**Journal of Industrial Microbiology & Biotechnology (2002) 29,** 281–285

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# Optimization of galacto-oligosaccharide production by *Bifidobacterium infantis* RW-8120 using response surface methodology

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**Oligosaccharide (OS) production, cell concentration ($2\times10^9$ colony-forming unit/ml), lactose concentration (25% wt/vol), reaction time (6 h), and temperature (50°C) were chosen as the central condition of the central composite design (CCD) for optimizing the production process using *Bifidobacterium infantis* RW-8120 in skim milk. Statistical analysis ($P<0.01$) revealed that the most relevant variable concerning OS production and yield was the lactose concentration. The coefficient of determination ($R^2$) is good for the second-order OS production model (0.92) and fairly good for the second-order nonlinear OS yield model (0.816). An increase of lactose concentration and temperature resulted in a higher OS production. The optimal values for OS production appear to be near the area associated with the central points of the modeling design except for the lactose concentration, which was 40% (wt/vol) of the final volume.**
*Journal of Industrial Microbiology & Biotechnology* (2002) **29,** 281–285 doi:10.1038/sj.jim.7000319

**Keywords:** prebiotic; probiotic strains; bifidobacteria; central composite design; galacto-oligosaccharides; lactose concentration; optimization

## Introduction

Bifidobacteria are dominant among the hundreds of species capable of inhabiting the intestinal tract. The indigenous microflora of infants is dominated by bifidobacteria, which become established shortly after birth. The number of bifidobacteria decreases with increasing age of the individual and eventually becomes the third most abundant genus (accounting for 25% of the total adult gut flora). The genus *Bifidobacterium* also constitutes a significant proportion of the probiotic cultures used in the food industry. The utilization of strains belonging to *Bifidobacterium animalis*, *B. longum*, *B. bifidum*, and *B. infantis* as probiotic starter cultures is due to their important role in the large intestine, namely, improvement of the microbial balance of the human gastrointestinal tract [7,8]. To increase the number of beneficial bacteria in the human intestine, an interesting approach is to selectively stimulate their growth by supplementing food with ingredients that can only be metabolized by such bacteria. Certain oligosaccharides (OS), the so-called prebiotics, have been shown to exert this growth-stimulating effect on probiotic bacteria, including bifidobacteria [13].

Galacto-oligosaccharides are food components known to promote growth of bifidobacteria *in vivo* [4]. Galacto-oligosaccharides are produced from lactose by glycosyl transfer of one or more D-galactosyl units onto the D-galactose moiety of lactose catalyzed by β-galactosidase [10]. Commercial products are made using β-galactosidases isolated from such sources as bacteria and fungi [16]. In dairy products, most of the probiotic bacteria and the prebiotic OS have been used in combination, called synbiotics [13]. Incubation of cell-associated β-galactosidases with high concentrations of lactose resulted in the production of a range of OS, together with glucose and galactose. Lactose hydrolysis and transgalactosylation properties of the enzyme have been studied in several probiotic bacteria including bifidobacteria [5,6,9,11, 14,15,17]. Enzymes with a β-galactosidase activity were extracted from pure cultures of *B. angulatum*, *B. bifidum* BB-12, *B. adolescentis* ANB-7, *B. infantis* DSM-20088, and *B. pseudolongum* DSM-20099 and used in glycosyl transfer reactions to synthesize OS from lactose [12]. Hung and Lee [9] reported that of 29 strains of bifidobacteria studied, *B. infantis* HL96 was selected for a strong ability to synthesize OS.

The aim of the present work was to study the effects of lactose concentration, cell concentration, reaction time, and temperature on galacto-oligosaccharide production using *B. infantis* RW-8120 to establish the most appropriate reaction conditions in milk.

## Materials and methods

### *Bacterial strain, culture conditions, and OS fermentation*

*B. infantis* RW-8120 was obtained from the Food Research and Development Centre collection (Saint-Hyacinthe, Quebec, Canada). Stock cultures were prepared in brain–heart infusion (Difco Laboratories, Detroit, MI, USA) supplemented with 10% glycerol and frozen at −80°C. Lactobacilli MRS broth (Difco Laboratories) supplemented with 0.05% L-cysteine–HCl was

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Received 29 March 2002; accepted 9 August 2002

**Table 1** CCD of variables and observed and predicted responses for OS production and yield

| Run | $X_1$ | $X_2$ | $X_3$ | | $X_4$ | OS produced | | OS yield | |
|---|---|---|---|---|---|---|---|---|---|
| | Reaction time (h) | Temperature (°C) | Cells (cfu/ml) | Cell volume (ml) | Lactose (%) | Expected (%) | Predicted (%) | Expected (%) | Predicted (%) |
| 1 | 3 | 50 | $2\times10^9$ | 1.72 | 25 | 9.49 | 9.33 | 37.96 | 38.39 |
| 2 | 4 | 40 | $7\times10^8$ | 0.60 | 15 | 4.09 | 3.03 | 27.25 | 25.66 |
| 3 | 4 | 40 | $7\times10^8$ | 0.60 | 35 | 7.32 | 8.32 | 20.92 | 22.05 |
| 4 | 4 | 40 | $3.3\times10^9$ | 2.84 | 15 | 4.29 | 5.11 | 28.59 | 28.88 |
| 5 | 4 | 40 | $3.3\times10^9$ | 2.84 | 35 | 11.64 | 12.30 | 33.26 | 37.08 |
| 6 | 4 | 60 | $7\times10^8$ | 0.60 | 15 | 4.73 | 4.47 | 31.53 | 28.02 |
| 7 | 4 | 60 | $7\times10^8$ | 0.60 | 35 | 13.56 | 13.16 | 38.73 | 37.89 |
| 8 | 4 | 60 | $3.3\times10^9$ | 2.84 | 15 | 1.98 | 1.33 | 13.17 | 12.40 |
| 9 | 4 | 60 | $3.3\times10^9$ | 2.84 | 35 | 11.94 | 11.93 | 34.11 | 34.07 |
| 10 | 6 | 36 | $2\times10^9$ | 1.72 | 25 | 9.38 | 7.69 | 37.51 | 32.88 |
| 11 | 6 | 50 | $1.6\times10^8$ | 0.14 | 25 | 5.10 | 6.43 | 20.32 | 27.63 |
| 12 | 6 | 50 | $2\times10^9$ | 1.72 | 11 | 1.05 | 2.99 | 10.55 | 18.35 |
| 13 | 6 | 50 | $2\times10^9$ | 1.72 | 39 | 16.16 | 14.69 | 35.30 | 33.51 |
| 14 | 6 | 50 | $2\times10^9$ | 1.72 | 25 | 9.08 | 8.64 | 37.70 | 33.51 |
| 15 | 6 | 50 | $2\times10^9$ | 1.72 | 25 | 9.17 | 8.64 | 40.39 | 35.33 |
| 16 | 6 | 50 | $3.8\times10^9$ | 3.31 | 25 | 6.78 | 5.93 | 27.12 | 22.97 |
| 17 | 6 | 64 | $2\times10^9$ | 1.72 | 25 | 5.57 | 7.75 | 22.27 | 30.07 |
| 18 | 8 | 40 | $7\times0^8$ | 0.60 | 15 | 4.39 | 3.46 | 29.28 | 25.34 |
| 19 | 8 | 40 | $7\times10^8$ | 0.60 | 35 | 8.09 | 9.45 | 23.12 | 26.31 |
| 20 | 8 | 40 | $3.3\times10^9$ | 2.84 | 15 | 2.92 | 4.03 | 19.46 | 22.72 |
| 21 | 8 | 40 | $3.3\times10^9$ | 2.84 | 35 | 12.59 | 11.91 | 35.97 | 35.50 |
| 22 | 8 | 60 | $7\times10^8$ | 0.60 | 15 | 3.86 | 3.91 | 25.74 | 24.34 |
| 23 | 8 | 60 | $7\times10^8$ | 0.60 | 35 | 15.06 | 13.30 | 43.04 | 38.79 |
| 24 | 8 | 60 | $3.3\times1^9$ | 2.84 | 15 | 1.20 | −0.74 | 7.98 | 2.88 |
| 25 | 8 | 60 | $3.3\times10^9$ | 2.84 | 35 | 8.79 | 10.56 | 25.13 | 29.14 |
| 26 | 9 | 50 | $2\times10^9$ | 1.72 | 25 | 8.00 | 8.63 | 32.00 | 34.67 |

used to cultivate the frozen microorganisms and recovered strains were subcultured once. Active cultures were incubated between 18 and 24 h at 37°C in an anaerobic chamber with a gas atmosphere of 5% $CO_2$, 10% $H_2$, and 85% $N_2$. Frozen stock cultures of *B. infantis* were produced according to the method described by Blanchette *et al* [2]. Tubes of 10 ml of stock cultures ($5.8\times10^{10}$ CFU/ml) containing *B infantis* RW-8120 were defrosted and volumes corresponding to the concentrations of the cells for each bioconversion were added to skim milk.

OS were synthesized in skim milk (10% wt/vol) containing 10.9–39.1% (wt/wt) lactose, at the specified temperatures and cell volume (Table 1), in a final volume of 50 ml. Bottles (150 ml) were agitated using an orbital shaker (100 rpm). Samples were taken at the specified intervals (Table 1). Samples (3 ml) were diluted 1:12 in $H_2SO_4$ (0.08 N) and analyzed by HPLC.

### HPLC analysis

Sugars were measured by HPLC using a refractive index detector (model 2410; Waters, Milford, MA, USA) maintained at 40°C, a UV detector (model 484; Waters) set at 210 nm, and an Ion-300 column (300 mm×7.8 mm i.d.; Transgenomic, Omaha, NE, USA) operated at 40°C and at a flow rate of 0.4 ml/min. The mobile phase was 0.02 N $H_2SO_4$. The HPLC system was calibrated between 0.062% (w/w) and 3.00% for stachyose, raffinose, lactose, glucose, galactose, citric acid, acetic acid, and lactic acid. A standard curve was prepared by diluting compounds in 0.08 N

**Table 2** Estimates of regression coefficients of the variable for OS production and OS yield

| Coefficient | OS production | | | | OS yield[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | Estimate | SE[b] | *t* value | *P* level | Estimate | SE | *t* value | *P* level |
| $b_0$ | −14.7188 | 18.7608 | −0.7846 | 0.4493 | −2.7067 | 17.8621 | −0.1515 | 0.9043 |
| $b_1$ | 0.0724 | 2.2343 | 0.0324 | 0.9747 | −3.3984 | 2.1438 | −1.5852 | 0.3583 |
| $b_2$ | 0.5203 | 0.6423 | 0.8100 | 0.4351 | 1.0506 | 0.6089 | 1.7255 | 0.3344 |
| $b_3$ | 8.9838 | 3.0630 | 2.9330 | 0.0136 | 28.1398 | 2.9057 | 9.6843 | 0.0655 |
| $b_4$ | −0.1876 | 0.4114 | −0.4560 | 0.6573 | −0.4341 | 0.3735 | −1.1623 | 0.4523 |
| $b_{11}$ | 0.0416 | 0.1510 | 0.2755 | 0.7881 | 0.3778 | 0.1454 | 2.5982 | 0.2339 |
| $b_{22}$ | −0.0046 | 0.0061 | −0.7456 | 0.4716 | −0.0102 | 0.0058 | −1.7504 | 0.3304 |
| $b_{33}$ | −0.9778 | 0.4887 | −2.0008 | 0.0707 | −3.2647 | 0.4632 | −7.0480 | 0.0897 |
| $b_{44}$ | 0.0010 | 0.0062 | 0.1654 | 0.8716 | −0.0296 | 0.0054 | −5.4997 | 0.1145 |
| $b_{12}$ | −0.0123 | 0.0224 | −0.5518 | 0.5921 | −0.0421 | 0.0212 | −1.9811 | 0.2976 |
| $b_{13}$ | −0.1688 | 0.1997 | −0.8451 | 0.4160 | −0.6514 | 0.1896 | −3.4365 | 0.1803 |
| $b_{14}$ | 0.0087 | 0.0224 | 0.3897 | 0.7042 | 0.0572 | 0.0212 | 2.6962 | 0.2261 |
| $b_{23}$ | −0.1164 | 0.0399 | −2.9125 | 0.0141 | −0.4205 | 0.0379 | −11.0921 | 0.0572 |
| $b_{24}$ | 0.0085 | 0.0045 | 1.9040 | 0.0834 | 0.0337 | 0.0042 | 7.9337 | 0.0798 |
| $b_{34}$ | 0.0425 | 0.0399 | 1.0630 | 0.3105 | 0.2636 | 0.0379 | 6.9518 | 0.0910 |

[a]Yields are expressed as the percent (wt/wt) OS content as determined by HPLC of the total lactose added to milk.
[b]Standard error.

**Table 3** Analysis of variance of the second-order OS production model

| Source of variation[a] | Sum of squares | *df*[b] | Mean squared | *F* value | *P* |
|---|---|---|---|---|---|
| $[t]$ | 1.12250785 | 1 | 1.12250785 | 277.162432 | 0.03819368 |
| $[t]^2$ | 0.24305724 | 1 | 0.24305724 | 60.0141322 | 0.08172565 |
| $[T^\circ]$ | 0.00733258 | 1 | 0.00733258 | 1.8105126 | 0.40688136 |
| $[T^\circ]^2$ | 1.78040773 | 1 | 1.78040773 | 439.606846 | 0.03034023 |
| $[C]$ | 0.55454054 | 1 | 0.55454054 | 136.923591 | 0.0542734 |
| $[C]^2$ | 12.8215524 | 1 | 12.8215524 | 3165.8154 | 0.01131336 |
| $[L]$ | 343.491444 | 1 | 343.491444 | 84812.7023 | 0.00218599 |
| $[L]^2$ | 0.08764104 | 1 | 0.08764104 | 21.6397634 | 0.13480141 |
| $[t][T^\circ]$ | 0.97515625 | 1 | 0.97515625 | 240.779321 | 0.04097042 |
| $[t][C]$ | 2.28765625 | 1 | 2.28765625 | 564.853395 | 0.02677048 |
| $[t][L]$ | 0.48650625 | 1 | 0.48650625 | 120.125 | 0.05792455 |
| $[T^\circ][C]$ | 27.1701563 | 1 | 27.1701563 | 6708.68056 | 0.00777213 |
| $[T^\circ][L]$ | 11.6110563 | 1 | 11.6110563 | 2866.92747 | 0.01188834 |
| $[C][L]$ | 3.61950625 | 1 | 3.61950625 | 893.705247 | 0.02128732 |
| Lack of fit | 35.2284515 | 10 | 3.52284515 | 869.838308 | 0.02638091 |
| Pure error | 0.00405 | 1 | 0.00405 | | |
| Total | 441.919712 | 25 | | | |

[a]$t$, Reaction time; $T^\circ$, temperature (°C); $C$, cell concentration (ml); $L$, lactose (%).
[b]Degrees of freedom.

$H_2SO_4$. The sugars and the organic acids used for the standard curve were HPLC grade and were obtained from Sigma (St. Louis, MO, USA). Raffinose was used to quantify the presence of OS with a degree of polymerization (dp) of 3. A sample (0.5 g) of milk was blended with 5.5 g of 0.08 N $H_2SO_4$. The slurry was centrifuged at 27,000×*g* for 15 min at 4°C. The supernatant was then filtered through a 0.45-$\mu$m membrane filter (Millipore, Bedford, MA, USA) into vials and stored at 4°C in the Auto Injector (model 717; Waters).

### *Central composite designs (CCDs)*

In order to describe the nature of the response surface in the optimum region, a CCD with two levels was performed. The design consists of a $2^k$ factorial or fractional factorial design augmented by $2k$ axial (star) points at $(\pm\alpha,0,0,\ldots,0)$, $(0,0,\pm\alpha,\ldots,0)$, …, $(0,0,0,\ldots,\pm\alpha)$, and $n_o$ center points at $(0,0,\ldots,0)$, where $\alpha$ is the distance of the star point from the center. The experimental design was thus made up of a full $2^4$ factorial design with its four cube points, augmented with six replications of the center points (all factors at level 0) and the four star points, i.e., points having for one factor an axial distance to the center of $\pm\alpha$, whereas the other factor is at level 0. The axial distance was chosen to be 1.414 to make this design rotatable. For predicting the optimal point, a second-order polynomial function was fitted to the experimental results. For four factors, this equation is:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{34}X_3X_4$$

where $Y$, predicted response, stands for OS production or OS yield; $b_0$ is the intercept; $b_1-b_4$ are the linear coefficients; $b_{11}$, $b_{22}$, $b_{33}$, and $b_{44}$ are the quadratic coefficients; $b_{12}$, $b_{13}$, $b_{14}$, $b_{23}$, $b_{24}$, and $b_{34}$ are the cross-product coefficients; and $X_i$ is the coded independent variable.

The three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on the dependent ones. These graphs were drawn by imposing a constant value (i.e., the central points of the interval taken into consideration) to one independent variable.

### *Data analysis*

The responses obtained for each run (Table 1) were subjected to multiple nonlinear regression using the software STATISTICA (V5.0, 1995; StatSoft, Tulsa, OK) to obtain the coefficients of the second polynomial. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination $R^2$, and its statistical significance was checked by an $F$-test. The significances of the regression coefficient were tested by a $t$-test. Only coefficients with significant levels higher than 90% ($P<0.10$) were included in the final models. The $F$-test was used to evaluate the significance of the models.

## Results and discussion

### *Optimization of the process*

To optimize the production process of OS using *B. infantis* RW-8120, a cell volume of 1.72 ml corresponding to a cell concentration

**Table 4** Best-fit equations relative to the effects of different variables on OS production (%) and yield (%)

| Response | | Model[a] | $R^2$ | *F* value | SE[b] |
|---|---|---|---|---|---|
| OS production | Eq. (1) | $8.894[C]-0.977[C]^2-0.116[T^\circ][C]+0.009[T^\circ][L]$ | 0.920 | 9.067 | 1.790 |
| OS Yield | Eq. (2) | $28.140[C]-3.265[C]^2-0.420[T^\circ][C]+0.034[T^\circ][L]+0.264[C][L]$ | 0.816 | 3.486 | 6.017 |

Only terms with $P<0.10$ were included.
[a]$C$, cell concentration (ml); $T^\circ$, temperature (°C); $L$, lactose (%).
[b]Standard error.

($2\times10^9$ cfu/ml) in 50 ml of skim milk, lactose concentration (25% wt/vol), reaction time (6 h), and temperature (50°C) were chosen as the central condition of the CCD for optimizing the production process. In adding different volumes of stock cultures [tubes of 10 ml of stock cultures ($5.8\times10^{10}$ CFU/ml) containing the cells of *B infantis* RW-8120 were defrosted and volumes corresponding to the concentrations of the cells for each bioconversion were added to skim milk], we adjusted the cell concentration. The experimental design was repeated twice.

Table 1 shows the experimental conditions and the results for OS production and yield, according to the factorial design. The effect estimates and the associated $t$-values and significance level for each variable, as well as the interactions between them, were determined and reported in Table 2. Statistical analysis revealed that the most relevant variable ($P<0.01$) concerning the OS production and yield was the lactose concentration ($L$) (Table 3). The interaction between cell concentration ($C$) and temperature ($T°$) had a small effect, whereas the reaction time ($t$) was not statistically significant. Lactose concentration ($L$) variable was the only statistically significant factor for OS yield.

Only the estimates with significance level higher than 90% ($P<0.10$) were included in the final model (Table 2). Thus, the reduced models, which describe the responses as functions of the more significant variables, were generated. Therefore, the statistical models for OS production and yield can be expressed, respectively, by Eqs. (1) and (2) with 95% confidence (Table 4).

The coefficient of determination ($R^2$) is highly significant for the second-order OS production model (92.0%) and significant for the second-order nonlinear OS yield model (81.6%). Therefore, the first model can be used to generate response surfaces enabling analysis of the variable effects on OS production.

To determine optimal levels of the variables for OS production, three-dimension surface plots were constructed according to Eq. (1) in Table 4. Figure 1 shows the effect of lactose concentration $L$ and of the cell concentration $C$ on OS production. A quadratic effect of $C$ and a linear effect of $L$ on the response were observed. OS reached its maximum value at the $C$ level between 0.5 and 3 ml and at 40% of $L$. Figure 2 depicts the influence of lactose concentration $L$ and temperature $T°$; it can be seen as a linear effect for both $L$ and $T°$. Therefore, an increase of $L$ and $T°$ both resulted in a higher OS production. Finally, Figure 3 shows the effect of cell concentration and temperature; $C$ exerted a quadratic effect on OS production, whereas $T$ had a linear effect.

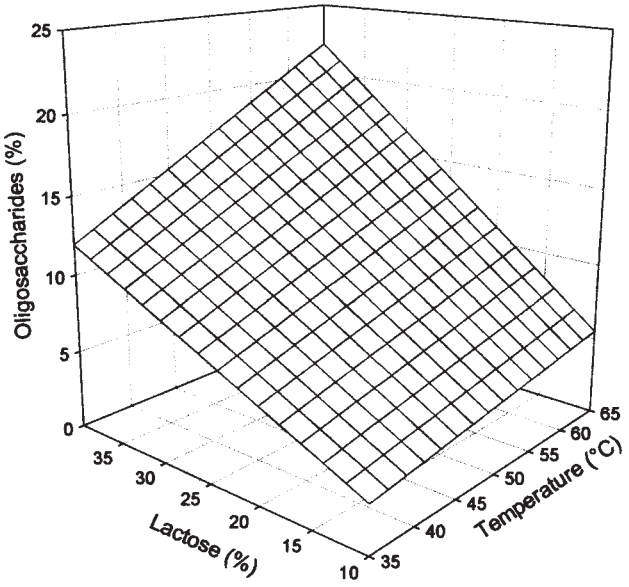


**Figure 2** Three-dimensional surface plots of OS production as the function of temperature ($T°$) and lactose ($L$) in Eq. (1). $C$ was kept at central point (1.72 ml).

## *Verification*

The optimal values for OS production appear to be near the area associated with $L$=40%, $C$=1.72 ml, $T°$=50°C, and $t$=6 h.

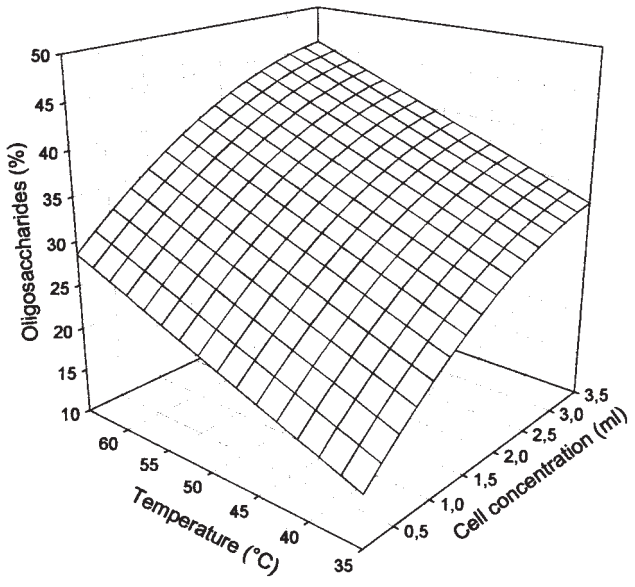


**Figure 1** Three-dimensional surface plots of OS production as the function of cell concentration ($C$) and lactose ($L$) in Eq. (1). $T°$ was kept at central point (50°C).

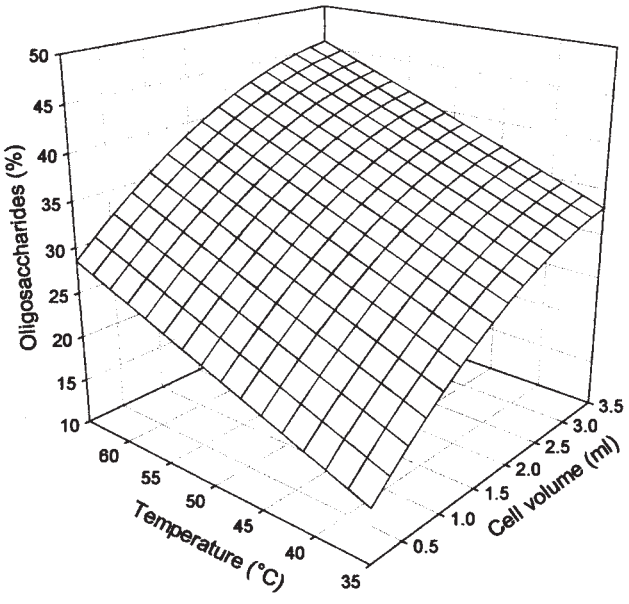


**Figure 3** Three-dimensional surface plots of OS production as the function of cell concentration ($C$) and temperature ($T°$) in Eq. (1). $L$ was kept at central point (25%).

Verification experiments were conducted under two different culture conditions. One combination was under the optimal conditions for OS production (experiment A: $L$=40%, $C$=1.72 ml, $T^{\circ}$=50°C, and $t$=6 h). The other experiments were done under the suboptimal conditions of variables for OS production (experiment B: $L$=40%, $T^{\circ}$=55°C; experiment C: $L$=35%, $T^{\circ}$=50°C; experiment D: $L$=35%, $T^{\circ}$=55°C; fixed $C$=1.72 ml and $t$=6 h for all experiments). Each combination was repeated three times. This allowed us to estimate adequately the variability of the process optimization results in the neighborhood of the observed maximum in the first experiment. This might allow results that would be representative of future production reproducibility and robustness. The ANOVA indicates that the effect of lactose concentration was significant ($P<0.05$) while the effect of temperature was not. Use of 40% lactose instead of 35% resulted in an 12% increase in OS production (13.2 % *vs.* 11.6% for 40 and 35% lactose, respectively), although no significant difference was observed for OS yield (33.1% *vs.* 33% for 40 and 35% lactose, respectively). However, there was no increase of OS yield (12.2% *vs.* 12.5%) between 50 and 55°C. Boon *et al* [3] noted that the effect of temperature is small compared to the influence of initial lactose concentration on the synthesis of OS. Albayrak and Yang [1] indicated that production of OS increased with increasing initial lactose concentration. OS yield decreased with increasing lactose conversion due to increasing dominance of hydrolysis at lower lactose concentrations.

Rabiu *et al* [12] observed a maximal yield of total OS at 30% (wt/wt) lactose using the enzyme extracted from *B. angulatum*. Production of OS was also carried out by Rabiu *et al* [12] with enzymes extracted from other bifidobacteria. Yields for bifidobacterial enzymes were comparable to those seen with *B. angulatum* and varied between 27% and 48%. OS yield values estimated in the present work (Table 1) are in agreement with those of Rabiu *et al* [12] although the strain of *B. infantis* tested by these authors had an OS yield of 47.6%. OS production of our strain varied according to the experimental condition and OS yield could reach 43%.

## Conclusion

Results from the present study indicate that an increase of lactose concentration and temperature resulted in a higher OS production by *B. infantis* RW-8120. The optimal value for OS production appears to be near the area associated with lactose concentration of 40%, a cell volume of 1.72 ml, a temperature of 50°C, and a reaction time of 6 h.

## References

1 Albayrak N and ST Yang. 2002. Production of galacto-oligosaccharides from lactose by *Aspergillus oryzae* beta-galactosidase immobilized on cotton cloth. *Biotechnol Bioeng* 77: 8–19.
2 Blanchette L, D Roy and S Gauthier. 1995. Production of cultured Cottage cheese dressing by bifidobacteria. *J Dairy Sci* 78: 1421–1429.
3 Boon MA, AE Janssen and AK van't Riet. 2000. Effect of temperature and enzyme origin on the enzymatic synthesis of oligosaccharides. *Enzyme Microb Technol* 26: 271–281.
4 Bouhnik Y, B Flourie, L D'Agay-Abensour, P Pochart, G Gramet, M Durand and JC Rambaud. 1997. Administration of transgalacto-oligosaccharides increases fecal bifidobacteria and modifies colonic fermentation metabolism in healthy humans. *J Nutr* 127: 444–448.
5 Desjardins ML, D Roy and J Goulet. 1990. Growth of bifidobacteria and their enzyme profiles. *J Dairy Sci* 73: 299–307.
6 Dumortier V, C Brassart and S Bouquelet. 1994. Purification and properties of a β-D-galactosidase from *Bifidobacterium bifidum* exhibiting a transgalactosylation reaction. *Biotechnol Appl Biochem* 19: 341–354.
7 Gomes AMP and FX Malcata. 1999. *Bifidobacterium* spp. and *Lactobacillus acidophilus*: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends Food Sci Technol* 10: 139–157.
8 Holzapfel WH, P Haberer, J Snel, U Schillinger and JH Huis in't Veld. 1998. Overview of gut flora and probiotics. *Int J Food Microbiol* 41: 85–101.
9 Hung MN and BH Lee. 1998. Cloning and expression of β-galactosidase gene from *Bifidobacterium infantis* into *Escherichia coli*. *Biotechnol Lett* 20: 659–662.
10 Mahoney RR. 1998. Galactosyl-oligosaccharide formation during lactose hydrolysis: a review. *Food Chem* 63: 147–154.
11 Moller PL, F Jorgensen, OC Hansen, SM Madsen and P Stougaard. 2001. Intra-and extracellular beta-galactosidases from *Bifidobacterium bifidum* and *B. infantis*: molecular cloning, heterologous expression, and comparative characterization. *Appl Environ Microbiol* 67: 2276–2283.
12 Rabiu BA, AJ Jay, GR Gibson and RA Rastall. 2001. Synthesis and fermentation properties of novel galacto-oligosaccharides by beta-galactosidases from *Bifidobacterium* species. *Appl Environ Microbiol* 67: 2526–2530.
13 Roberfroid MB. 1998. Prebiotics and synbiotics: concepts and nutritional properties. *Br J Nutr* 80: S197–S202.
14 Roy D, L Blanchette, L Savoie, P Ward and P Chevalier. 1992. α- and β-Galactosidase properties of *Bifidobacterium infantis*. *Milchwissenschaft* 47: 18–21.
15 Roy D, JL Berger and G Reuter. 1994. Characterization of dairy-related *Bifidobacterium* spp. based on their beta-galactosidase electrophoretic patterns. *Int J Food Microbiol* 23: 55–70.
16 Sako T, K Matsumoto and R Tanaka. 1999. Recent progress on research and applications of non-digestible galacto-oligosaccharides. *Int Dairy J* 9: 69–80.
17 Van Laere KM, TT Abee, HA Schols, G Beldman and AG Voragen. 2000. Characterization of a novel beta-galactosidase from *Bifidobacterium adolescentis* DSM 20083 active towards transgalactooligosaccharides. *Appl Environ Microbiol* 66: 1379–1384.