highest folate concentration (35.94 μg/L) (Table 2). As shown earlier, the biosynthesis of folate was a non-growth associated process, therefore the balanced carbon flux in cells is required through the folate pathway and other metabolite pathways (*5*,*7*,*12*).

Table 2. The performance and kinetic parameter values of folate biosynthesis by *L. plantarum* I-UL4 using different concentrations of lactose

Kinetic parameter	γ(lactose)/(g/L)			
	5	10	15	20
t/h	18	18	12	12
X_{max}/(g cell/L)	5.75	5.92	7.00	7.07
P_{max}/(μg folate/L)	34.22	35.94	31.10	30.47
S_i–S_f/(g substrate consumed/L)	3.89	6.84	12.11	15.24
μ/h^{-1}	0.37	0.39	0.39	0.34
$Y_{x/s}$/(g cell/g substrate)	1.48	0.87	0.58	0.46
$Y_{p/s}$/(μg folate/g substrate)	8.80	5.25	2.57	2.00
$Y_{p/x}$/(μg folate/g cell)	5.95	6.07	4.44	4.31
P_r/(μg folate/(L·h))	0.90	1.01	1.12	0.92

Optimization of meat extract concentration for folate production

The growth performance and the kinetic parameter values of *L. plantarum* I-UL4 cultivation using meat extract as a nitrogen source are shown in Table 3. When 15 g/L of meat extract were used, maximum cell concentration (X_{max}) of 4.75 g/L and the highest folate biosynthesis (52.82 μg/L) were obtained. The growth of *L. plantarum* I-UL4 and folate biosynthesis were reduced with meat extract concentration above 15 g/L in the medium.

Table 3. The performance and kinetic parameter values of folate biosynthesis by *L. plantarum* I-UL4 using different concentrations of meat extract

Kinetic parameter	γ(meat extract)/(g/L)			
	5	10	15	20
t/h	8	8	12	8
X_{max}/(g cell/L)	3.1	4.06	4.75	3.93
P_{max}/(μg folate/L)	44.14	49.62	52.82	48.71
S_i–S_f/(g substrate consumed/L)	7.89	7.67	7.62	7.52
μ/h^{-1}	0.31	0.4	0.32	0.38
$Y_{x/s}$/(g cell/g substrate)	0.39	0.53	0.62	0.52
$Y_{p/s}$/(μg folate/g substrate)	5.59	6.47	6.93	6.48
$Y_{p/x}$/(μg folate/g cell)	14.24	12.22	11.12	12.39
P_r/(μg folate/(L·h))	2.16	1.25	1.47	1.07

Further increment of nitrogen source in the medium showed an inhibitive effect on the growth performance of the strain and folate biosynthesis. This finding is not in agreement with previous studies (*7*,*8*) where batch cultures of *L. lactis* MG1363 supplemented with growth-inhibiting substances increased the folate biosynthesis. Such discrepancies may be due to the use of meat extract in the medium, which consists of nitrogenous compounds and growth factor.

Optimization of PABA concentration for folate production

The addition of PABA into the medium promoted good growth of *L. plantarum* I-UL4 and folate biosynthesis. PABA concentration of 0.1 to 10 μM significantly increased the biosynthesis of folate (Table 4). However, an addition of above 15 μM of PABA caused cell inhibition and decreased biosynthesis of folate. At the concentration of 50 to 100 μM of PABA, the growth was significantly inhibited and the substrate was not fully utilized by *L. plantarum* I-UL4. The strain efficiently produced high amount of folate (55.02 μg/L) at 10 μM of PABA. This result suggested that the biosynthesis of folate by *L. plantarum* I-UL4 was enhanced by the addition of PABA to the medium. In the absence of PABA, low folate levels were found to be produced by *L. lactis* strain (*7*) and PABA is an important precursor of folate biosynthesis (*5*).

Optimization of lactose, meat extract, and PABA by central composite design

A second experiment was conducted as a result of the aforementioned findings, in an attempt to further optimize the independent levels of lactose, meat extract and PABA with a face-centred central composite design (CCD) using Design Expert software. The results of 20 experiments to evaluate the effect of three factors of medium composition that influenced folate biosynthesis are shown in Table 5. Centre points with a coded value (0) were repeated six times in order to estimate pure error for the lack of fit test. Models with a significant lack of fit should not be used for predictions. The insignificant lack of fit is most desirable at $p>0.1$.

Table 4. The performance and kinetic parameter values of folate biosynthesis by *L. plantarum* I-UL4 using different concentrations of PABA

Kinetic parameter	*c*(PABA)/μM										
	0.1	0.3	1.0	3.0	5.0	7.0	10	15	20	50	100
t/h	12	18	18	12	18	12	12	12	12	8	6
X_{max}/(g cell/L)	3.36	4.42	4.30	4.76	4.09	3.76	4.36	2.74	1.86	1.08	0.95
P_{max}/(μg folate/L)	39.89	44.16	45.86	45.90	45.38	49.27	55.02	41.79	39.80	26.82	24.93
S_i–S_f/(g substrate consumed/L)	7.36	7.51	7.22	7.15	6.07	6.59	6.62	5.50	5.76	6.13	5.20
μ/h^{-1}	0.23	0.19	0.21	0.22	0.21	0.20	0.28	0.21	0.21	0.13	0.15
$Y_{x/s}$/(g cell/g substrate)	0.46	0.59	0.60	0.67	0.67	0.57	0.66	0.50	0.32	0.18	0.18
$Y_{p/s}$/(μg folate/g substrate)	5.42	5.88	6.35	6.42	7.48	7.48	8.34	7.60	6.91	4.38	4.79
$Y_{p/x}$/(μg folate/g cell)	11.87	9.99	10.67	9.64	11.10	13.10	12.66	15.25	21.40	24.83	26.24
P_r/(μg folate/(L·h))	0.99	1.85	1.38	1.00	0.43	0.95	2.11	0.48	0.34	1.05	1.27

Table 5. Central composite design with a real value and response to folate concentration (actual and predicted values)

Run	γ(A)/(g/L)	γ(B)/(g/L)	*c*(C)/μM	γ(folate)/(μg/L)	
				Obtained value	Predicted value
1	5	5	3	38.70	40.80
2	20	5	3	50.11	48.06
3	5	20	3	47.94	45.66
4	20	20	3	54.86	55.84
5	5	5	10	47.64	46.88
6	20	5	10	49.91	52.41
7	5	20	10	47.80	50.07
8	20	20	10	60.39	58.51
9	12.5	12.5	6.5	53.00	52.43
10	12.5	12.5	6.5	52.73	52.43
11	12.5	12.5	6.5	51.09	52.41
12	12.5	12.5	6.5	52.73	52.41
13	1.90	12.5	6.5	47.27	46.42
14	23.11	12.5	6.5	57.11	57.52
15	12.5	1.90	6.5	44.14	42.97
16	12.5	23.11	6.5	49.99	50.72
17	12.5	12.5	1.55	49.05	50.03
18	12.5	12.5	11.45	57.64	56.22
19	12.5	12.5	6.5	50.97	52.41
20	12.5	12.5	6.5	53.56	52.41

A – lactose, B – meat extract, C – PABA

The results from the CCD showed that the optimal concentrations of lactose, meat extract and PABA for folate biosynthesis were 20 g/L, 20 g/L and 10 μM, respectively. The Design Expert software uses an optimization method that allows the criteria for all variables and responses to be set. This optimization method takes into consideration a combination of criteria in the calculation of the optimum points. Therefore, based on the setting for criteria of lactose (in range), meat extract (in range), PABA (in range) and response (maximize), the optimum point of lactose (20 g/L), meat extract (16.57 g/L) and PABA (10 μM) was suggested.

The maximum response predicted from the model was 58.51 μg/L. Repeated experiments were performed to verify the predicted optimum value. A maximum folate concentration (60.39 μg/L) was obtained from run no. 8. Although the actual experimental response value at the optimum point was slightly higher than the predicted value, statistically, there was no significant difference. The maximum folate concentration obtained from the optimized medium composition was compared with the MRS standard medium, and it was apparent that the optimized medium formulation significantly improved folate biosynthesis by about twofold. It is interesting to note that *L. plantarum* has an ability to produce about 45 μg/L of folate (*4*) as well as other beneficial metabolites including bacteriocin (*26*). In this study, even higher production of folate by *L. plantarum* I-UL4 was obtained.

Regression analysis was performed to fit the response function with the experimental data. The data obtained (Table 6) were fitted to a quadratic polynomial model, and the obtained full actual model is shown in Eq. 2:

$$Y=52.42+3.93A+2.74B+2.19C-0.22A^2-2.78B^2+ +0.36C^2+0.73AB-0.43AC-0.42BC \quad /2/$$

where Y represents the predicted responses; A, B and C are coded values of lactose, meat extract and PABA concentration, respectively.

Table 6. Regression coefficient and the significance for the response of folate biosynthesis

Factor	Degree of freedom	Coefficient estimate	Standard error	Computed *t*-distribution	p>|*t*|
Intercept	1	52.42	0.93	56.37	0.0012
A	1	3.93	0.62	6.34	0.0011
B	1	2.74	0.62	4.42	0.0017
C	1	2.19	0.62	3.53	0.0065
A^2	1	–0.22	0.76	–0.29	0.7775
B^2	1	–2.78	0.76	–3.66	0.0052
C^2	1	0.36	0.76	0.47	0.6511
AB	1	0.73	0.76	0.96	0.3632
AC	1	–0.43	0.76	–0.57	0.5826
BC	1	–0.42	0.76	–0.55	0.5955

The student's *t*-distribution and the corresponding p-values along with the second order coefficient are shown in Table 6. p-Value is used as a tool to determine the significance of each coefficient and higher p-value indicated higher significance of corresponding coefficient (*10*). The parameter was estimated and the corresponding p-values showed that A (lactose), B (meat extract) and C (PABA) had significant effect on the biosynthesis of folate. Positive coefficient for A, B and C was indicated as a linear effect to the response. Table 6 shows some of the model terms of response of folate biosynthesis (A^2, C^2, AB, AC and BC), which had p-value>0.05. Therefore, they were eliminated from the model and simplified quadratic model equation most suitably described the folate biosynthesis as follows:

$$Y=52.42+3.93A+2.74B+2.19C-2.78B^2 \qquad /3/$$

The independent variables were fitted to the second order. Table 7 shows the results of the analysis of variance (ANOVA) to indicate the adequacy of the fitted model. The low p-value ($p<0.001$) indicated that the obtained equation was appropriate and suitable after model reduction. The determination of R^2 coefficient, correlation and model significance (F-value) were used to analyze the adequacy of the model. The quality of fit of the equation was expressed by the determination coefficient, R^2. Common study showed that a good R^2 should be at least 80 %. The value of coefficient $R^2=0.9063$ indicated that the model could explain about 91 % of the variability and it was attributed to the independent variables. Model significance (F-value) is a measure of variation of the data around the mean. Also, the F-value indicates that the present model can serve as a good prediction of the experimental results. Central composite rotatable design (CCRD) analysis was unable to support a full cubic model; the results from the model were accompanied by aliased statement, which indicated that not all parameters could be uniquely estimated.

Fig. 1 depicts the three-dimensional plot showing the effect of lactose (A) and meat extract (B) as responses to folate biosynthesis. Based on the p-value derived from the CCD analysis (Table 6), it shows no interactions between these two variables. Biosynthesis of folate was increased when high concentration of meat extract was added to the medium, while keeping lactose at low concentration.

Fig. 2 shows the combined effects of meat extract (B) and PABA (C) as response to folate biosynthesis. From the analysis, the independent variables affect the biosynthesis, but there were no interactions between meat extract and PABA, based on p-value results. This indicates that all independent variables cannot be interacting due

Table 7. Analysis of variance (ANOVA) for the quadratic model of folate biosynthesis

Source	Sum of squares	Degree of freedom	Mean	F-value	Prob>F
Linear	332.68	3.00		14.82	<0.0001
Cross product	7.16	3.00		0.27	0.8442
Quadratic	63.42	3.00		4.56	0.0331
Cubic	35.64	4.00		7.36	0.0252
Total	52127.26	13.00	2606.36		
Pure error	4.86	4.00	1.22		
$R^2=0.9063$					

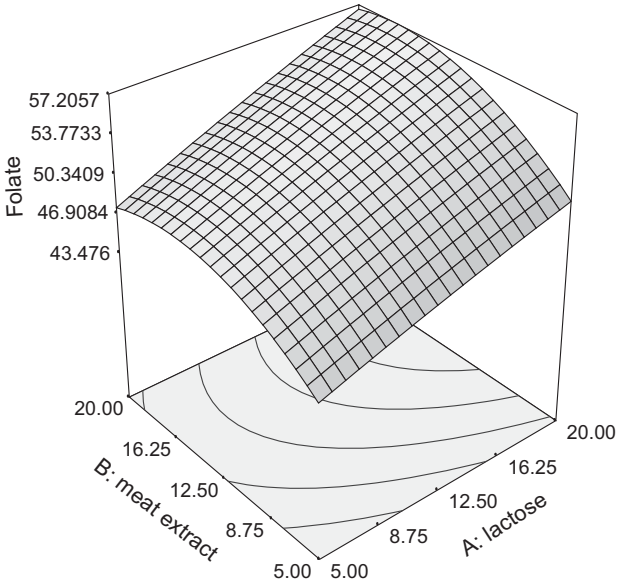

Fig. 1. Response surface plot of folate biosynthesis as a function of lactose and meat extract concentrations

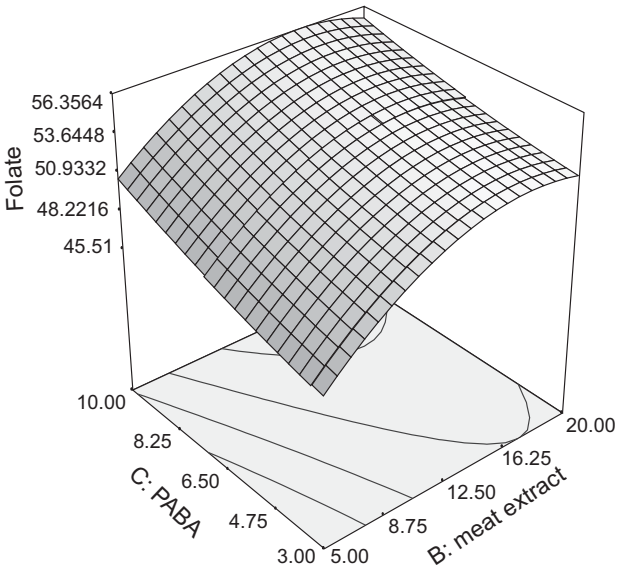

Fig. 2. Response surface plot of folate biosynthesis as a function of meat extract and PABA concentrations

to the insignificant model of p-value test. Low concentration of meat extract and the addition of high concentration of PABA to the medium induced high biosynthesis of folate. The addition of PABA stimulated the biosynthesis of folate. In *L. plantarum* I-UL4, folate biosynthesis was shown to be dependent on the concentration of PABA in the medium. Generally, with the addition of PABA, the biosynthesis of folate was increased by about twofold compared to the standard MRS medium.

Conclusion

Biosynthesis of folate was strain-dependent and all investigated LAB strains were able to grow well and produce folate. The highest folate biosynthesis was obtained in *L. plantarum* I-UL4 culture using standard MRS medium. A better understanding of the relationship among lactose, meat extract and PABA was obtained by RSM, which was used as a statistical tool to improve the folate biosynthesis of *L. plantarum* I-UL4. RSM analyses demonstrated that optimum biosynthesis of folate can be successfully predicted and the combination of lactose (20 g/L), meat extract (16.57 g/L) and PABA (10 μM) significantly improved folate biosynthesis. The optimized medium formulation could be used for the cultivation process of *L. plantarum* I-UL4 for folate biosynthesis in a bioreactor system associated with a process control strategy.

Nomenclature

t: time at maximum cell concentration
X_{max}: maximum cell concentration (g cell/L)
P_{max}: maximum folate concentration (μg folate/L)
S_i–S_f: substrate consumed (g substrate/L)
S_i: initial substrate concentration (g substrate/L)
S_f: final substrate concentration (g substrate/L)
μ: specific growth rate (h^{-1})
$Y_{x/s}$: growth yield coefficient (g cell/g substrate)
$Y_{p/s}$: folate yield based on substrate utilized (μg folate/g substrate)
$Y_{p/x}$: folate biosynthesis per cell (μg folate/g cell)
P_r: folate productivity (μg folate/(L·h))

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References

1. K.M. Forssén, M.I. Jägerstad, K. Wigertz, C.M. Witthöft, Folates and dairy products: A critical update, *J. Am. Coll. Nutr.* (Suppl. 2), *19* (2000) 100–110.
2. R.R. Eitenmiller, W.O. Landen: *Vitamin Analysis for the Health and Food Sciences*, CRC Press LLC, New York, USA (1999).
3. R. Póo-Prieto, D.B. Haytowitz, J.M. Holden, G. Rogers, S.F. Choumenkovitch, P.F. Jacques, J. Selhub, Use of the affinity/HPLC method for quantitative estimation of folic acid in enriched cereal-grain products, *J. Nutr. 136* (2006) 3079– 3083.
4. J.G. Le Blanc, G. Savoy de Giori, E.J. Smid, J. Hugenholtz, F. Sesma: Folate Production by Lactic Acid Bacteria and Other Food-Grade Microorganisms. In: *Communicating Current Research and Educational Topics and Trends in Applied Microbiology*, A. Méndez-Vilas (Ed.), Formatex, Badajoz, Spain (2007) pp. 239–339.
5. A. Pompei, L. Cordisco, A. Amaretti, S. Zanoni, D. Matteuzzi, M. Rossi, Folate production by bifidobacteria as a potential probiotic property, *Appl. Environ. Microbiol. 73* (2007) 179–185.
6. R.G. Crittenden, N.Z. Martinez, M.J. Playne, Synthesis and utilization of folate by yogurt starter cultures and probiotic bacteria, *Int. J. Food Microbiol. 80* (2003) 217–222.
7. W. Sybesma, M. Starrenburg, L. Tijsseling, M.H.N. Hoefnagal, J. Hugenholtz, Effects of cultivation conditions on folate production by lactic acid bacteria, *Appl. Environ. Microbiol. 69* (2003) 4542–4548.
8. W. Sybesma, M. Starrenburg, M. Kleerebezem, I. Mierau, W.M. de Vos, J. Hugenholtz, Increased production of folate by metabolic engineering of *Lactococcus lactis*, *Appl. Environ. Microbiol. 69* (2003) 3069–3076.
9. G.E.P. Box, K.B. Wilson, On the experimental attainment of optimum conditions, *J. Roy. Stat. Soc. B, 1* (1951) 1–45.
10. D.C. Montgomery, R.H. Myers: *Response Surface Methodology: Process and Product Optimization Using Designed Experiments*, John Wiley & Sons, Inc, New York, USA (1997) pp. 427–510.
11. N.N. Nawani, B.P. Kapadnis, Optimization of chitinase production using statistics based experimental designs, *Process Biochem. 40* (2005) 651–660.
12. M.Y. Lin, C.M. Young, Folate levels in cultures of lactic acid bacteria, *Int. Dairy J. 10* (2000) 409–413.
13. S. Sirisansaneeyakul, T. Luangipat, W. Vanichsriratana, T. Srinophakun, H.H.H. Chen, Y. Chisti, Optimization of lactic acid production by immobilized *Lactococcus lactis* IO-1, *J. Ind. Microbiol. Biotechnol. 34* (2007) 381–391.
14. H.L. Foo, T.C. Loh, F.L. Law, Y.Z. Lim, C.N. Kufli, G. Rusul, Effects of feeding *Lactobacillus plantarum* I-UL4 isolated from Malaysian tempeh on growth performance, faecal flora and lactic acid bacteria and plasma cholesterol concentrations in post-weaning rats, *Food Sci. Biotechnol. 12* (2003) 403–408.
15. H.L. Foo, T.C. Loh, P.W. Lai, Y.Z. Lim, C.N. Kufli, G. Rusul, Effects of adding *Lactobacillus plantarum* I-UL4 metabolites in drinking water of rats, *Pak. J. Nutr. 2* (2003) 283–288.
16. B.E. Terzaghi, W.E. Sandine, Improved medium for lactic streptococci and their bacteriophages, *Appl. Environ. Microbiol. 29* (1975) 807–813.
17. J.C. De Man, M. Rogosa, M.E. Sharpe, Cultivation medium for lactobacilli, *J. Appl. Biotechnol. 21* (1977) 130–135.
18. L. Rodrigues, J. Teixeira, R. Oliveira, H.C. van der Mei, Response surface optimization of the medium components for the production of biosurfactants by probiotic bacteria, *Process Biochem. 41* (2006) 1–10.
19. G.L. Miller, Use of dinitrosalycilic acid reagent for determination of reducing sugar, *Anal. Chem. 31* (1959) 426–428.
20. D.W. Horne, D. Patterson, *Lactobacillus casei* microbiological assay of folic acid derivatives in 96-well microtiter plates, *Clin. Chem. 34* (1988) 2357–2359.
21. A.R. Berry, C.M.M. Franco, W. Zhang, A.P.J. Middelberg, Growth and lactic acid production in batch culture of *Lactobacillus rhamnosus* in a defined medium, *Biotechnol. Lett. 21* (1999) 163–167.

22. M. Rosfarizan, A.B. Ariff, M.A. Hassan, M.I.A. Karim, Kojic acid production by *Aspergillus flavus* using gelatinized and hydrolyzed sago starch as carbon sources, *Folia Microbiol. 43* (1998) 459–464.

23. B. Bibal, G. Goma, Y. Vayssier, A. Pareilleux, Influence of pH, lactose and lactic acid on the growth of *Streptococcus cremoris*: A kinetic study, *Appl. Microbiol. Biotechnol. 28* (1988) 340–344.

24. M.S. Kalil, H.S. Alshiyab, W.M.W. Yusoff, Effect of nitrogen source and carbon to nitrogen ratio on hydrogen production using *C. acetobutylicum*, *Am. J. Biochem. Biotechnol. 4* (2008) 393–401.

25. V.M.G. Lima, N. Krieger, M.I.M. Sarquis, D.A. Mitchell, L.P. Ramos, J.D. Fontana, Effect of nitrogen and carbon sources on lipase production by *Penicillium aurantiogriseum*, *Food Technol. Biotechnol. 41* (2003) 105–110.

26. S.D. Todorov, L.M.T. Dicks, Effect of growth medium on bacteriocin production by *Lactobacillus plantarum* ST194BZ, a strain isolated from boza, *Food Technol. Biotechnol. 43* (2005) 165–173.