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Original Article

THE PREBIOTIC INFLUENCE OF INULIN ON GROWTH RATE AND ANTIBIOTIC SENSITIVITY OF *LACTOBACILLUS CASEI*

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ABSTRACT

Objective: This research study is focused on the prebiotic effect of inulin on the antibiotic sensitivity of *Lactobacillus casei* and on the determination of functionality of specific growth rate (μ) of the probiotic bacteria on the concentrations of lactose (C_L = 10-30 g/l) and inulin (C_I = 0.164-0.625 g/l) along with the optimization of growth condition through Response Surface Methodology (RSM).

Methods: The sensitivity of *Lactobacillus casei* towards norfloxacin was determined using well diffusion method. Using the initial values of μ (h^{-1}) of *Lactobacillus casei* at different values of C_L (g/l) and C_I (g/l), the functionality of μ on the concentrations of the carbon sources have been derived, and the optimum condition has been identified.

Results: Although *Lactobacillus casei* is sensitive to norfloxacin, resistance is developed in the presence of inulin. Quadratic model equation $\mu = 0.83+0.054*C_L-0.035*C_I-0.049*C_L*C_I-0.29*C_L^2-0.33*C_I^2$ is valid and the optimum value of specific growth rate is 0.8285 h^{-1} at C_L = 20 g/l and C_I = 0.32 g/l.

Conclusion: The interesting observation of the development of antibiotic resistance of *Lactobacillus casei* in the presence of inulin suggests that the intake of probiotic *Lactobacillus casei*, may be done along with prebiotic inulin when a patient is treated with antibiotics like norfloxacin. Moreover, the model equation correlating the functionality of growth rate of *Lactobacillus casei* on lactose and inulin will be helpful in fortifying the probiotic milk products and drugs with prebiotics like inulin.

Keywords: *Lactobacillus casei*, Prebiotic, Inulin, Antibiotic sensitivity, Statistical growth model, Optimization of specific growth rate, Response Surface Methodology

INTRODUCTION

Synergistic combinations of probiotic bacteria and prebiotic carbohydrates are new concepts in food processing [1]. Prebiotic bio-molecules enhance the growth rate of probiotics which in turn act against pathogenic bacteria through the secretion of bacteriocin [2]. Thus, prebiotics may be combined with dairy products namely yoghurt etc. which contain an array of probiotic bacteria [3]. Among different probiotics, inulin, a fructan consisting of glycosidic bonds like fructosyl-fructose with a terminal glucose unit is one of the most popular one [4-7]. Ideally, the prebiotic molecule should not support the growth of pathogens. Although the synbiotic combinations of suitable probiotic-prebiotic pairs are expected to be beneficial for human health, the assessment should be made about the sensitivity of the combinations of broad spectrum antibiotics. The genus *Lactobacillus* (probiotic bacteria) is inherently resistant to tetracycline, vancomycin, erythromycin, streptomycin, clindamycin, gentamicin and oxacillin, but, there may exist some broad spectrum antibiotics against which probiotic strains are susceptible [8].

The combination with prebiotic may also influence the sensitivity of probiotics. No such study elucidating this fact is reported in the literature. Therefore, addressing all these issues regarding the application of synbiotics in food processing, the following novel objectives have been set in the present research by selecting the combination of *Lactobacillus casei (L. casei)* and inulin as the probiotic bacteria and prebiotic biomolecule respectively. These are determination of the sensitivity of *Lactobacillus casei* against a broad spectrum antibiotic, namely, norfloxacin with and without inulin; determination of a statistical growth model; optimization of the growth of *Lactobacillus casei* against the concentration of inulin and lactose using Response Surface Methodology. The functionality of specific growth rate of *Lactobacillus casei* on the concentration of carbohydrate sources, determined for the first time in this report, is very important for the overall production of the microorganism as well as for the metabolic products generated during growth.

MATERIALS AND METHODS

Chemicals

Beef extract (10 g/l) (Merck, India), Yeast extract (5 g/l), Peptone (10 g/l) (Himedia, India), Sodium acetate (5 g/l) (Himedia, India), dipotassium hydrogen phosphate (2 g/l) (Himedia, India), Tri-ammonium citrate (2 g/l) (Himedia, India), Magnesium sulphate (0.05 g/l) (Himedia, India), Manganese sulphate (0.05 g/l) (Himedia, India) and Lactose (20 g/l) (Merck, India) were used in the present research study. These are the composition of Modified de-Man Rogosa Sharp (MMRS) medium. Glucose is used as carbon source for standard de-Man Rogosa Sharp (MRS) medium.

Microorganisms

Lactobacillus casei (2651 1951 RPK) culture purchased from NCIM, Pune were used.

Prebiotic

Food grade commercial inulin purchased from Himedia, India.

Pre-adaptation of culture

Adaptation of the strain to Modified de-Man Rogosa Sharp (MMRS) medium containing a high concentration of lactose (50 g/l) and inulin (50 g/l) was performed by repetitive subculturing for three times. The pre-culture process was conducted in an incubator at 37 °C using 250 mL Erlenmeyer flasks for 18 h, based on sufficient growth ($5x10^{10}$cfu/ml).

The cell from the last adaptation experiment was stored at 4°C for use in further experiments. The pre-adaptation is needed to activate the culture at the high level of concentrations of lactose and inulin exceeding the upper limits of present concentration ranges for the two carbohydrate sources and to maintain the culture in the exponential phase.

Kinetic study

Batch experiments were conducted to determine the kinetics of growth of *Lactobacillus casei* using both carbohydrate sources, namely inulin and lactose simultaneously by varying the initial concentration of lactose, i.e., 10 g/l, 20 g/l, 30 g/l at each initial inulin concentration of 0.00 g/l, 0.164 g/l, 0.32 g/l and 0.63 g/l.

Optimization study

The multivariate functionality of μ_{ave} (average specific growth rate) on two independent variables has been determined by conducting 13 batch mode experiments using the Central Composite Design (CCD) technique to set the experimental conditions.

Experimental design and optimization

The Face-centered Central Composite Design (FCCD) was created by entering the factors viz. the concentration of lactose and concentration of inulin in terms of ±1 levels to perform RSM using Design-Expert 8.1 (Stat-Ease, Inc., Minneapolis, USA). The experiments were conducted randomly to avoid systematic biasness. Accordingly, a design layout was created using 13 experimental runs with six center points. In a typical experimental run, all the operating variables were pre-set at a predetermined design value as per the experimental design layout shown in table 1. In order to investigate the effects of individual parameters as well as their interactive effects on the response variable, a general second order polynomial response surface model was selected and is expressed by Eq. (1):

$$Y_k = b_{k0} + \sum_{i=1} b_{ki}X_i + \sum_{i=1} b_{kii}X_i^2 + \sum_{i=1}\sum_{j=1} b_{kij}X_iX_j \text{---------------} (1)$$

Where, Y_k is response variable, b_{k0} is a constant intercept, b_{ki}, b_{kii} and b_{ij} are the linear, quadratic and interaction regression coefficients respectively. X_i and X_j represent the coded values of the process variables (factors). The regression Eq. (2) was considered for multiple simultaneous optimizations in order to maximize Y_k using the numerical optimization program of the same Design Expert software.

Antibiotic sensitivity

The sensitivity of the selected microorganisms, *Lactobacillus casei* towards a common antibiotic namely, norfloxacin was also tested using well diffusion method. MRS agar plate containing 20 g/l glucose and MMRS agar plate containing 20 g/l glucose and commercial inulin each was prepared. In each plate 0.1 g norfloxacin was applied in the well. The *Lactobacillus casei* cell culture was spread with a glass spreader. The plates were kept in an incubator at 37 °C for 24 h.

Scanning electron microscopy (SEM)

SEM analysis of 24 h batch culture of *Lactobacillus casei* using two types of MMRS media, one containing 20 g/l lactose and the other containing 20 g/l lactose and 20 g/l commercial inulin, were used to observe the morphological changes of the bacterial cell.

RESULTS AND DISCUSSION

Growth pattern of *Lactobacillus casei* in presence of inulin

Time histories of cell concentration have been plotted in fig.1 with inulin (0-0.624 g/l) as a parameter at initial lactose concentration of 20 g/l. From the analysis of the figure, it is evident that the cell concentration at any reaction time increase with the increase of inulin concentration and passes through the maximum at the inulin concentration of 0.32 g/l. Same trends have also been obtained for other concentrations of lactose (not shown).

From fig.1 it is evident that at 0.624 g/l inulin concentration specific growth rate of *Lactobacillus casei* is lower than at 0.32 g/l inulin concentration due to substrate inhibition. As inulin is a heavier molecule, even the enzymatic conversion to its monomers is slow and hence there is an accumulation of inulin on outer cell wall at high concentration levels. Due to the formation of a thick layer, a mass transfer limitation may creep into the system.

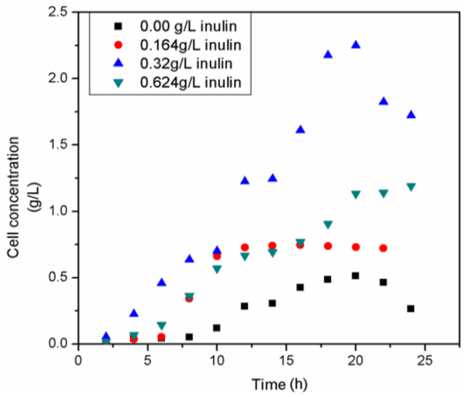

Fig. 1: Growth curves of *Lactobacillus casei* for 20 g/l lactose concentration using concentrations of inulin as a parameter. ■: without inulin, ●:0.164 g/l inulin, ▲: 0.32 g/l inulin ▼ :0.625 g/l inulin

Dependence of specific growth rate (μ) on concentrations of lactose and inulin

According to the standard MRS medium composition for lactic acid bacteria, carbohydrate concentration is used 20 g/l. Under this study lactose was used as the carbohydrate source. In order to carry out the optimization of specific growth rate (μ) through Response Surface Methodology, the range of concentration of lactose (C_L= 10-30 g/l) was chosen around the standard carbohydrate concentration value (20 g/l) of MRS medium. From preliminary observation, it was seen that inulin has inhibitory effect at high concentrations and hence a low concentration range was chosen for inulin under the present study. The values of μ obtained at different values of concentration of lactose (C_L) and concentration of inulin (C_I) have been shown in table 1.

In accordance with the statistical analysis model fit summary (table 2), a quadratic model was selected as the best fitted with lower standard deviation (0.060) and lower PRESS value (0.18), predicted residuals sum of squares (1.27), higher adjusted R^2 (0.9666), regression coefficient (0.9108), predicted R^2 regression coefficient (0.8613) and adequate precision in comparison to linear and 2FI, i.e., Two-Factor Interaction model. The data obtained from ANOVA, "analysis of variance" (table 3), for quadratic model shows an insignificant lack of fit (sum of squares = 1.27>0.05), larger F value (Fischer test value) (70.39), high R^2value (0.9805) and low C. V., i. e, "coefficient of variance" value (0.89). The model equation showing the dependence of specific growth rate simultaneously on initial concentration of lactose and inulin is as follows:

$$\mu = 0.83+0.054*C_L-0.035*C_I-0.049*C_L*C_I-0.29*C_L2-0.33*C_I^2 \text{--------------} (2)$$

Where C_L and C_I are the concentrations of lactose and inulin and μ (R1) is specific growth rate. From Eq. 2, it is evident that the factors C_L, C_I and C_L^2 affected the change in weight of the sample positively.

Optimization

In order to understand the effect of lactose concentration and inulin concentration on specific cell growth rate, the result obtained by response surface methodology technique have been presented in three-dimensional plots shown in fig.2a. It is observed that the maximum specific growth rate of *Lactobacillus casei* is obtained at 20 g/l of lactose concentration and 0.32 g/l of inulin concentration. The contour plot shown in fig.2b clearly confirms the optimum values of the response variables. Solutions for optimal conditions of specific growth rate are shown in table 4.

Effect of inulin on antibiotic sensitivity

The photographs of petri-plates of *Lactobacillus casei*, used for the determination of antibiotic (norfloxacin) sensitivity through well diffusion technique with and without inulin are shown in fig.3a and fig.3b. By the measurement of zone of inhibition it is clear that while it measures 0.055 m in absence of inulin, no inhibition zone is observed in the presence of inulin. Thus, it is revealed that while the strain of *Lactobacillus casei* used in the present investigation is inherently susceptible towards norfloxacin, resistance to the antibiotic is developed in the presence of inulin. The results signify that norfloxacin act against the *Lactobacillus casei* strain used under the present investigation. The antibacterial effect of norfloxacin, a fluoroquinolone, may be through the inhibitory action on the enzyme DNA gyrase, essential for replication, repair and transcription of DNA and topoisomerase essential for entangling DNA of the bacteria under attack [9].

However, the exact mechanism may be determined by further investigation. The development of resistance in the presence of inulin may be due to the formation of a coating of inulin around the cell wall as observed (fig. 3b) in the case of growth of the same microorganism on lactose and inulin.

Table 1: Experimental design matrix

S. No.	C_L g/l	C_I g/l	C_L g/l	C_I g/l	Response (μ) (h^{-1})
1	20.00	0.32	0	0	0.8285
2	30.00	0.62	+1	+1	0.092
3	10.00	0.62	-1	+1	0.1823
4	20.00	0.75	0	+1	0.151
5	20.00	0.13	0	-1	0.2173
6	5.9	0.32	-1	0	0.12
7	20.00	0.31	0	0	0.8285
8	30.00	0.00	+1	-1	0.283
9	20.00	0.32	0	0	0.8285
10	10.00	0.00	-1	-1	0.1787
11	20.00	0.32	0	0	0.8285
12	20.00	0.32	0	0	0.8285
13	30.41	0.32	+1	0	0.4143

Here, C_L= Concentration of lactose, C_I= Concentration of inulin, μ = Specific growth rate.

Table 2: Model Fit summary statistics for final specific growth rate

Std. Dev.	0.060
Mean	0.44
C. V.%	13.38
PRESS	0.18
R-Squared	0.9805
AdjR Squared	0.9666
PredR-Squared	17.684

Here, C. V. = Coefficient of variance, The Analysis of Variance (ANOVA) table is shown in table 3.

Table 3: Analysis of variance (ANOVA) for response surface quadratic model

Source	Sum of squars	df	Mean square	F value	p-value Prob>F
Model	1.25	5	0.25	70.39	<0.001(significant)
C_L	0.023	1	0.023	6.53	0.0378
C_I	9.882E-003	1	9.882E-003	2.79	0.1387
C_LC_I	9.467E-003	1	9.467E-003	2.67	0.1460
C_L2	*0.59*	1	0.59	166.47	<0.0001
C_I2	*0.77*	1	0.77	217.31	<0.0001
Residual	0.025	7	3.541E-003		
Lack of Fit	0.025	3	8.262E-003		
Pure Error	0.000	4	0.000		
Cor total	1.27	12			

Here, F value= Fischer test value, p-value= lack of fit value

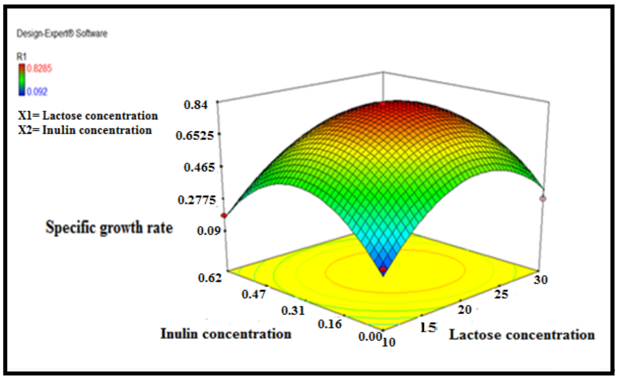

Fig. 2a: Three-dimensional plots for optimization of specific growth rate of *Lactobacillus casei*. Here R1= Response (μ or specific growth rate)

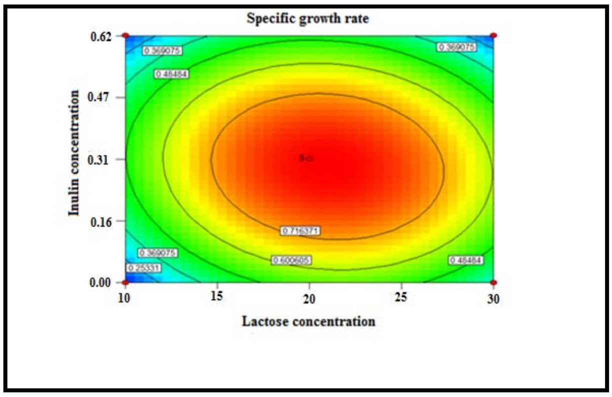

Fig. 2b: Contour plot for optimization of specific growth rate of *Lactobacillus casei*

Table 4: Solutions for optimal conditions of specific growth rate

Number	Lactose concentration (C_L)	Inulin concentration (C_I)	Desirability
1	20.00	0.32	1.0 (selected)
2	10.00	0.62	0.909
3	10.00	0.00	0.909

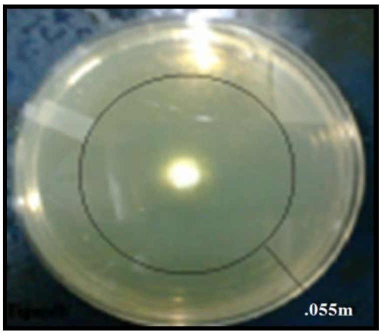

Fig. 3a: Effect of norfloxacin on *Lactobacillus casei* in de-Man Rogosa Sharp (MRS) agar plate. The marked area indicates the zone of inhibition (.055 m)

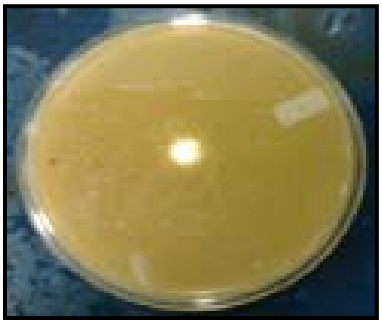

Fig. 3b: Effect of norfloxacin on *Lactobacillus casei* in Modified de-Man Rogosa Sharp (MMRS) agar plate in presence of inulin

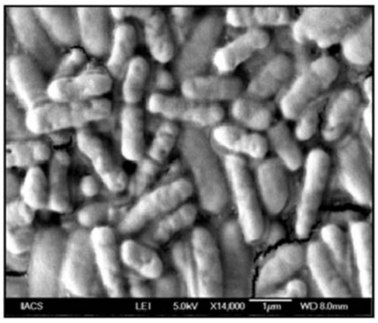

Fig. 4a: SEM image of *Lactobacillus casei* cell without inulin

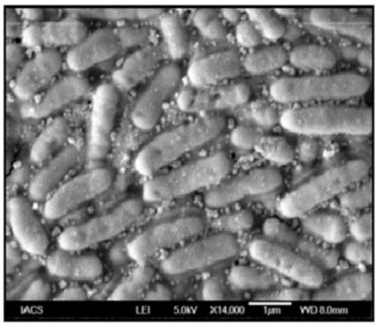

Fig. 4b: SEM image of *Lactobacillus casei* cell in presence of inulin

Scanning electron microscopy image

The SEM images (fig. 4a and 4b) of *Lactobacillus casei* grown on lactose and on lactose-inulin mixture respectively reveal that in the presence of inulin, there is a formation of a distinct layer of inulin over the cell wall. This may be due to the fact that inulin, being a large molecule cannot be transported inside the cells, and they are decomposed enzymatically to glucose and fructose which are in turn assimilated by *Lactobacillus casei* [10].

CONCLUSION

From the experimental findings, it is ascertained that the presence of inulin imparts resistance in *Lactobacillus casei* against norfloxacin, a broad spectrum antibiotic. Thus, inulin may be mixed with dairy products using *Lactobacillus casei* to avoid the destruction of probiotic cells in the human body by the antibiotic. From response surface methodology, it is observed that the specific growth rate is maximum (0.8285 h^{-1}) at concentrations of lactose and inulin of 20 g/l and 0.32 g/l respectively.

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CONFLICT OF INTERESTS

Declare none

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