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Shilpi Gupta
Technological University Dublin, shilpi.19may@gmail.com

Sabrina Cox
Technological University Dublin, sabrina.cox@tudublin.ie

Nissreen Abu-Ghannam
Technological University Dublin, nissreen.abughannam@tudublin.ie

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Process optimization for the development of a functional beverage based on lactic acid fermentation of oats

Shilpi Gupta, Sabrina Cox, Nissreen Abu-Ghannam*

School of Food Science and Environmental Health, Dublin Institute of Technology, Cathal Brugha St., Dublin 1, Ireland

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ABSTRACT

Oats (*Avena sativa*) have received considerable interest for their high content of soluble and insoluble fibre and for their high fermentability upon applying probiotic lactic acid bacteria (LAB). In the present study, Box–Behnken optimization design was used to optimize three different levels of oat, sucrose and starter culture concentration on the final viable cell population of *Lactobacillus plantarum* for the development of a fermented drink. A second-order polynomial response surface equation was developed indicating the effect of the studied variables on *L. plantarum* growth. Contour maps generated using the response surface equation showed that the experimental variables significantly affected the growth of the *L. plantarum*. The optimized factors (5.5% oats, 1.25% sugar and 5% inoculum) were then applied to prepare a fermented drink to obtain a growth of 10.4 log CFU/ml. The shelf life of the fermented drink was monitored over a period of 21 days. Physical parameters such as colour and viscosity were also measured along with the microbiological count, pH and titrable acidity. β-Glucan level remain unchanged during the fermentation and also during the entire storage period.

1. Introduction

Increasing awareness among consumers about the health benefits of high-fibre diets has emphasised the importance of developing enriched-fibre food products. Cereals contain biologically active ingredients like dietary and functional fibres, contributing to about 50% of the fibre intake in western countries [1,2]. Cereals are one of the most suitable substrates for the development of foods containing probiotic microorganisms (in most cases lactic acid bacteria or bifidobacteria) [3,4] and may also have prebiotic properties due to the presence of non-digestible components of cereal matrix. During cereal fermentations several volatile compounds are formed which contribute to a complex blend of flavours [5]. The presence of aromas such as diacetyl acetic acid and butyric acid make fermented cereal based products more appealing [6]. Among the cereals, the interest in oats as a food ingredient has increased in recent years due to different dietary fibre types, such as mixed-linked (1→3),(1→4)-β-glucan (β-glucan), arabinoxylans and cellulose, in addition to relatively high levels of protein, lipids (unsaturated fatty acids), vitamins, antioxidants and phenolic compounds [1,7]. These β-glucans have been reported to be effective in lowering the plasma cholesterol and postprandial serum glucose level [8]. In addition, β-glucan is fermented by intestinal microflora which results in the formation of short-chain fatty acids which are protective to colon mucosa (as a prebiotic) [9]. The recommended daily intake of β-glucan for achieving positive health effects is 3 g per day [10,11].

Strains of several *Lactobacillus* species, used as probiotics, have proven to exert a range of health promoting activities such as immunomodulation, enhancement of resistance against pathogens and reduction of blood cholesterol levels [12]. Growth and product formation during fermentation by microorganisms can be affected by medium composition, presence of oxygen, pH and product concentration. Thus, it is important to optimize the concentration of the nutrients in order to get maximum growth of *Lactobacillus plantarum*. The 'one-at-a-time-approach' is frequently used to obtain high yields of the desired metabolic products in a microbial system. However, this method is extremely time consuming and disregards the complex interactions among various physicochemical parameters [13]. Response surface methodology (RSM) is a collection of mathematical and statistical techniques for searching optimum conditions of factors for desirable responses, and evaluating the relative significance of several affecting factors even in the presence of complex interactions. Box–Behnken is a spherical, revolving RSM design that consists of a central point and the middle points of the edges of the cube circumscribed on the sphere. The design leads to the generation of contour plots by linear or quadratic effects of key variables and a model equation is derived that fits the experimental data to calculate the optimal response of the system.

Oats are good substrates for the growth of probiotic strains and due to the presence of non-digestible components may also serve as prebiotics [14]. However, a systematic approach is needed in

* Corresponding author. Tel.: +353 1 402 7570; fax: +353 1 878 8978.
E-mail address: nissreen.abughannam@dit.ie (N. Abu-Ghannam).

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Table 1
Level and code of independent variables, sucrose concentration, oat concentration and inoculum level used for Box–Behnken experimental design.

Independent variable	Coded symbol	Levels		
		−1	0	+1
Oat concentration (%)	X_1	4	5.5	7
Sugar concentration (%)	X_2	1	1.5	2
Inoculum level	X_3	1	5.5	10

order to identify the factors, which would facilitate the growth of LAB initially so that these findings can form a platform for fermentation of oats later with probiotic strains. Thus the present work focuses on using lactic acid bacteria to study the applicability of oats for the development of a functional drink. The effects of different oat, sucrose and starter concentrations on the growth of lactic acid bacteria, *L. plantarum*, for the development of a fermented drink were optimized by Box–Behnken designs. Thereafter, the shelf life of the fermented drink prepared from the parameters optimized by Box–Behnken design was studied by evaluating the cell viability, β-glucan, titrable acidity, colour and pH. At the same time, the level of β-glucan in the developed drink was compared to the levels recommended by FDA in an oat based functional product.

2. Materials and methods

2.1. Oat material

Odlums Healthy Heart porridge oatflakes were purchased from a local superstore in Dublin city. The oats were ground in a Russell Hobbs grinder to produce oat flour.

2.2. Culture

L. plantarum ATCC 8014 was purchased from Medical Supply Company, Dublin, Ireland. The culture was maintained at −70 °C in 20% glycerol stocks and grown in Man Rossa de Sharpe (MRS; Scharlau Chemie, Spain) broth at 37 °C.

2.3. Box–Behnken design experiments

RSM was applied to investigate the influence of oat, sugar and the inoculum concentrations on the growth of *L. plantarum* (Table 1) using Design Expert (Version 5.0.9) software (Stat-Ease Corporation, USA). Box–Behnken design consists of three interlocking 2^2 factorial designs having points, all lying on the surface of a sphere surrounding the centre of the design [15]. In order to statistically optimize the medium components and evaluate main effects, interaction effects and quadratic effects of the three factors on the growth of *L. plantarum*, a design with three factors and three levels including five replicates at the centre point was used (Table 2). The Box–Behnken design was specifically selected since it requires fewer runs than a central composite design in cases of three or four variables. This cubic design is characterized by set of points lying at the midpoint of each edge of a multidimensional cube and centre point replicates ($n = 5$) whereas the 'missing corners' help the experimenter to avoid the combined factor extremes.

The non-linear computer-generated quadratic model is given as

$$Y = \beta_0 + \sum_{i=0}^{3} \beta_i X_i + \sum_{j=0}^{3} \beta_{ii} X_i^2 + \sum_{i=0}^{3} \sum_{j=0}^{3} \beta_{ij} X_i X_j \quad (1)$$

where Y is the measured response associated with each factor level combination; β_0 is an intercept; β_i is the regression co-efficient computed from the observed experimental values of Y; and X_i is the coded level of independent variables. The terms X_i X_j and X_i^2 represent the interaction and quadratic terms, respectively. The independent variables selected are shown in Table 1 along with their low, medium, and high levels.

2.4. Fermentation

2.4.1. Inoculum development

Overnight grown cells of *L. plantarum* were used as inoculum such that a cell concentration of 4×10^8, 2.2×10^9 and 4×10^9 colony forming units (CFU)/ml could be attained at a 1, 5.5 and 10% in the fermented drink.

2.4.2. Fermentation of oat based beverage

To prepare the cereal based drink, the oat substrate and the sugar were mixed as per the nutrient illustration given in Table 2. This was then heated at 95 °C in a thermostatically controlled water bath for 10 min with stirring at regular intervals. The samples were cooled to 37 °C before the addition of the required amounts of starter culture (Table 2). The fermentation was carried out in 250 ml Erlenmeyer flasks containing 50 ml oat beverage and incubated at 37 °C with orbital shaking at 150 rpm for 8 h and analysis of the beverage was carried out for log CFU/ml and β-glucan.

Validity of the optimized results was checked by growing the culture under optimized conditions for 8 h and checking for the log CFU/ml.

Table 2
Box–Behnken design with experimental and predicted values of *L. plantarum*.

Expt no.	Oat (%)	Sugar (%)	Inoculum (%)	*L. plantarum* (log CFU/ml)	
				Experimental	Predictied
1	4	1	5.5	9.78	9.87
2	7	1	5.5	9.8	9.79
3	4	2	5.5	10.38	10.39
4	7	2	5.5	9.86	9.77
5	4	1.5	1	10.16	10.10
6	7	1.5	1	9.79	9.82
7	4	1.5	10	10.25	10.22
8	7	1.5	10	9.75	9.81
9	5.5	1	1	10.3	10.28
10	5.5	2	1	10.49	10.55
11	5.5	1	10	10.41	10.35
12	5.5	2	10	10.56	10.58
13	5.5	1.5	5.5	10.5	10.41
14	5.5	1.5	5.5	10.5	10.41
15	5.5	1.5	5.5	10.4	10.41
16	5.5	1.5	5.5	10.46	10.41
17	5.5	1.5	5.5	10.2	10.41

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2.5. Shelf life evaluation

The optimized values of oat, sugar and inoculum concentrations were then selected to carry out shelf life analysis of the fermented product. Fermentation with the optimized composition was carried out in 250 ml Duran bottles containing 50 ml media for 8 h and then the fermented beverage was refrigerated at 4 °C. One bottle of fermented beverage was withdrawn for sampling at regular intervals for 21 days and analyzed for pH, titrable acidity, viable cell count, colour, viscosity and β-glucan content.

2.6. Analytical methods

2.6.1. Viable cell enumeration

Enumeration of viable cells of *L. plantarum* ATCC 8014 was performed by estimation of colony forming unit number on MRS-agar plates (medium pH 5.7) after incubation at 37 °C for 48 h.

2.6.2. Titrable acidity and pH

Titrable acidity was determined by titrating 10 ml of each sample with 0.1 N NaOH, using phenolphthalein as an indicator. An Orion 520A (AGB Scientific Limited) pH meter was used for the measurement of pH.

2.6.3. Estimation of β-glucan

The β-glucan content was evaluated using an enzymatic kit according to the manufacturer's instructions (Megazyme International Ireland Ltd.).

2.6.4. Colour analysis

The Hunter Lab co-ordinates [L^* (lightness), a^* (red–green) and b^* (yellow–blue)] were measured by a spectrocolorimeter (ColorQuest XE, Hunter Associate Laboratory, Inc., Reaston, VA) and a CIE standard illuminant C. Hunter Lab colour meter was calibrated using black and white references. The samples were placed in a transparent glass cuvette to measure the colour parameters. The total amount of colour change in order to see the effect of heat was estimated as per Eq. (2) [16].

$$\Delta E = \sqrt{(L_0 - L)^2 + (a_0 - a)^2 + (b_0 - b)^2} \quad (2)$$

where L_0, a_0 and b_0 would be the colour parameters of oat drink. This total change takes into account the change in each of the colours described by the L^*, a^* and b^* parameters.

2.6.5. Viscosity analysis

Viscosity measurements were taken using a Brookfield viscometer model RVT. Approximately 50 ml sample was placed in a beaker and the viscosity measurements were taken using spindle 6 of the viscometer at 10 rpm. The viscosity readings were calculated using the Brookfield finder factor to convert the RVT viscosity readings to units of centipoise (cP).

2.6.6. Detection of Enterobacteria and moulds

Microbiological analyses of the fermented oat drink were carried out at the end of 8 h to check for the presence of *Enterobacteria* and moulds. Serial dilutions were prepared in Maximum Recovery Diluent (MRD) and 100 μl of the broth was spread on the following media: (1) Eosin Methylene Blue (EMB; Scharlau Chemie, Spain) agar for enumeration of *Enterobacteria* such as *E. coli*; (2) Malt Extract Agar (MEA; Scharlau Chemie, Spain) for the presence of moulds.

Table 3
Co-efficients of the response function for predicting *L. plantarum* log CFU/ml from Eq. (3) by regression analysis and their significance values obtained by ANOVA.

Term	Regression co-efficient	*t*-Value	*P*-value
Intercept	10.41		
X_1	−0.17	−4.44	0.0022
X_2	0.13	3.24	0.0119
X_3	0.029	0.75	0.4776
$X_1 \times X_1$	−0.45	−8.55	<0.0001
$X_2 \times X_2$	−0.00225	−0.042	0.9673
$X_3 \times X_3$	0.030	0.57	0.5852
$X_1 \times X_2$	−0.14	−2.47	0.0385
$X_1 \times X_3$	−0.032	−0.60	0.5680

2.7. Statistical analysis

All the experiments were carried out in triplicate and replicated at least twice. Results are expressed as average ± standard deviation (SD). Data from the Box–Behnken factorial design were subjected to a second-order multiple regression analysis using least-squares regression to obtain the parameter estimated for the mathematical model. The regression analysis and analysis of variance (ANOVA) for Box–Behnken design were carried out using the Design Expert software. Analysis of variance (ANOVA) for other experiments was done using the STATGRAPHICS Centurion XV (StatPoint Technologies, Inc., Warrenton, VA). Values of $P < 0.05$ were considered as statistically significant.

3. Results and discussion

3.1. Statistical analysis of results obtained by experimental design

Development of a new functional food demands several important factors to be considered; the most important of which are the bioactive components. For a fermented oat based product the important bioactives would be the final viable cell population and the β-glucan level. Thus, the fermentation parameters were optimized in order to attain a high growth of the LAB. Since the main ingredients of the oat based beverage were oats, sugar and inoculum, these were the factors that were selected for optimization. For all the experiments proposed by Box–Behnken design the fermentation was carried out over a period of 8 h as short fermentation times are preferable in order to minimize the risk of contamination. Moreover, a preliminary experiment was carried out wherein 5.5% oat substrate and 1% sucrose were dissolved in 50 ml deionized water. The oat beverage was heated at 95 °C in a thermostatically controlled water bath for 10 min and inoculated with 5% inoculum. The viable cell population was monitored for 10 h. The viable cells increased from an initial level of 1.8×10^9 to 3.02×10^{10} over the 8 h fermentation period after which it started reducing.

The 17 experiments proposed by the Box–Behnken design with three factors and three levels (Table 1) including five replicates at the centre point were used for fitting a second-order response surface. The five centre point runs provided a measure of process stability and inherent variability. The effect of different concentrations of the three factors on the growth of *L. plantarum* is shown in Table 2. Experimental results for *L. plantarum* growth were fitted to a full quadratic second-order polynomial equation by applying multiple regression analysis (Eq. (3)) and the regression co-efficients obtained to predict polynomial model for *L. plantarum* growth are summarized in Table 3.

$$\text{Log CFU/ml} = +10.41 - 0.17X_1 + 0.13X_2 - 0.029X_3 - 0.045X_1^2 - 0.0022X_2^2 + 0.03X_3^2 - 0.14X_1X_2 - 0.032X_1X_3 - 0.01X_2X_3 \quad (3)$$

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Table 4
Analysis of variance and regression analysis for *L. plantarum* growth.

Source	Sum of squares	DF	Mean square	*F*-value	Prob > *F*
Model	1.32	9	0.15	10.8	0.0024
Residual	0.095	7	0.014		
Lack of fit	0.032	3	0.011	0.68	0.609
Pure error	0.063	4	0.016		
Total	1.41	16			

DF: degrees of freedom; *F*: variance ratio; *P*: probability.

when the values of $X_1 - X_3$ were substituted in the above equation, the predicted log CFU/ml for *L. plantarum* (Y) was obtained. A comparison of predicted and experimentally obtained values can be seen in Table 2.

In order to determine the significance of the quadratic model, ANOVA analysis was conducted. The *P*-values were used as a tool to check the significance of each co-efficient, which also indicated the interaction strength of each parameter. The smaller the *P*-values are, the bigger the significance of the corresponding co-efficient [17]. Corresponding *P*-values suggest that, among the test variables used in this study, X_1 (oat concentration), X_2 (sugar concentration), $(X_1)^2$ (oat × oat) and X_1X_2 (oat × sugar) are significant model terms with *P*-values less than 0.05. Therefore, they can act as limiting nutrients and a small variation in their concentrations will alter the growth of *L. plantarum* to a considerable extent. Other terms, such as X_3 (inoculum concentration), $(X_2)^2$ (sugar × sugar), $(X_3)^2$ (inoculums × inoculum) are insignificant. The goodness of fit of the model was examined by *F*-test and the determination co-efficient R^2. The greater the *F*-value is from unity, the more certain it is that the factors explain adequately the variation in the data around its mean, and the estimated factor effects are real. The analysis of variance (Table 4) showed that this regression model was highly significant ($P < 0.01$) as is evident from the Fisher, *F*-test (F_{model}, the ratio of mean square regression to mean square residual is 10.8) and has a very low probability value [$(P_{model} > F) = 0.0024$]. The value of 0.609 for lack of fit implies that it is not significant comparing to the pure error and that the model equation was adequate for predicting the *L. plantarum* growth. The fitness of the model was further confirmed by a satisfactory value of determination co-efficient, which was calculated to be 0.9328, indicating that 93.28% of the variability in the response could be predicted by the model. Furthermore, the predicted log CFU/ml by the final quadratic model, along with the corresponding values observed is given in Table 2. The agreement between the log CFU/ml predicted by the model and the experimental data is very strong as shown by a high value of correlation co-efficient, R (0.9658). The low co-efficient of variation (CV = 1.14%) suggested that the model was precise and reliable. The residuals were examined to check the adequacy of the model. The residuals were plotted against the predicted value as shown in Fig. 1. The "horizontal band" indicated no abnormality, i.e. no unusual behaviour [18], confirming the adequacy of the regression model.

3.2. Optimization of the components of the oat based drink

The three-dimensional response surfaces were generated to study the interaction among the three factors tested and to visualize the combined effects of factors on the growth of LAB in oat based media (Fig. 2). The effect of interaction of the three components on growth of *L. plantarum* was tested by contour plots for all possible combinations of factors, keeping one factor constant at a time. The interactions between the variables can be inferred from the shapes of the contour plots [19]. Circular contour plots indicate that the interactions between the variables are negligible. In contrast, elliptical ones indicate the evidence of the interactions (Fig. 2).

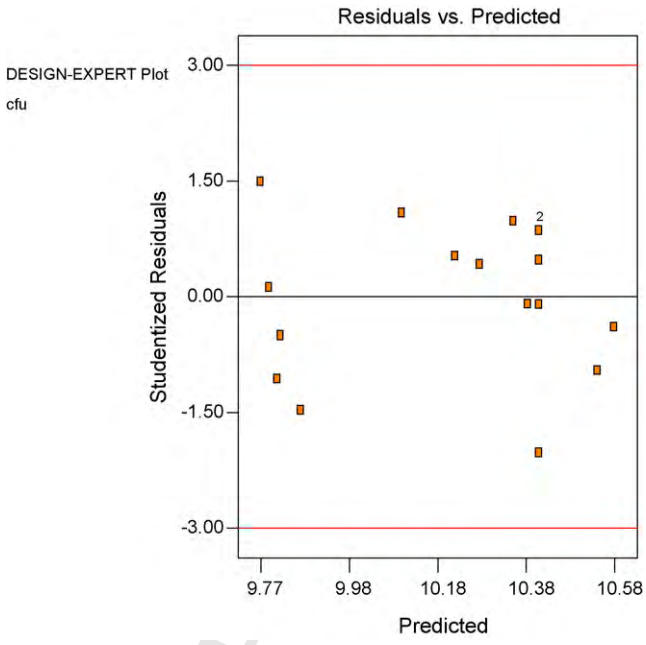

Fig. 1. Plot of studentized residuals against the predicted value for *L. plantarum*.

Concentration of oats and sugar are the dominant factors which control the biosynthesis of *L. plantarum*. Hence, a strong interaction between them for growth of bacteria is inevitable.

The optimum production of *L. plantarum* and the optimized values of selected variables were obtained by solving the regression equation (3). The analysis determined that the maximum response was 10.4 log CFU/ml with the corresponding optimal values of the test variables in uncoded units as 5.5% oats, 1.25% sugar and 5% inoculum. All the optimal points were located inside the experimental region and varied around their centre points to different extent. The verification of the results using the optimized concentrations was accomplished by carrying out the fermentation for a period of 8 h. A log CFU/ml of 10.4 was obtained showing the validity of the predicted model for the growth of *L. plantarum*.

The growth of LAB in the oat based beverage was comparable with the results obtained from previous workers in oats and other cereals. Kedia et al. [20] reported a maximum growth of *L. plantarum*

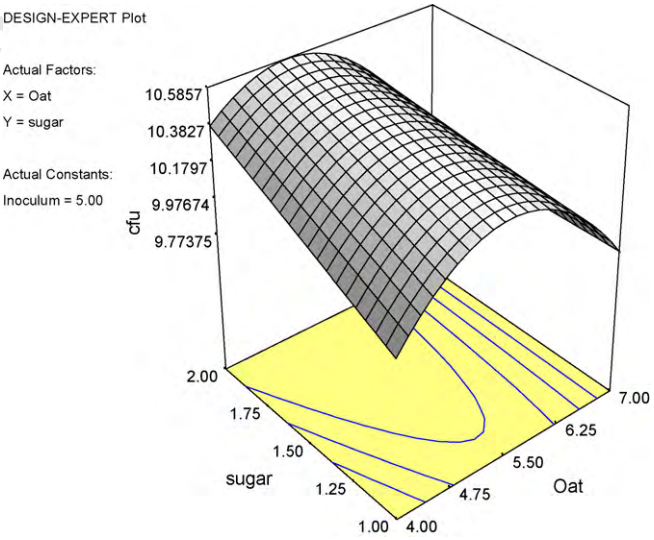

Fig. 2. Contour plot showing the effect of oat and sugar concentration (%) on the growth of *L. plantarum*.

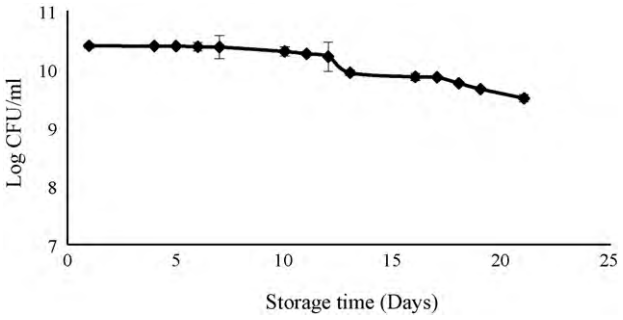

Fig. 3. Effect of storage time at 4 °C on the viable cell count of oat based drink.

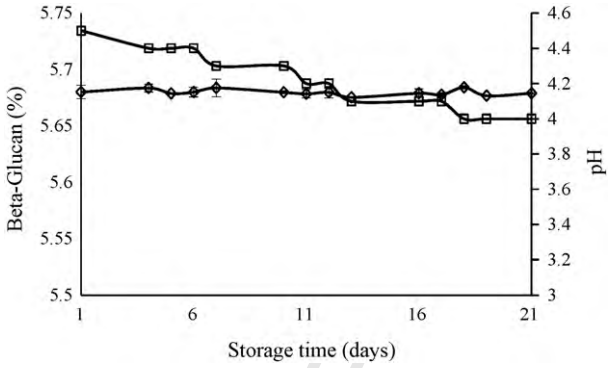

Fig. 4. Effect of storage time on the β-glucan level (◊) and pH (□) of oat based drink.

of 9.16 $\log_{10}$ CFU/ml in white oat flour. Patel et al. [21] reported a maximum growth of *L. plantarum* in malt, barley and wheat of 9.15 $\log_{10}$ CFU/ml, 8.46 $\log_{10}$ CFU/ml and 8.39 $\log_{10}$ CFU/ml, respectively. Angelov et al. [22] and Mårtensson et al. [23] have also reported a successful production of beverage from oats. The results in the present study are higher than those reported by Helland et al. [24] who studied the growth and metabolism of four probiotic strains in milk- and water-based puddings containing both maize and rice flour. In their study all the strains showed good growth and survival in milk-based puddings (8–9.1 log CFU g^{-1}), but *L. rhamnosus* GG was the only strain with an acceptable survival in water-based puddings (8 log CFU g^{-1}). Arora et al. [25] also reported only 8.88 CFU g^{-1} of *L. acidophilus* in the fermented food mixture formulated from germinated barley flour, whey powder and tomato pulp (2:1:1 w/w).

It is of a great concern that the β-glucan level in the products should be unaffected throughout the fermentation process and storage of the final product as the β-glucan is the main active component for the cholesterol-lowering properties recognized in oats. It has been reported that changes in properties of β-glucan may arise from certain processing conditions such as shearing, damage due to mechanical processing and excessive heat treatment [26]. Hence, in order to see the effect of heating (95 °C for 10 min) or fermentation, the content of β-glucan was estimated for all the combinations given by Box–Behnken design. Samples were analyzed before and after sample preparation and fermentation and were statistically analyzed by two-way ANOVA. Results showed that were no significant ($P < 0.05$) difference in the β-glucan content before and after the different processing steps. The average concentration of β-glucan in fermented drink containing 4, 5.5 and 7% oats varied from 5.65 to 5.68%. In contrast to the results in the present study, Lambo et al. [1] reported a reduction in the β-glucan level after 20 h of fermentation with lactic acid bacteria. Mårtensson et al. [23] also reported no change in the β-glucan level after fermentation with *Lactobacillus reuteri* and *Lactobacillus acidophilus*. However, a reduction in the β-glucan level was reported after fermentation with *Bifidobacterium bifidum*.

3.3. Shelf life analysis

The oat based drink was fermented for a period of 8 h and then stored at 4 °C. After the completion of fermentation samples were withdrawn to check for the presence of *Enterobacter* and moulds. There was a complete absence of any *Enterobacteria* or moulds in the fermented drink. For shelf life analysis samples were withdrawn after every 3 days. The viable cell count at the end of the 8 h fermentation period was found to be 10.4 log CFU/ml. The stability of *L. plantarum* during storage was monitored (Fig. 3) and a reduction of 0.9 log CFU/ml was seen at the end of the 21 days storage period. The reduction of 0.9 log CFU/ml was not found to be significant ($P > 0.05$). These results indicate that *L. plantarum* is capable of surviving under high acidic conditions during storage at 4 °C. High survival rates of *L. plantarum* in fermented products during storage under refrigerated conditions have been reported in earlier studies [26,27]. Mårtensson et al. [23] reported high survival of *L. reuteri* in oat based non-dairy products after 30 days of storage.

The level of β-glucan at the end of 8 h fermentation period was found to be 5.68% which remained constant throughout the 21 days storage period ($P > 0.05$) (Fig. 4). Similar results were reported by Angelov et al. [22] as well. According to FDA, the recommended level of β-glucan in a probiotic based functional drink should be 0.75 g. Hence, it is concluded that in order to get the desired level a consumption of 250 ml of the drink prepared in the present study is required.

The change in colour of the drink over 21 days was also monitored. There were slight variations in the colour over the entire period of shelf life. The change in colour decreased by a value of 0.31 which was not significant ($P > 0.05$). Thus, storage did not affect the colour of the oat based drink which is important with regards to sensory characteristics. Another important parameter is the viscosity. A drink must have suitable viscosity in order for it to be drinkable and be sensory appealing. Also, it has been reported that β-glucan causes an increase in the viscosity of the solutions [28,29]. Thus, the viscosity of the drink, prepared in the present study, with optimized parameters was measured and found to be 1000 mPa s and no significant ($P > 0.05$) change was found in the viscosity of the drink over the entire storage period.

A pH level above 4.0 is generally required for a fermented beverage throughout storage. Results from the storage period (Fig. 4) showed that the pH on day 1 was 4.5 and remained above 4.0 for 21 days. Single factor AONA analysis showed that the slight pH change observed during 21 days was not significant ($P > 0.05$). Similar results were reported by Rozada et al. [30] during the fermentation of a malt based beverage by *Bifidobacterium breve*. Helland et al. [24] reported a reduction in the pH levels to 3.4–4.4 in cereal based products after 21 days storage. The only factor that changed significantly over storage was the titrable acidity. The titrable acidity increased by 12.5% over the 3 weeks storage period which was expected due to the slight increase in the pH over the storage period.

4. Conclusion

The evaluation of cereals such as oats as a support for development of functional food is a challenge for the food industry. The present work shows the applicability of RSM, including an experimental design, regression analysis, and model generation for the optimization of an oat based beverage. An empirical model to simulate *L. plantarum* growth was developed in terms of fermentation conditions (factors) by RSM and an ANOVA test was performed which showed a good fitting of the model. Application of RSM in the development of functional foods helps in cutting

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down on time and resources for finding the optimum concentration of different components and allows better understanding of the interaction between the variables. Oats and sugar concentration were found to be the factors with the greatest influence on *L. plantarum* growth. The oat based drink was successfully fermented with the optimized parameters and found to be stable for 21 days with a reduction of less than 1 log CFU/ml. The content of β-glucan remained unchanged during the processing, fermentation and storage. Further studies are needed which should involve a sensory panel's subjective analysis to correlate with the objective results of the present study.

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