Optimization of cholesterol removal, growth and fermentation patterns of Lactobacillus acidophilus ATCC 4962 in the presence of mannitol, fructo-oligosaccharide and inulin: a response surface methodology approach

Optimization of Cholesterol Removal, Growth and Fermentation Patterns of *Lactobacillus acidophilus* ATCC 4962 in Presence of Mannitol, FOS and Inulin: A Response Surface Methodology Approach

M. T. Liong and N. P. Shah*

School of Molecular Sciences, Victoria University, Werribee Campus, PO Box 14428, Melbourne City Mail Centre, Victoria 8001, Australia

Running headline: Optimizing removal of cholesterol

***Corresponding author:**

Professor Nagendra P. Shah

Phone: +61-3-9216-8289

Fax: +61-3-9216-8284

Email: nagendra.shah@vu.edu.au

ABSTRACT

Aims: To optimize cholesterol removal by *L. acidophilus* ATCC 4962 in the presence of prebiotics, and study the growth and fermentation patterns of the prebiotics.

Methods and Results: *L. acidophilus* ATCC 4962 was screened in the presence of six prebiotics, namely sorbitol, mannitol, maltodextrin, hi-amylose maize, fructooligosaccharide (FOS) and inulin in order to determine the best combination for highest level of cholesterol removal. The first-order model showed that the combination of inoculum size, mannitol, FOS and inulin was best for removal of cholesterol. The second-order polynomial regression model estimated the optimum condition of the factors for cholesterol removal by *L. acidophilus* ATCC 4962 to be 2.64% w/v inoculum size, 4.13% w/v mannitol, 3.29% w/v FOS and 5.81% w/v inulin. Analyses of growth, mean doubling time and short-chain-fatty-acid (SCFA) production using quadratic models indicated that cholesterol removal and the production of SCFA were growth associated.

Conclusions: Optimum cholesterol removal was obtained from the fermentation of *L. acidophilus* ATCC 4962 in the presence of mannitol, FOS and inulin. Cholesterol removal and the production of SCFA appeared to be growth associated and highly influenced by the prebiotics.

Significance and impact of the study: Response surface methodology (RSM) proved reliable in developing the model, optimizing factors, and analyzing interaction effects. The results provide better understanding on the interactions between probiotic and prebiotics for the removal of cholesterol.

Key words: optimization; cholesterol removal; inoculum size; prebiotic; RSM

INTRODUCTION

Interest in the usage of probiotics for human health dated back to 1908 when Metcnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (O'Sullivan et al. 1992). More recently, probiotics have been defined as 'cultures of live microorganisms that, applied in animals or humans, benefit the host by improving properties of indigenous microflora' (Arihara and Itoh, 2000). They mainly consist of lactobacilli, streptococci, enterococci, lactococci and bifidobacteria. Over the years, lactobacilli have been associated with the improvement of lactose intolerance, increase in natural resistance to infectious disease in gastrointestinal tract, suppression of cancer, improved digestion and reduction in serum cholesterol level (Gibson and Roberfroid, 1995). For hypercholesterolemic individuals, significant reductions in plasma cholesterol levels are associated with a significant reduction in the risk of heart attacks (Lourens-Hattingh and Viljoen, 2001). Various studies reported that lactobacilli could lower total cholesterol and low-density-lipoprotein (LDL) cholesterol (Anderson and Gilliland, 1999; Sanders, 2000).

Prebiotics are defined as nondigestable substances that exert biological effect on humans by selectively stimulating the growth or bioactivity of beneficial microorganisms either present, or therapeutically introduced to the intestine (Tomasik and Tomasik, 2003). Several non-starchy polysaccharides such as fructooligosaccharides, lactulose and β-cyclodextrin have been considered to have prebiotic properties. Recently, polyols such as mannitol, sorbitol and xylitol have been included to the prebiotics group (Klahorst, 2000). Prebiotics have been linked with cholesterol reducing effects. It was previously found that hepatocytes isolated from oligofructose-fed rats had a slightly lower capacity to synthesize triacylglycerol from radiolabeled acetate. This led to the hypothesis that decreased de novo lipogenesis in the

liver, through lipogenic enzymes, is the key to reduction of VLDL-triglyceride secretion in rats fed with oligosaccharides (Robertfroid and Delzenne, 1998). Administration of oligofructose was postulated to modify lipogenic enzyme gene expression, observed by a 50% reduction of activity of acetyl-CoA carboxylase, malic enzyme and ATP citrate lyase (Delzenne and Kok, 2001).

Probiotics and prebiotics simultaneously present in a product are called either synbiotics or eubiotics. Such a combination aids survival of the administered probiotic and facilitates its inoculation into the colon. Additionally, the prebiotic induces growth and increases activity of positive endogenic intestinal flora (Tomasik and Tomasik, 2003). Experiments with rats showed that synbiotics protect the organism from carcinogens significantly better than either prebiotics or probiotics individually (Gallaher and Khil, 1999). However, there is little information on suitable combinations of probiotics and prebiotics specifically targeting removal or lowering of cholesterol.

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving and optimizing processes. It also has important applications in design, development and formulation of new products, as well as improvement of existing product designs (Myers and Montgomery, 1995). Response surface models may involve main effects and interactions or have quadratic and possibly cubic terms to account for curvature. It has been successfully utilized to optimize compositions of microbiological media (Oh et al. 1995), improving fermentation processes (Lee and Chen, 1997) and product development (Gomes and Malcata, 1998). Conventional methods (such as one factor at one time) have been applied previously to evaluate the *in vitro* performance of probiotics and/or prebiotics to remove cholesterol. However, these methods require a large number of experiments to describe the effect of individual factors, were time consuming, and no statistical method

was established to distinguish the interaction effects from main effects. Thus, the aim of this study was to optimize cholesterol removal by using *L. acidophilus* ATCC 4962 in the presence of mannitol, FOS and inulin, through the approach of response surface.

MATERIALS AND METHODS

Bacteria and media preparation

L. acidophilus ATCC 4962 is a human derived strain that was obtained from the Australia Starter Culture Collection Center (ATCC) (Werribee, Australia). All stock cultures were stored in 40% glycerol at –80 °C, and transferred successively three times in sterile de Mann, Rogosa, Sharpe (MRS) broth using 1% inoculum and 20 h incubation at 37 °C prior to use. The culture was then centrifuged at 4 °C for 15 min at 2714 x g (Sorvall RT7, Newtown, Conn., U.S.A.). The supernatant was discarded while the pellet was washed twice with sterile distilled water, resuspended by vortexing in 50 ml of 0.1 M phosphate buffer (pH 6.8), and recentrifuged at 2714 x g at 4 °C for 15 min. After discarding the supernantant, 50 ml of 0.1 M phosphate buffer (pH 6.8) containing 2.0 % (w/v) of food grade cryoprotectant Unipectin^{TM} RS 150 (Savannah Bio Systems, Balwyn East, Australia) was added to the pellet. The mixture was vortexed, poured into large petri dishes and freeze-dried (Dynavac FD300, Airvac Engineering Pty. Ltd., Rowville, Australia) at -88 °C for 40 h for primary freezing and 8 h for secondary freezing. After freeze-drying, the hygroscopic cultures were transferred into sterile sealed bags and stored at -18 °C until used. Six types of commercially available prebiotics were used, namely sorbitol (Sigma Chemical Co., St. Louis, MO, U.S.A.), mannitol (Sigma), maltodextrin (Grain Processing Corp., Muscatine, IA, U.S.A.), hi-amylose maize (Starch Australasia Ltd., Lane Cove, NSW, Australia), inulin (Orafti Pty. Ltd., Tienen, Belgium) and FOS (Orafti). FOS used was Raftilose P95 that

contained 5% of glucose, fructose and sucrose. It contained oligofructose with DP ranging from 2 to 7, with an average DP of 4. Inulin used was Raftiline ST with a purity of 92%, an average DP of 10. Hi-amylose maize contained > 70% amylose, and 32.5% total dietary fiber.

All prebiotics were used at concentrations as per the experimental design (Table 1). Prebiotics were prepared in phosphate buffer (0.1 M, pH 6.0) containing ammonium citrate (2.0 g L^{-1}), sodium acetate (5.0 g L^{-1}), magnesium sulfate (0.1 g L^{-1}), manganese sulfate (0.05 g L^{-1}), dipotassium phosphate (2.0 g L^{-1}) and Tween 80 (1.0 ml L^{-1}). Freeze-dried cells of *L. acidophilus* ATCC 4962 were inoculated at appropriate levels as described in the experimental design.

Cholesterol removal

Freshly prepared media containing prebiotics were added with water-soluble filter-sterilized cholesterol (polyoxyethanyl-cholesteryl sebacate), at a final concentration of 70-100 μg ml^{-1}, inoculated with appropriate levels of freeze-dried *L. acidophilus* ATCC 4962 (Table 1), and incubated anaerobically at 37 °C for 48 h. After the incubation period, cells were centrifuged and the remaining cholesterol concentration in the spent broth was determined using the OPA colorimetric method as described previously (Rudel and Morris, 1973).

Growth of *L. acidophilus* ATCC 4962 in the presence of prebiotics

The growth was determined using the plate count method. Bacilli generally divide in one plane, and can produce chains of cells due to the failure to separate completely. Thus, at the end of the fermentation time, fermentation broth containing probiotic cultures sonicated for 5 s to disrupt clumps of lactobacilli (Bermudez et al.

2001) before serial dilutions were performed. Subsequent serial dilution blanks were vortexed for 30 s individually. One milliliter sample was taken after the incubation period, and 10-fold serial dilutions were made using peptone water diluent. MRS agar was used for plating and the plates were incubated anaerobically at 37 °C for 24 h in an anaerobic jar (Becton Dickinson Microbiology Systems®, Sparks, MD, U.S.A.) with a Gas Generating Kit® (Oxoid, Ltd.). Growth was calculated as log10 colony forming units (CFU ml^{-1}) and expressed as percentage difference between initial growth values obtained at time = 0 and at the end of the incubation period.

Mean doubling time

Mean doubling time was calculated as described previously (Shin et al. 2000). The specific growth rate (μ) of the cultures was obtained using the following equation:

$$\mu = (\ln X_2 - \ln X_1) / (t_2 - t_1)$$

where X_2 and X_1 are the cell density at time t_2 and t_1, respectively. Mean doubling time (T_d) was calculated as:

$$T_d = \ln 2/\mu$$, and expressed in min.

Short chain fatty acids (SCFA) determination

The fermentation of prebiotics was determined by measuring short chain fatty acids as the end products of fermentation using high performance liquid chromatography (HPLC, Varian Australia Pty. Ltd., Mulgrave, Australia). At the end of the incubation period, fermentation broths containing *L. acidophilus* ATCC 4962 and the prebiotics used were centrifuged at 2714 x g at 4 °C for 15 min, and the supernatant was prepared for HPLC analysis using the method as described previously (Dubey and Mistry, 1996). Briefly, 5 ml of supernatant was added to 100 μL of 15.5 N HNO_3 and 5

ml of 0.009 N H_2SO_4. The mixture was vortexed for 10 sec and recentrifuged at 14 000 x g for 10 min. The supernatant was filtered (0.20 μm) and stored at 4 °C until analysed. SCFA was expressed as the total acetic, butyric and propionic acids.

Experimental design and statistical analyses

Screening experiments to select prebiotics were performed with seven independent factors namely, inoculum size of *L. acidophilus* ATCC 4962 (X_1), sorbitol (X_2), mannitol (X_3), maltodextrin (X_4), hi-amylose maize (X_5), inulin (X_6) and FOS (X_7), using a two level partial factorial design 2^{7-2} resulting in 64 experimental runs (including duplicates) and 5 middle point runs. The units and the coded levels of the independent factors are shown in Table 1. First order empirical equation was used to exclude insignificant factors and to generate steepest ascent. Optimization was performed using a rotatable central composite design (CCD) with an alpha value of ± 2.00 for four factors. The treatment combinations of CCD were allocated in 2 blocks, with the first block representing the first day of the experiment and contained all factorial runs accompanied by 4 center runs. The second block, representing the second day of the experiment, contained all axial runs accompanied by 2 center runs. These modeling and statistical analyses were performed using the Design Expert version 5.07 software (Stat-Ease Corp., Minneapolis, MN, U.S.A.). All data presented are means of triplicate experiments, n = 3.

RESULTS

Screening of factors and steepest ascent

Results from the two-level partial factorial design are shown in Table 1, while analysis of variance (ANOVA) for the evaluation of the first-order model is shown in Table 2. ANOVA showed that the model used was suitable, lack-of-fit test was insignificant with only 9.58% total variation that was not explained by the model. Removal of cholesterol was significantly influenced by inoculum size of *L. acidophilus* ATCC 4962 (X_1), mannitol (X_3), FOS (X_6) and inulin (X_7), while the other prebiotics were found to have insignificant influence and were not included in the ANOVA. Thus, further optimization processes will only involve these four factors. A first-order equation (coded term) was generated from this first-degree order model, for response of cholesterol removal (Y), with the significant factors now redefined as inoculum size (X_1), mannitol (X_2), FOS (X_3) and inulin (X_4):

$$Y = 33.28 + 3.50X_1 + 1.17X_2 + 0.83X_3 + 1.17X_4.$$

From the equation and coefficient estimate, inoculum size (X_1) produced greatest effect and was used as the fundamental scale in the next step, steepest ascent. In this study, the steepest ascent design was based on the increase of 0.50 (% w/v) concentrations for X_1. This produced 5 design units (0.50/0.10 = 5). Thus, movement for X_2 was 1.67 design units [(1.17/3.50)(5) = 1.67], for X_3 was 1.19 design units [(0.83/3.50)(5) = 1.19] and for X_4 was 2.53 design units [(1.17/3.50)(5) = 2.53]. The following steepest ascent coordinates were generated as shown in Table 3. Steepest ascent coordinates showed that removal of cholesterol decreased after the fifth step, with highest value of 50.938 $\mu g\ ml^{-1}$, from the combination of inoculum size (2.20% w/v), mannitol (4.36% w/v), FOS (3.40% w/v) and inulin (6.08% w/v). This combination was used as the middle point for optimization experiments.

Optimization of cholesterol removal

Optimization was performed using CCD with fixed middle point of inoculum size (2.20% w/v), mannitol (4.30% w/v), FOS (3.40% w/v) and inulin (6.00% w/v). Design matrix for CCD and responses are shown in Table 4, while the adequacy and fitness were evaluated by ANOVA and regression coefficients (Table 5). ANOVA results indicated that the quadratic regression to produce the second-order model was significant. Lack-of-fit test was insignificant and a good coefficient regression was obtained. Inoculum size, mannitol, FOS and inulin significantly influenced cholesterol removal.

The effect of each factors were further assessed using perturbation plots, to show how the response changes as each factor moves from the chosen reference point, with all other factors held constant at reference values (Oh et al. 1995). In this study, as one particular chosen factor was assessed, the other factors were held constant at the optimum point. Figure 1 shows the perturbation plot of the factors used in this study. Although all factor showed significant quadratic effect, the curve with the most prominent change was the perturbation curve of inoculum size, compared to the other factors that were fixed at their maximum levels. Thus, we believe that inoculum size was the most significant factor that contributed to the removal of cholesterol with the most obvious quadratic effect. Although the P-values of both FOS and inulin showed similar levels of significance, it could be clearly seen from the perturbation plot that the response curve of inulin was less prominent than that of FOS.

The best explanatory equation to fit the second-order model and subsequently produce the response surface was expressed as:

$$Y_0 = c + c_1X_1 + c_2X_2 + c_3X_3 + c_4X_4 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{44}X_4^2 + c_{12}X_1X_2 + c_{13}X_1X_3 + c_{14}X_1X_4 + c_{23}X_2X_3 + c_{24}X_2X_4 + c_{34}X_3X_4$$

where $c \ldots c_{23}$ are regression coefficients and X_1, X_2, X_3, X_4 are the coded independent factors. Here, the second-order regression model involved four factors, thus producing four linear, four quadratic and six interaction terms. Response surface was generated (Figure 2) based on the second-order equation:

$$Y_0 = 56.58 + 6.38X_1 - 0.63X_2 - 1.49X_3 - 1.19X_4 - 7.34X_1^2 - 6.42X_2^2 - 5.97X_3^2 - 5.75X_4^2 - 0.72X_1X_2 + 0.34X_1X_3 - 0.034X_1X_4 + 1.51X_2X_3 - 0.50X_2X_4 - 1.01X_3X_4$$

An optimum point was produced with optimum cholesterol removal obtained at 58.142 $\mu g\ ml^{-1}$. The combination that produced the optimum point was (X_1, X_2, X_3, X_4) = (0.437, -0.082, -0.115, -0.092). The original levels that correlated with those coded values were found to be inoculum size at 2.64% w/v, mannitol at 4.14% w/v, FOS at 3.28% w/v and inulin at 5.82% w/v.

All these predictions by the regression model were further ascertained by a validation experiment. We compared the cholesterol removal patterns over a 24 h period using four different media: the optimum medium (inoculum size: 2.60% w/v; mannitol: 4.10% w/v; FOS: 3.30% w/v; inulin: 5.80% w/v), the center-point medium (inoculum size: 2.20% w/v; mannitol: 4.30% w/v; FOS: 3.40% w/v; inulin: 6.00% w/v), the high-point medium (inoculum size: 3.20% w/v; mannitol: 6.30% w/v; FOS: 4.40% w/v; inulin: 8.00% w/v) and the low-point medium (inoculum size: 1.20% w/v; mannitol: 2.30% w/v; FOS: 2.40% w/v; inulin: 4.00% w/v). The cholesterol removal curves are shown in Figure 3. Although the exact cholesterol removal quantities were different from the predictions, the patterns were in tandem with predictions by the model. Highest cholesterol was removed from the optimum medium, and lower from the center-point medium. Least cholesterol was removed from both high-point and low-point media, as supported by the response surface of cholesterol removal (Figure 2).

Growth, mean doubling time and production of SCFA

We further studied patterns of growth, mean doubling time and production of SCFA from the fermentation of prebiotics, at the experimental regions used to obtain optimum removal of cholesterol. The response obtained using the CCD is shown in Table 6. The statistical analyses with coefficient estimates and the significance of each response model are presented in Table 7.

The response surface of growth (Y_1) is shown in Figure 4, and was generated based on the following coded factor equation:

$$Y_1 = 41.97 + 2.49X_1 - 0.12X_2 - 1.49X_3 – 3.35X_4 – 3.90X_1^2 – 4.05X_2^2 – 2.77X_3^2 - 0.50X_4^2 - 0.22X_1X_2 + 1.66X_1X_3 + 1.63X_1X_4 + 0.89X_2X_3 - 0.08X_2X_4 + 0.53X_3X_4$$

The response surface clearly indicated that an optimum point (45.21%) was produced with X_1, X_2, X_3 and X_4 at 2.23% w/v, 4.21% w/v, 3.04% w/v and 4.00% w/v, respectively. Growth increased with increasing inoculum size level from 1.20% w/v to 2.23% w/v. Further increase in concentrations of inoculum size beyond 1.69% w/v generated a decrease in growth. Similarly, increasing concentrations of mannitol and FOS from 2.30% w/v to 4.21% w/v and 2.40% w/v to 3.04% w/v, respectively, increased growth, but further increase in the prebiotics concentration generated a decrease in growth. Inulin produced highest growth at its lowest concentration of 4.00% w/v, and produced lowest growth at its highest concentration of 8.00% w/v. It appeared that growth of *L. acidophilus* ATCC 4962 was influenced by inulin in a linear manner, while inoculum size, mannitol and FOS showed significant quadratic effects. Other than main quadratic effects, interactions between inoculum size and FOS, and inoculum size and inulin produced strongest influence towards growth, while the other interactions were insignificant.

In this study, patterns of mean doubling time (Y_2) were studied using the response surface (Figure 5) that was generated from the equation:

$$Y_2 = 291.21 + 1.53X_1 + 0.32X_2 – 0.28X_3 – 1.31X_4 – 0.97X_1^2 + 0.095X_2^2 – 0.40X_3^2 - 0.60X_4^2 + 0.34X_1X_2 + 0.42X_1X_3 – 0.16X_1X_4 + 0.66X_2X_3 - 0.21X_2X_4 + 0.70X_3X_4$$

Inoculum size, FOS and inulin showed significant quadratic effect, while mannitol did not (Table 7). FOS mainly contributed to the interaction effects, with only interaction terms involving FOS showed significant influence on mean doubling time. All these significant interaction terms also showed positive regression coefficients, indicating that either a decrease or increase in both factors will contribute to an increase in mean doubling times.

The SCFA (Y_3) was obtained as a total of individual fatty acids, namely acetic, butyric and propionic acids. A response surface (Figure 6) was generated from the second-order equation:

$$Y_3 = 60.03 + 6.67X_1 + 0.62X_2 + 2.30X_3 + 3.29X_4 – 6.08X_1^2 - 9.65X_2^2 – 10.69X_3^2 - 12.34X_4^2 + 0.66X_1X_2 + 3.80X_1X_3 + 4.84X_1X_4 + 1.45X_2X_3 + 1.29X_2X_4 + 3.20X_3X_4$$

All factors produced significant quadratic effects on production of SCFA. Response surfaces produced showed that the production of SCFA appeared to be growth associated.

DISCUSSION

Various factors normally affect the response surfaces that are produced. Thus, screening experiments are needed to segregate important main effects from less important ones (Montgomery, 1996). In this study, first degree order equation was

generated and significance of factors was tested using screening experiments. A complete replication of the seven factors using a 2^x factorial design would need 128 experimental runs. However, only seven degree of freedoms would be needed to estimate main effects, and 21 degree of freedoms would estimate two-factor interaction effects, while the remaining 99 degree of freedoms would estimate error or/and three or higher-factor interaction effects (Cox and Reid, 2000). Thus, a partial two-level factorial design (2^{7-2}) was applied in this study. Partial factorial designs are capable of identifying important factors using less number of experimental runs without loss of information on main factor effects and their interactions (Li et al. 2002). Following the screening of significant factors, design points were subjected to steepest ascent before subsequent optimization steps. Steepest ascent or steepest descent involved the generation of mathematical movements along an ascending or descending path until no improvement occurred (Montgomery, 1996).

A significant quadratic regression, insignificant lack-of-fit and a small total variation (4.60%) that was not explained by the model, suggested that the model accurately represented data in the experimental region. This also indicated that second-order terms were sufficient and higher-order terms were not necessary (Oh et al. 1995). It must also be noted that the *t* value of the quadratic term of inoculum size (X_1^2) was higher than others (Table 5), indicating that the quadratic effect of inoculum size had the strongest effect on cholesterol removal, which was also confirmed using the perturbation plot. Validation experiments showed that the predicted value was 58.142 μg/ml while the actual experimental result was 52.941 μg/ml. However, it must be noted that the conditions for both were slightly different. The predicted value was obtained at the predicted 2.64% w/v inoculum size, 4.14% w/v mannitol, 3.28% w/v FOS and 5.82% w/v inulin, while the actual experiments were conducted with 2.60%

w/v inoculum size, 4.10% w/v mannitol, 3.30% w/v FOS and 5.80% w/v inulin. Under such dissimilarity, the difference between the prediction and actual data was only 8.95%. The obvious difference of cholesterol removal between the optimum, high-point, low-point and center-point media proved the validity of the model and the reproducibility of the prediction.

From Table 5, it must be noted that the coefficient estimates of the interaction terms of (X_2, X_4) and (X_3, X_4) had negative signs (X_{24} = -0.50, X_{34} = -1.01). These negative signs may imply that for an increase of the response, the coded levels of (X_2, X_4) and (X_3, X_4) must have different signs, either one must be higher than zero and the other lower than zero (Oh et al. 1995). However, it must be noted that the optimum was achieved at (X_2 = -0.082, X_4 = -0.092) and (X_3 = -0.115, X_4 = -0.092), which would produce a positive sign instead. This may be due to other terms that may dominate this particular interaction term (Oh et al. 1995). Considering that the lack-of-fit test was insignificant, other higher terms would not have contributed to this, thus, we postulate that the linear term might have played a role.

The response surface of growth showed similar patterns with the response surface of removal of cholesterol, indicating a strong correlation between removal of cholesterol and growth. Previous studies also showed that cholesterol assimilation by strains of *L. acidophilus* during refrigerated storage of nonfermented milk was associated with bacterial growth and their viability, and was growth dependent (Piston and Gilliland, 1994; Pereira and Gibson, 2002). This has led us to postulate that cholesterol removal *in-vitro* was growth associated. Significant interaction terms of inoculum size with FOS and inulin showed that these two prebiotics strongly encouraged growth of *L. acidophilus* ATCC 4962. Comparing these two, a higher coefficient of regression for X_1X_3 than X_1X_4 indicated that FOS was more preferred

than inulin. Studies using bifidobacteria showed that the bifidogenic effects of inulin and FOS are independent of chain lengths or GF_n type. FOS of the GF_2 and GF_3 moiety were also found to be more rapidly consumed compared to GF_4 (Kaplan and Hutkins, 2000). All these may have contributed to the preference of *L. acidophilus* ATCC 4962 on FOS than on inulin, and the fact that linear decrease in concentration of inulin contributed to an increase in growth.

Mean doubling time was used as a measure of the effectiveness of a specific carbon source in modulating bacterial growth rate (Bruno et al. 2002). Of all factors, FOS contributed significantly in the interaction patterns of mean doubling time, and higher growth rates (lower mean doubling time) were obtained at lower concentration of FOS (Figure 5). It was previously reported that both the uptake and hydrolysis of FOS are induced by higher oligosaccharides but repressed by products of their hydrolysis (Kaplan and Hutkins, 2003). In this experiment, it appeared that at higher concentration of FOS, more product of hydrolysis were produced and repressed bacterial growth rate, producing a higher mean doubling time. It must also be noted that the interaction between FOS and inulin produced lower mean doubling times when one factor was at lower levels and the other at higher levels . This indicated that when FOS was at its lower level, *L. acidophilus* ATCC 4962 utilized a higher level of inulin for higher growth rate and vice versa. It appeared that although *L. acidophilus* ATCC 4962 preferred FOS over inulin, but under conditions of substrate limitation, inulin was beneficially utilized for the modulation of growth rate.

The major products of metabolism of prebiotics are short chain fatty acids (SCFA), carbon dioxide and hydrogen, and bacterial cell mass (Cummings et al. 2001). Although much work has been done on SCFA production and the significance of the individual acids, no particular pattern of SCFA production from prebiotic fermentation

401 has emerged as yet. Hence, in this study, we analyzed the SCFA production from
402 fermentation of mannitol, FOS and inulin by *L. acidophilus* ATCC 4962. Production of
403 SCFA appeared to be growth associated and correlated with the patterns of cholesterol
404 removal. Although all factors significantly affected the production of SCFA, mannitol
405 exhibited the strongest effect (Table 7). While the utilization of FOS and inulin has been
406 widely reported, the utilization of mannitol to produce high concentration of SCFA was
407 less studied and was also found to be strain dependent. Lactic acid bacteria that
408 produced NADH oxidase would have the alternative NADH-H^+-oxidizing mechanism,
409 resulting in higher ability to grow on substrates more chemically reduced than glucose,
410 such as mannitol (Stanton et al. 1999). This may contribute to the better growth of *L.*
411 *acidophilus* ATCC 4962 in the presence of mannitol and subsequently produced higher
412 amount of SCFA and higher cholesterol removal. Previous study showed that strains of
413 *L. acidophilu*s that utilized mannitol also exhibited capability of cholesterol uptake
414 (Gupta et al. 1996).

415 In conclusion, cholesterol removal was optimized after selecting a combination
416 of inoculum size and prebiotic, with the predicted optimum removal of 58.142 μg ml^{-1}
417 obtained at 2.64% w/v inoculum size, 4.14% w/v mannitol, 3.28% w/v FOS and 5.82%
418 w/v inulin. Validation experiment showed that RSM was reliable in developing a
419 model, optimization of factors, and analysis of interaction effects. Analysis of growth,
420 mean doubling time and production of SCFA showed that cholesterol removal and the
421 production of SCFA was growth associated.
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Tomasik, P. J. and Tomasik, P. (2003) Probiotics and prebiotics. *Cereal Chemistry* **80**, 113-117.

525 Table1. Treatment combinations and response for screening experiments.

Standard order	Factors*							Response
	Inoculum size (% w/v)	Sorbitol (% w/v)	Mannitol (% w/v)	FOS (% w/v)	Hi-maize (% w/v)	Inulin (% w/v)	Maltodextrin (% w/v)	Cholesterol assimilated (μg ml^{-1})
1	-1	-1	-1	-1	-1	1	1	31.36
3	1	-1	-1	-1	-1	-1	-1	33.13
5	-1	1	-1	-1	-1	-1	-1	25.52
7	1	1	-1	-1	-1	1	1	36.09
9	-1	-1	1	-1	-1	-1	1	27.71
11	1	-1	1	-1	-1	1	-1	39.17
13	-1	1	1	-1	-1	1	-1	32.53
15	1	1	1	-1	-1	-1	1	36.15
17	-1	-1	-1	1	-1	-1	-1	27.50
19	1	-1	-1	1	-1	1	1	39.01
21	-1	1	-1	1	-1	1	1	31.51
23	1	1	-1	1	-1	-1	-1	34.90
25	-1	-1	1	1	-1	1	-1	34.58
27	1	-1	1	1	-1	-1	1	36.15
29	-1	1	1	1	-1	-1	1	30.64
31	1	1	1	1	-1	1	-1	39.58
33	-1	-1	-1	-1	1	1	-1	28.70
35	1	-1	-1	-1	1	-1	1	34.22
37	-1	1	-1	-1	1	-1	1	26.30
39	1	1	-1	-1	1	1	-1	36.20
41	-1	-1	1	-1	1	-1	-1	28.49
43	1	-1	1	-1	1	1	1	38.54
45	-1	1	1	-1	1	1	1	31.09
47	1	1	1	-1	1	-1	-1	34.01
49	-1	-1	-1	1	1	-1	1	25.25
51	1	-1	-1	1	1	1	-1	38.23
53	-1	1	-1	1	1	1	-1	30.16
55	1	1	-1	1	1	-1	1	35.73
57	-1	-1	1	1	1	1	1	33.59
59	1	-1	1	1	1	-1	-1	36.82
61	-1	1	1	1	1	-1	-1	31.61
63	1	1	1	1	1	1	1	40.52
65	0	0	0	0	0	0	0	32.81
66	0	0	0	0	0	0	0	31.98
67	0	0	0	0	0	0	0	33.02
68	0	0	0	0	0	0	0	31.88
69	0	0	0	0	0	0	0	33.96

526 *Inoculum size: 0.10-0.30% w/v; Sorbitol: 0.50-1.50% w/v; Mannitol: 0.50-1.50% w/v;
527 Maltodextrin: 0.50-1.50% w/v; Hi-amylose maize: 0.50-1.50% w/v; FOS: 0.50-1.50%
528 w/v; Inulin: 0.50-1.50% w/v.
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Table 2. Analysis of variance and coefficient estimates for the evaluation of the first-order model.

Source of variation	Sum of squares	DF*	Mean square	F-value	P-value
Model†	1115.371	4	278.84	148.73	< 0.0001
Curvature	1.41	1	1.41	0.75	0.3890
Residual	118.11	63	1.87		
Lack-of-fit	49.73	27	1.84	0.97	0.5269
Pure error	68.38	36	1.90		
Correlation total	1234.90	68			

Factor:	Coefficient estimate	DF	Standard error	t-value	P-value
Inoculum size (X_1)	3.50	1	0.17	20.43	0.0001‡
Mannitol (X_3)	1.17	1	0.17	6.83	0.0001‡
FOS (X_6)	0.83	1	0.17	4.85	0.0001‡
Inulin (X_7)	1.77	1	0.17	10.36	0.0001‡

*DF: degree of freedom.
†R^2 = 0.9042.
‡Significant at alpha 0.05.

Table 3: Coordination path of steepest ascent for all chosen factors in coded and natural levels.

Step	Coded factors*				Natural factors†				Cholesterol removed (μg ml^{-1})
	ξ_1	ξ_3	ξ_6	ξ_7	X_1	X_2	X_3	X_4	
1) Base	0	0	0	0	0.20	1.00	1.00	1.00	16.478
Δ	5	1.67	1.19	2.53	(5)(0.1) = 0.5	(1.67)(0.50) = 0.84	(1.19)(0.50) = 0.60	(2.53)(0.50) = 1.27	
2) Base + Δ	5	1.67	1.19	2.53	0.70	1.84	1.60	2.27	36.563
3) Base + 2Δ	10	3.34	2.38	5.06	1.20	2.68	2.20	3.54	44.375
4) Base + 3Δ	15	5.01	3.57	7.59	1.70	3.52	2.80	4.81	50.781
5) Base + 4Δ	20	6.68	4.76	10.12	2.20	4.36	3.40	6.08	50.938
6) Base + 5Δ	25	8.35	5.95	12.65	2.70	5.20	4.00	7.35	48.813
7) Base + 6Δ	30	10.02	7.14	15.18	3.20	6.04	4.60	8.62	47.497

*ξ_1: inoculum size (% w/v), ξ_3: mannitol (% w/v), ξ_6: FOS (% w/v); ξ_7: inulin (% w/v).

†X_1: inoculum size (% w/v), X_2: mannitol (% w/v), X_3: FOS (% w/v); X_4: inulin (% w/v).

576 Table 4. Combination matrix of the central composite design (CCD) using coded levels

577 for the response of cholesterol removal.

Standard run	Block*	Factors				Cholesterol removal ($\mu g\ ml^{-1}$)†
		Inoculum size (X_1)	Mannitol (X_2)	FOS (X_3)	Inulin (X_4)	
1	1	-1	-1	-1	-1	30.367
2	1	1	-1	-1	-1	46.304
3	1	-1	1	-1	-1	29.586
4	1	1	1	-1	-1	41.461
5	1	-1	-1	1	-1	26.461
6	1	1	-1	1	-1	42.086
7	1	-1	1	1	-1	31.929
8	1	1	1	1	-1	47.086
9	1	-1	-1	-1	1	28.023
10	1	1	-1	-1	1	40.367
11	1	-1	1	-1	1	23.648
12	1	1	1	-1	1	39.117
13	1	-1	-1	1	1	18.023
14	1	1	-1	1	1	38.179
15	1	-1	1	1	1	24.273
16	1	1	1	1	1	34.351
17	1	0	0	0	0	53.179
18	1	0	0	0	0	63.648
19	1	0	0	0	0	56.304
20	1	0	0	0	0	60.054
21	2	-2	0	0	0	15.211
22	2	2	0	0	0	33.414
23	2	0	-2	0	0	32.164
24	2	0	2	0	0	23.804
25	2	0	0	-2	0	34.586
26	2	0	0	2	0	24.976
27	2	0	0	0	-2	25.523
28	2	0	0	0	2	35.836
29	2	0	0	0	0	60.836
30	2	0	0	0	0	50.523

578 *1, first day of experiment; 2, second day of experiment.

579 †All factorial and axial points are means of duplicates.

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Table 5. Analysis of variance of the second-order model* and coefficient estimates for the response Y_0 and factors X_1, X_2, X_3 and X_4.

Source	Sum of squares	DF	Mean square	F-value	P-value
Model†	4302.42	14	307.32	10.78	0.0001
Residual	399.17	14	28.51		
Lack-of-Fit	284.11	10	28.41	0.99	0.5541
Pure error	115.07	4	28.77		
Total	4870.60	29			

Factor‡	Coefficient estimate	DF	Standard error	t-value	P-value
Intercept	c = 56.58	1	2.21		
X_1	c_1 = 6.38	1	1.09	5.85	0.0001§
X_2	c_2 = -0.63	1	1.09	-0.58	0.5735
X_3	c_3 = -1.49	1	1.09	-1.36	0.1938
X_4	C_4 = -1.19	1	1.09	-1.10	0.2915
X_1^2	c_{11} = -7.34	1	1.02	-7.20	0.0001§
X_2^2	c_{22} = -6.42	1	1.02	-6.30	0.0001§
X_3^2	c_{33} = -5.97	1	1.02	-5.86	0.0001§
X_4^2	C_{44} = -5.75	1	1.02	-5.64	0.0001§
X_1X_2	c_{12} = -0.72	1	1.33	-0.54	0.5993
X_1X_3	c_{13} = 0.34	1	1.33	0.250	0.8044
X_1X_4	C_{14} = -0.034	1	1.33	-0.026	0.9799
X_2X_3	C_{23} = 1.51	1	1.33	1.13	0.2774
X_2X_4	C_{24} = -0.50	1	1.33	-0.38	0.7120
X_3X_4	C_{34} = -1.01	1	1.33	-0.76	0.4615

*Y_0 = 56.58 + 6.38X_1 - 0.63X_2 - 1.49X_3 - 1.19X_4 - 7.34X_1^2 - 6.42X_2^2 - 5.97X_3^2 - 5.75X_4^2 - 0.72X_1X_2 + 0.34X_1X_3 - 0.034X_1X_4 + 1.51X_2X_3 - 0.50X_2X_4 - 1.01X_3X_4

†R^2= 0.9540.

‡X_1: inoculum size (% w/v), X_2: mannitol (% w/v), X_3: FOS (% w/v), X_4: inulin (% w/v).

§Significant at alpha 0.05.

600 Table 6. Combination matrix of the central composite design (CCD) using coded levels

601 for the factors and five responses.

Standard run	Block*	Factors†				Responses‡		
		X_1	X_2	X_3	X_4	Y_1	Y_2	Y_3
1	1	-1	-1	-1	-1	39.629	288.677	6.308
2	1	1	-1	-1	-1	35.996	290.797	13.064
3	1	-1	1	-1	-1	38.381	288.303	8.220
4	1	1	1	-1	-1	33.925	290.649	16.503
5	1	-1	-1	1	-1	28.365	284.406	5.992
6	1	1	-1	1	-1	35.774	288.435	16.711
7	1	-1	1	1	-1	30.550	286.989	8.915
8	1	1	1	1	-1	36.249	290.791	15.324
9	1	-1	-1	-1	1	28.398	287.901	5.131
10	1	1	-1	-1	1	32.935	288.418	24.531
11	1	-1	1	-1	1	23.948	285.813	11.966
12	1	1	1	-1	1	32.318	288.530	17.959
13	1	-1	-1	1	1	20.730	286.911	7.239
14	1	1	-1	1	1	32.278	288.579	35.922
15	1	-1	1	1	1	24.742	286.840	7.448
16	1	1	1	1	1	31.398	291.750	62.947
17	1	0	0	0	0	38.706	290.243	67.026
18	1	0	0	0	0	48.981	291.175	53.419
19	1	0	0	0	0	38.739	290.372	46.826
20	1	0	0	0	0	42.216	291.505	67.139
21	2	-2	0	0	0	19.677	284.734	36.543
22	2	2	0	0	0	31.106	292.091	45.701
23	2	0	-2	0	0	24.825	292.169	31.714
24	2	0	2	0	0	24.734	293.195	22.015
25	2	0	0	-2	0	32.519	291.310	23.119
26	2	0	0	2	0	27.326	290.102	22.252
27	2	0	0	0	-2	46.054	290.716	16.866
28	2	0	0	0	2	31.942	289.108	15.285
29	2	0	0	0	0	45.946	290.791	44.787
30	2	0	0	0	0	38.688	291.465	72.814

602 *1, first day of experiment; 2, second day of experiment.

603 †X_1 = inoculum size, X_2 = mannitol, X_3 = FOS, X_3 = inulin.

604 ‡Y_1 = growth (%), Y_2 = mean doubling time (min), Y_3 = SCFA (mmol l^{-1}).

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609 Table 7. Regression coefficients of the second-order equation* for the five responses†.

Coefficient	Y_1	Y_2	Y_3
c	41.97	291.21	60.03
c_1	2.46‡	1.53‡	6.67‡
c_2	-0.12	0.32	0.62
c_3	-1.49‡	-0.28	2.30
c_4	-3.35‡	-0.31	3.29
c_{11}	-3.90‡	-0.97‡	-6.08‡
c_{22}	-4.05‡	0.095	-9.65‡
c_{33}	-2.77‡	-0.40‡	-10.69‡
c_{44}	-0.50	-0.60‡	-12.34‡
c_{12}	-0.22	0.34	0.66
c_{13}	1.66‡	0.42‡	3.80
c_{14}	1.63‡	-0.16	4.84
c_{23}	0.89	0.66‡	1.45
c_{24}	-0.08	-0.21	1.29
c_{34}	0.53	0.70‡	3.20
R^2	0.9173	0.9377	0.8448
P-value	0.0001	0.0001	0.0016

610 *$Y = c + c_1X_1 + c_2X_2 + c_3X_3 + c_{11}X_1^2 + c_{22}X_2^2 + c_{33}X_3^2 + c_{12}X_1X_2 + c_{13}X_1X_3 +$
611 $c_{23}X_2X_3$.
612 †Y_1 = growth (%), Y_2 = mean doubling time (min), Y_5 = SCFA (mmol l^{-1}).
613 ‡Significant at alpha = 0.05.
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Figure 1. Perturbation plot of inoculum size (A), mannitol (B), FOS (C) and inulin (D).

Figure 2. Response surface for cholesterol removal (μg ml^{-1}) from the effects of (A) FOS and mannitol, and (B) inoculum size and inulin. Factors that were not included in the axes were fixed at their respective optimum levels.

Figure 3. Cholesterol removal by *L. acidophilus* ATCC 4962 in the optimum (■), center-point (●), high-point (▲) and low-point (♦) media, for the validation experiments. Factors combination for optimum medium were: inoculum size 2.60% w/v, mannitol 4.10% w/v, FOS 3.30% w/v and inulin 5.80% w/v. Center-point medium were: inoculum size 2.20% w/v, mannitol 4.30% w/v, FOS 3.400% w/v and inulin 6.00% w/v. High-point medium were: inoculum size 3.20% w/v, mannitol 6.30% w/v, FOS 4.40% w/v and inulin 8.00% w/v, and low-point medium were inoculum size 1.20% w/v, mannitol 2.30% w/v, FOS 2.40% w/v and inulin 4.00% w/v. Error bars represent standard error of means; n = 3.

Figure 4. Response surface for growth (%) from the effects of (A) FOS and mannitol, and (B) inoculum size and inulin. Factors that were not included in the axes were fixed at their respective optimum levels.

Figure 5. Response surface for mean doubling time (min) from the effects of (A) inoculum size and FOS, and (B) FOS and inulin. Factors that were not included in the axes were fixed at their respective optimum levels.

Figure 6. Response surface for the production of SCFA (mmol l^{-1}) from the effects of (A) FOS and mannitol, and (B) inoculum size and inulin. Factors that were not included in the axes were fixed at their respective optimum levels.

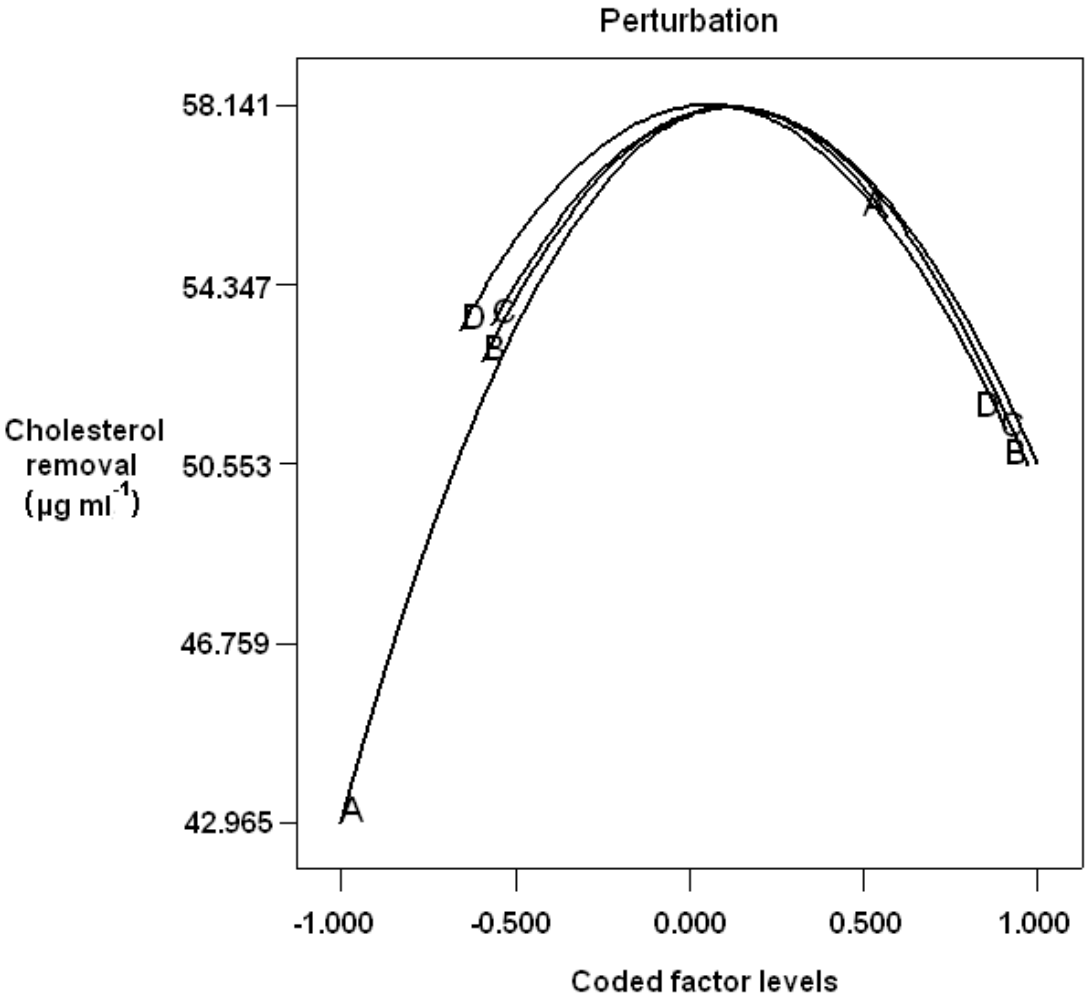
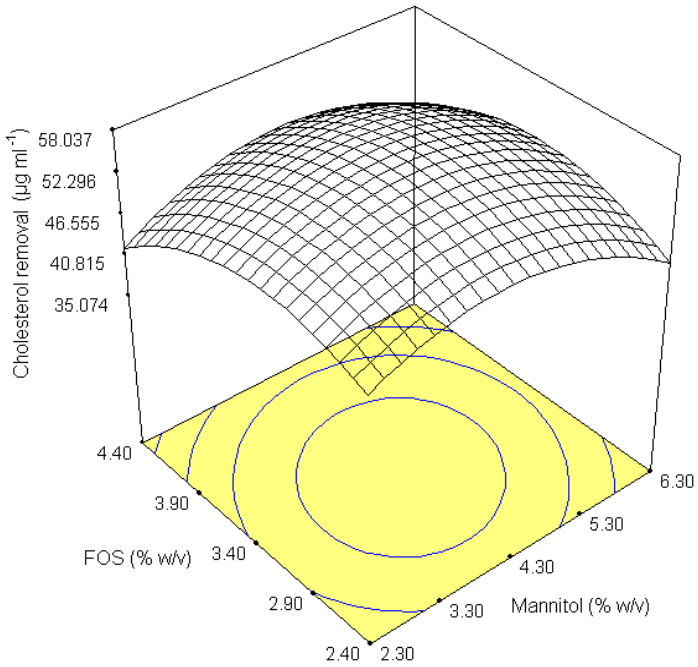
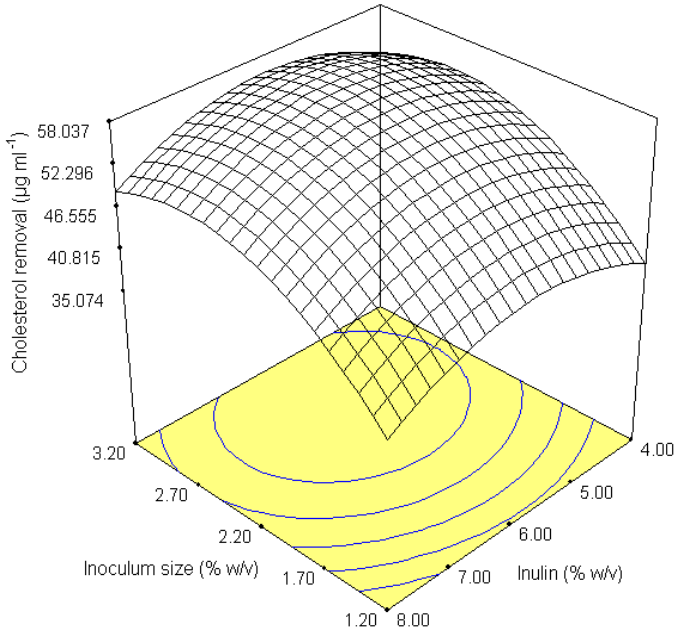

Perturbation
58.141
54.347
50.553
46.759
42.965
Cholesterol removal (µg ml-1)
A
D
C
B
-1.000
-0.500
0.000
0.500
1.000
Coded factor levels

Figure 1.

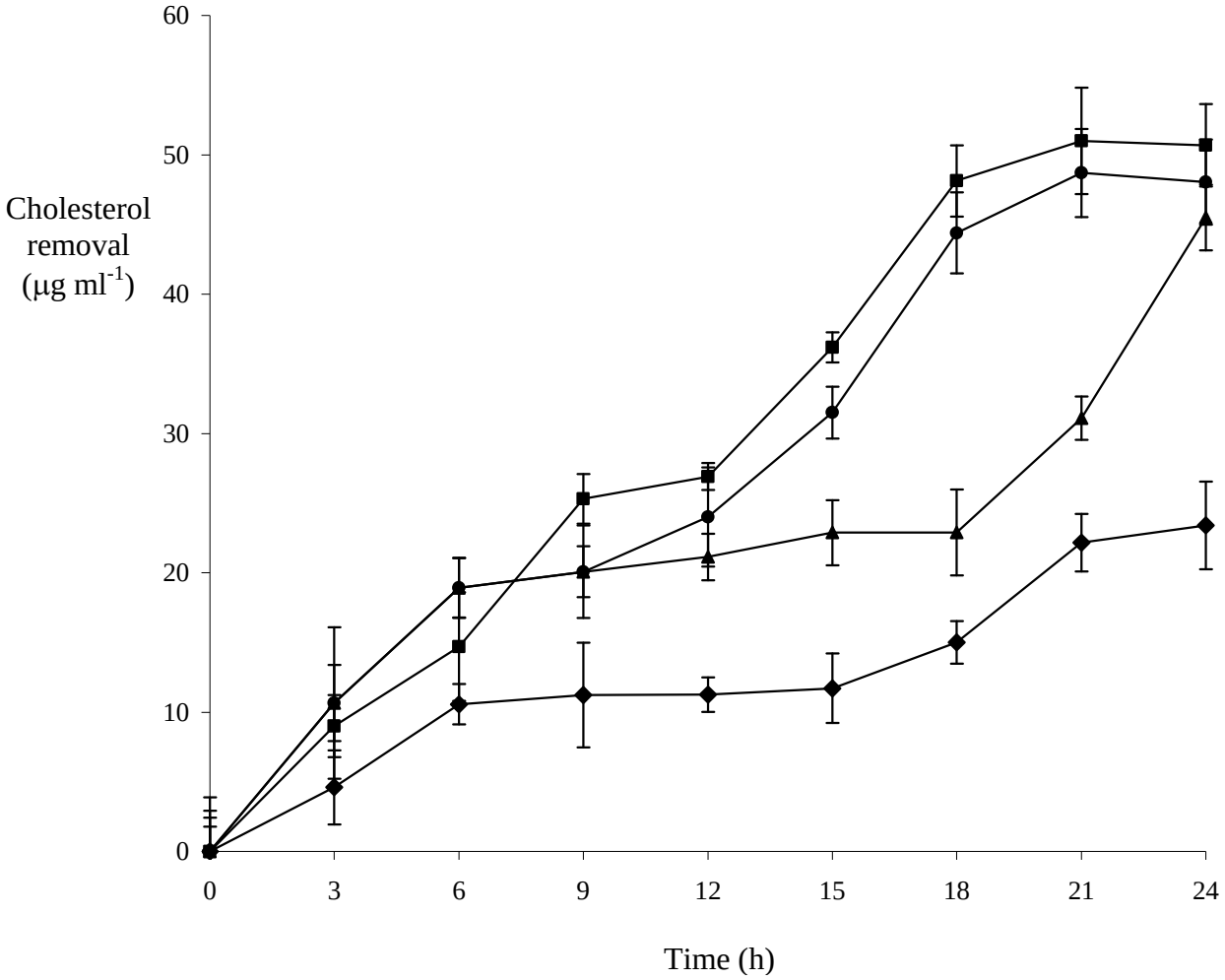

Cholesterol removal (µg ml-1)
58.037
52.296
46.555
40.815
35.074
4.40
3.90
3.40
2.90
2.40
FOS (% w/v)
2.30
3.30
4.30
5.30
6.30
Mannitol (% w/v)

(A)


Cholesterol removal (µg ml-1)
58.037
52.296
46.555
40.815
35.074
3.20
2.70
2.20
1.70
1.20
Inoculum size (% w/v)
8.00
7.00
6.00
5.00
4.00
Inulin (% w/v)

(B)

Figure 2.



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688 **Figure 3.**

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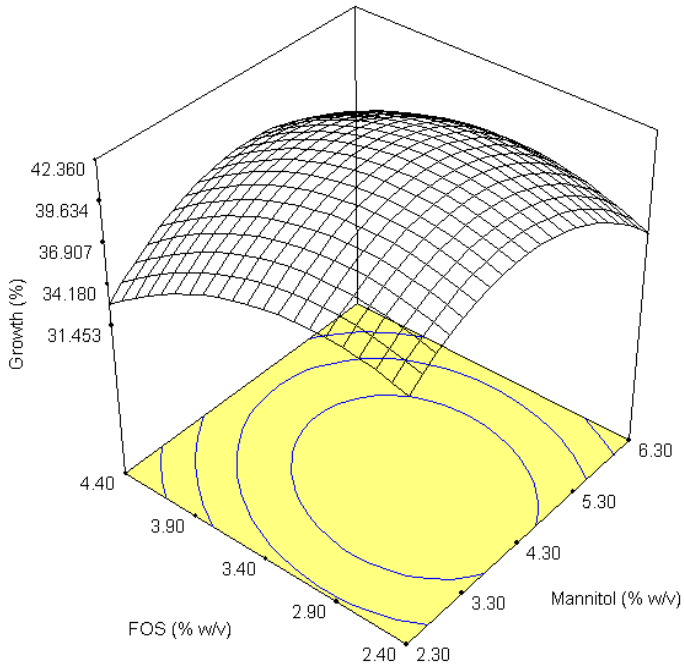
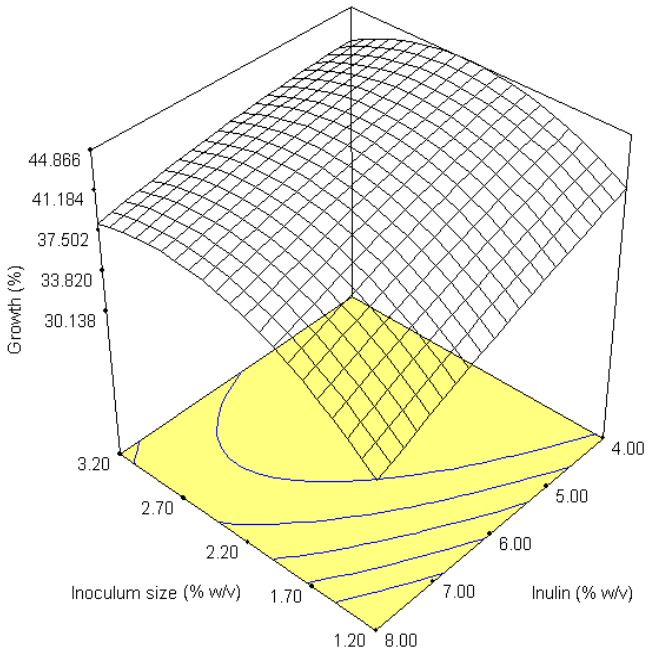
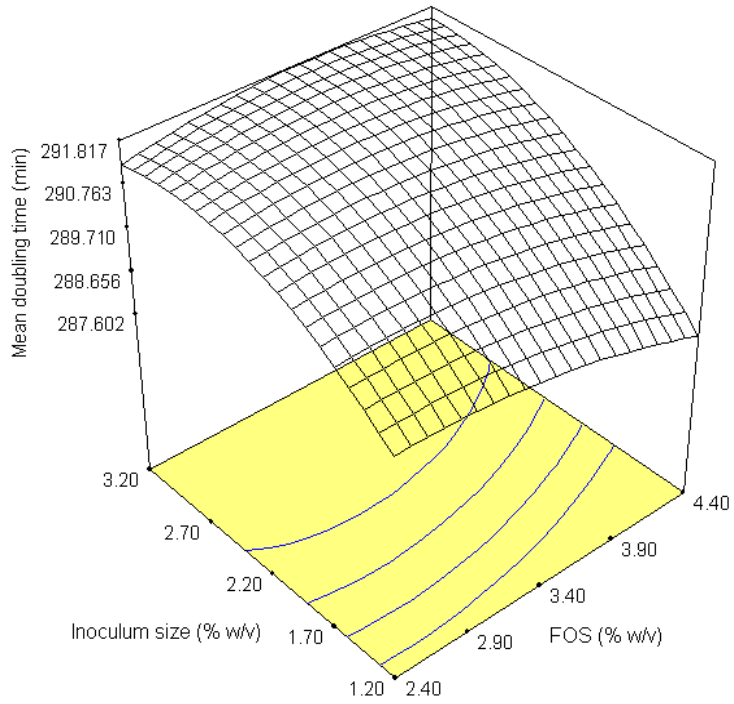

42.360
39.634
36.907
34.180
31.453
Growth (%)
4.40
3.90
3.40
2.90
2.40
FOS (% w/v)
2.30
3.30
4.30
5.30
6.30
Mannitol (% w/v)

(A)

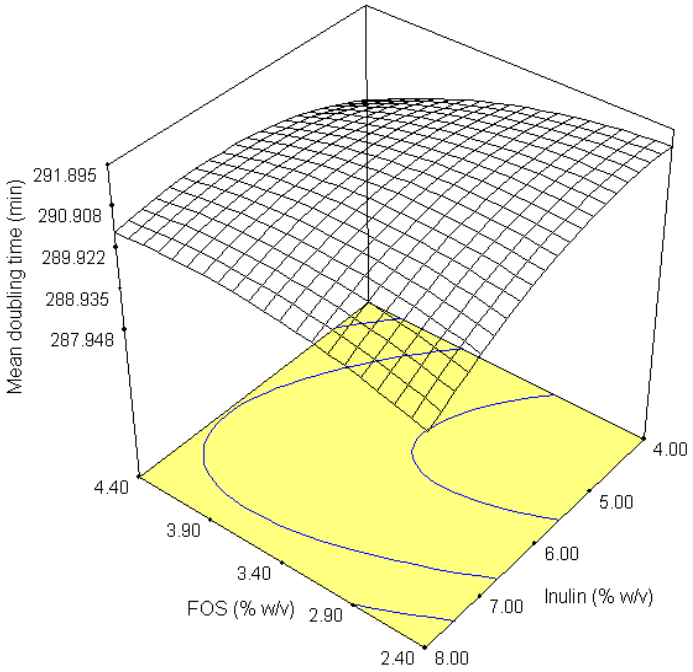

44.866
41.184
37.502
33.820
30.138
Growth (%)
3.20
2.70
2.20
1.70
1.20
Inoculum size (% w/v)
4.00
5.00
6.00
7.00
8.00
Inulin (% w/v)

(B)

Figure 4.

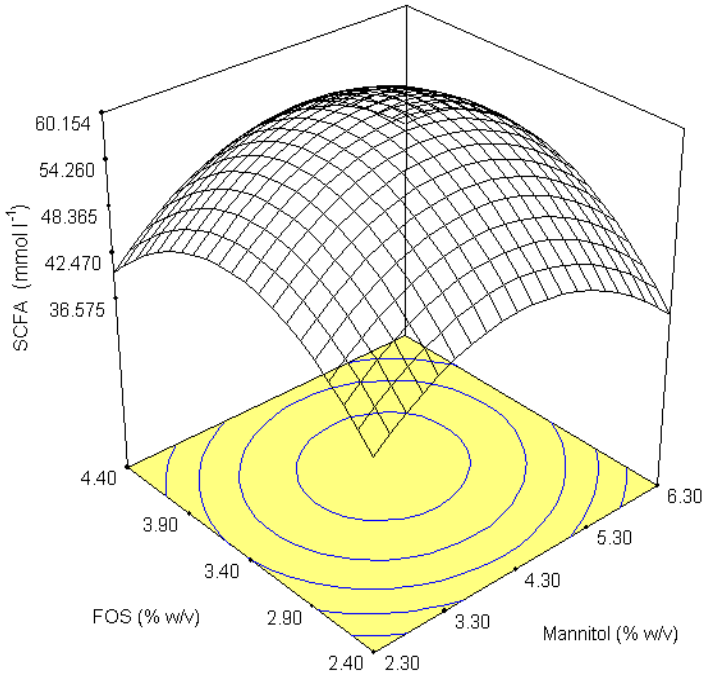

Mean doubling time (min)
291.817
290.763
289.710
288.656
287.602
3.20
2.70
2.20
1.70
1.20
Inoculum size (% w/v)
2.40
2.90
3.40
3.90
4.40
FOS (% w/v)

(A)

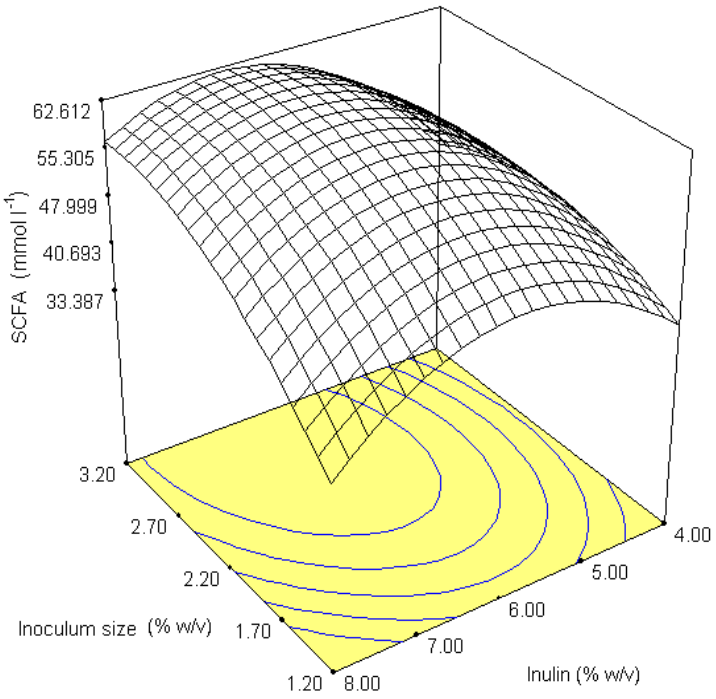

Mean doubling time (min)
291.895
290.908
289.922
288.935
287.948
4.40
3.90
3.40
2.90
2.40
FOS (% w/v)
4.00
5.00
6.00
7.00
8.00
Inulin (% w/v)

(B)

Figure 5.


60.154
54.260
48.365
42.470
36.575
SCFA (mmol l-1)
4.40
3.90
3.40
2.90
2.40
FOS (% w/v)
2.30
3.30
4.30
5.30
6.30
Mannitol (% w/v)

(A)


62.612
55.305
47.999
40.693
33.387
SCFA (mmol l-1)
3.20
2.70
2.20
1.70
1.20
Inoculum size (% w/v)
8.00
7.00
6.00
5.00
4.00
Inulin (% w/v)

(B)

Figure 6.