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Release of β-galactosidase by Permeabilization of Indigenously Isolated Lactobacillus acidophilus Using Lysozyme

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Enzymatic lysis, using lysozyme, has been shown in this paper to be an effective cell lysis method to release β-galactosidase from *Lactobacillus acidophilus* indigenously isolated from *Eleusine coracana*. The lytic process was developed using response surface methodology (RSM) to optimize lysozyme concentration, cell density and incubation time critical for the release of β-galactosidase. The optimized permeabilization condition (lysozyme: $33.63 \cdot 10^4$ U mL^{-1}; cell density: 4.7 % w/v on wet basis; incubation time: 10h 30min.) resulted in 1.2 fold increase in release of β-galactosidase when compared with an optimized ultrasonication process. These optimized conditions were used to determine the release constants of β-galactosidase and total protein release as a function of temperature. The enzyme and protein release constants were used further to calculate the location factor of β-galactosidase.

Key words:
Lactobacillus acidophilus, β-galactosidase, enzymatic permeabilization, response surface methodology, location factor, lysozyme

Introduction

There is a constant increase in the demand for biomolecules produced by microorganisms for a wide range of commercial applications. Although many such microbial enzymes and proteins are produced extracellularly, an equally high proportion of such potentially useful products are retained within the cells.[1–2] One such enzyme, β-galactosidase (EC 3.2.1.23), has found wide application for lactose hydrolysis in the dairy industry. We have earlier reported an indigenous strain of gram positive *Lactobacillus acidophilus* isolated from *Eleusine coracana* to be a potential producer of intracellular β-galactosidase.[3–4]

Intracellular location (periplasmic, cytoplasmic or cell-wall bound) can hinder recovery of such biomolecules and increase complications in their downstream processing. It therefore becomes necessary to select an efficient cell disruption or permeabilization method that ensures high enzyme recovery and cost-effective processing.[4] Various physical, chemical, enzymatic, or mechanical methods are available for cell disruption to release intracellular enzymes and proteins.[1,5] Widely used mechanical methods such as bead milling, high-pressure homogenization and ultrasonication cause non-specific disruption of the entire cell wall structure to small fragments and may cause product loss due to high heat generation associated with such processes.[6] Chemical and enzymatic permeabilization, on the other hand, through interaction with specific cell wall components cause selective release without generation of small cell fragments.[1,7]

Enzymatic lysis involves use of enzymes that offer advantages like high specificity in their lysis mechanism and mild reaction conditions. The permeabilization process thereby ensures minimal damage and maximum stability of the released protein with the residual cell debris being large enough to assist easy separation of the protein from the cell mass.[1,5] To achieve this, the selection of an appropriate enzyme for permeabilization plays a key role, which, in turn is largely influenced by the type of microorganism (Gram-positive or Gram-negative) and intracellular location of the desired product. Lysozyme (EC 3.2.1.17), also known as muramidase or N-acetylmuramide glycanhydrolase, are glycoside hydrolases that specifically permeabilize bacterial cell wall by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in their peptidoglycan layer. The presence of a pronounced peptidoglycan layer (50–80 % of the cell wall) and lack of the outer membrane make Gram-positive bacteria highly susceptible to permeabilization with lysozyme. Moreover, lysozyme derived from egg white is relatively cheap and currently is the only bacteri-

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olytic enzyme available commercially for large-scale application.[1,5]

A number of statistical methods are available for optimization of biological processes. Evolutionary Optimization (EVOP) was used by Saptarshi and Lele[8] to develop a disruption process that ensured high recovery of L-asparaginase from *E. caratovora*. However, Response Surface Methodology (RSM), by far has been most commonly employed for developing, improving, and optimizing various types of biochemical and biotechnological processes.[9–11] RSM is a widely used and efficient statistical tool to determine the optimal level of each factor and study interactions between each factor and their effect on product yield.[12–13] Furthermore, it is suitable for accurately describing a global optimum for exact conditions in a multifactorial system with minimum number of experiments thereby enabling cost reduction, without neglecting the interaction among the parameters.[14–15] Such a multivariate approach not only improves statistical interpretation, but also evaluates relative significance of several influencing factors even in the presence of complex interactions. The primary advantage of RSM lies in optimization of vital parameters by characterization and modeling of relationship between the independent variables, through the execution of a minimum number of well planned and designed experiments.[16–17] On account of such robustness and efficiency, Kim et al.[18] used RSM for optimization of lysis of *E. coli* in a microfluidic device by using a combination of surfactants and lysozyme. Similarly, de Faria et al.,[19] optimized the permeabilization of *Kluyveromyces lactis* by using glass beads for recovery of β-galactosidase.

In this paper, we report permeabilization optimization of *Lactobacillus acidophilus* by lysozyme for efficient release of intracellular β-galactosidase. RSM was used as a statistical tool for the permeabilization process with the objective to achieve a global optimum for maximum release of the enzyme. Process conditions that largely influenced enzyme release, namely cell density, lysozyme concentration and incubation time, were chosen in the optimization experiments. Further, kinetics of β-galactosidase release by using the optimized conditions was evaluated as a function of temperature and corresponding release constants were determined. These release constants obtained through enzymatic permeabilization were used to calculate the location factor of the enzyme. Location factor of β-galactosidase has been reported in *L. acidophilus*[4] and *Kluveromyces species*[20–21] using ultrasonication as the lysis method. However, to our knowledge, no reports are available on optimization of β-galactosidase release from *L. acidophilus* using lysozyme and calculating enzyme location factor thereafter.

Materials and methods

Maintenance of the culture and preparation of seed culture:

Indigenously isolated *Lactobacillus acidophilus* used in this study was maintained on MRS slants and sub-cultured every month. A seed culture was prepared by inoculating 100 mL MRS broth with a fresh slant (24 h old). The cells were grown at 25 °C for 18 h and harvested in sterile centrifuge tubes by centrifugation at 5000 rpm (2850 · g) at 15 °C for 15 minutes using Remi Centrifuge (Mumbai, India). The cell pellet was suspended in an appropriate amount of sterile saline so that optical density of the resulting suspension was 1.0, when measured at 660 nm wavelength on a spectrophotometer (Spectronic Genesys 5 UV-VIS spectrophotometer, Thermo Electron Corp, USA). This cell suspension was used as the seed culture.

Production of β-galactosidase:

Production of β-galactosidase by *L. acidophilus* was carried out in 250 mL Erlenmeyer flasks containing 100 mL of media comprising 10 g L^{-1} lactose, 20 g L^{-1} yeast extract, 10 g L^{-1} mycological peptone, 50 mg L^{-1} magnesium sulphate and 25 mg L^{-1} manganese sulphate procured from Himedia Labs, Mumbai, India, and 4 g L^{-1} tri-ammonium citrate, 2.5 g L^{-1} potassium acetate and 4 g L^{-1} dipotassium hydrogen phosphate procured from S. D. Fine Chemicals, Mumbai, India. The pH of the medium was adjusted to pH 6.5 before sterilization and the inoculated contents were incubated at 25 °C for 24 h under stationary conditions without mixing. The cells were then harvested by centrifugation at 5000 rpm (4420 · g) at 15 °C for 15 minutes using Beckman J2-MC Centrifuge, USA (Rotor JA 10). The harvested cells were washed twice by re-suspending the cells in phosphate buffered saline (0.9 % w v^{-1} sodium chloride in 100 mmol L^{-1} phosphate buffer; pH 7.0) followed by centrifugation at 10000 rpm (12100 · g) at 15 °C for 10 minutes using Beckman J2-MC Centrifuge, USA (Rotor JA 20).

Design of statistical experiments for cell permeabilization by lysozyme

The release of intracellular β-galactosidase was largely dependent on cell density, lysozyme concentration and incubation time of the lysozyme-cell mass mixture at constant temperature. It is crucial to select an appropriate range of the variables under study. The range selected should be wide enough to achieve a global optimum. A narrow range of the variables can result in missing the ‘global-optimum’ of the process since it may lie outside the range. Moreover, the model generated by the software,

based on the experimental runs, can give a robust prediction of experiments performed only within the range of study. Based on this, preliminary experiments were performed to obtain an in-depth knowledge of the process, enabling the selection of the best range for developing a design and eventually arriving at the global optimum for release of β-galactosidase through permeabilization of the *L. acidophilus* cell wall by lysozyme. In this experimental study, the effect of cell density was studied between 3 % and 6 % wv^{-1} on wet basis, lysozyme concentration between 10–30 U mL^{-1} and incubation time between 6–12 h. The extremes of the experimental run were calculated by the software, which are given in detail in the following section.

In order to evaluate the response of these independent variables, a central composite rotatable design (CCRD) was created using the Design Expert Software Version 6.0.10 trial version (State Ease, Minneapolis, MN). This design is a collection of mathematical and statistical techniques beneficial in providing a mathematical solution along with intricate analysis of the response as a function of the interactions between the independent variables.[17] The relation between the actual values and coded values of the variables was calculated as per the following equation:

$$x_i = \frac{X_i - X_{cp}}{\Delta X_i} \quad (1)$$

Table 1 – *Coded and uncoded values of the experimental variables*

Independent variables	Coded values				
	–1.68	–1	0	1	1.68
Lysozyme (Units mL^{-1} · 10^4)	3.18	10.00	20.00	30.00	36.82
Cell density (g % wet basis)	1.98	3.0	4.5	6.0	7.02
Incubation time (h)	3.95	6.0	9.0	12.0	14.05

where: x_i is a dimensionless value of an independent variable; X_i is the real value of an independent variable; X_{cp} is the real value of an independent variable at the center point; and ΔX_i is a step change of real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i. Based on equation 1, the coded and actual values of the variables used in this design of experiments is shown in Table 1, while the entire design of experiments, along with the β-galactosidase yield as the response obtained in each individual experimental set up, is depicted in Table 2. The relationship of the independent variables and the response were calculated by the second order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i \neq j} \beta_{iij} X_{ji} \quad (2)$$

Table 2 – *Experimental design of CCRD with β-galactosidase activity*

Std order	Lysozyme (units mL^{-1} · 10^4)	Cell density (% g wet wt)	Incubation time (h)	Experimental yield (U gDCW^{-1})	Predicted yield (U gDCW^{-1})
1	–1	–1	–1	310.83	302.27
2	1	–1	–1	461.77	457.77
3	–1	1	–1	329.62	306.72
4	1	1	–1	461.76	461.83
5	–1	–1	1	404.59	414.74
6	1	–1	1	501.84	534.97
7	–1	1	1	425.02	439.24
8	1	1	1	540.31	559.09
9	–1.68	0	0	338.97	348.12
10	1.68	0	0	603.25	579.65
11	0	–1.68	0	445.01	431.68
12	0	1.68	0	456.83	455.70
13	0	0	–1.68	298.51	324.48
14	0	0	1.68	541.27	500.84
15	0	0	0	535.82	530.74
16	0	0	0	537.89	530.74
17	0	0	0	541.01	530.74
18	0	0	0	528.56	530.74
19	0	0	0	524.93	530.74
20	0	0	0	513.77	530.74

where, Y is the predicted response; β_0 a constant; β_i the linear coefficient; β_{ii} the squared coefficient; β_{ij} the cross-product coefficient and k the number of factors. Calculation of the second order polynomial coefficients, response estimation of dependent variables and analysis of response surface plots were carried out by tools provided by the same software. The model proposed by the software was validated by running the experiments at the predicted levels of parameters for enzymatic release of intracellular β-galactosidase and by comparing the experimental enzyme activity with the predicted one.

Enzyme and protein release rate constant (K_a and K_p)

The experiment was performed in 50 mL Erlenmeyer flasks with a total reaction volume of 20 mL. The reaction mixture comprised of 4.7 % wv^{-1} (on wet basis) *L. acidophilus* cell suspension in phosphate buffer (100 mmol L^{-1}; pH 7.0) and 33.63 · 10^4 U mL^{-1} lysozyme. Each reaction mixture was incubated in orbital incubator shakers at 100 rpm at three different temperatures, i.e. 25, 32 and 37 °C. An aliquot of 0.5 mL was removed after every 2 h (0, 2, 4, 6 and 8) from the reaction mixture, except for the last one that was removed after 10 h and 30 minutes. The aliquots removed at each time interval were immediately centrifuged for 20 minutes at 12000 rpm (16500 · g) using Plastocraft Superspin R-V/FA, India at 5 °C and the resulting cell-free supernatant was immediately analyzed for the release of the enzyme and total protein. The values obtained from these experiments were used to calculate the enzyme release rate constants at each temperature and their respective enzyme location factor. A brief theory explaining this calculation is described in the following section of the manuscript.

Enzymatic release of intracellular biomolecules depends largely on the temperature of incubation of the enzyme with the microbial cell mass, besides the time and concentration of the enzyme. At a given constant cell concentration, enzymatic concentration, and temperature, the release of intracellular protein generally obeys first-order kinetics:

$$\ln\left[1-\left(\frac{R_a}{R_{am}}\right)\right]=-K_a t \quad (3)$$

$$\ln\left[1-\left(\frac{R_p}{R_{pm}}\right)\right]=-K_p t \quad (4)$$

where R_{am} and R_{pm} are the maximum amount of enzyme (U $gDCW^{-1}$) and protein (mg $gDCW^{-1}$) released, respectively, R_a and R_p is the amount of enzyme (U $gDCW^{-1}$) and protein (mg $gDCW^{-1}$) released respectively in time *t* (h), K_a and K_p are the disruption rate constants (h^{-1}) for the enzyme and protein, respectively, and t is the incubation time (h). The values of the disruption rate constants, K_a and K_p, were determined from the slope of the plot of $\ln[1-(R_a/R_{am})]$ versus *t* and $\ln[1-(R_p/R_{pm})]$ versus *t*, respectively.

The concept of location factor (LF) of the enzyme, introduced by Umakoshi et al.,[20] was applied by us to determine the location of intracellular β-galactosidase in *L. acidophilus*. According to the theory, when the enzyme is periplasmic, the release rate of enzyme (K_a) is greater than or equal to that of protein (K_p) so that the ratio becomes greater than or equal to unity. On the other hand, when the enzyme is cytoplasmic, the release rate of enzyme would be less than or equal to that of protein and hence, the location factor value will be less than one. Thus, the location factor (*LF*) value can be obtained by taking a ratio of the release rate constants of the enzyme (K_a) to protein (K_p).[20–21]

$$LF=\frac{K_a}{K_p} \quad (5)$$

Assay of β-galactosidase:

A modified method of Dickson and Martin,[22] involving the hydrolysis of substrate o-nitrophenyl-β-galactopyranoside (ONPG) to o-nitrophenol (ONP) was used to measure β-galactosidase activity. The cell-free supernatant was appropriately diluted to 1 mL using 100 mmol L^{-1} phosphate buffer solution (pH 7.0) for the assay. The reaction was initiated by adding 0.2 mL ONPG solution (2 mg mL^{-1}), prepared in 100 mmol L^{-1} phosphate buffer (pH 7.0). The mixture was incubated in a water bath at 50 °C for 10 minutes, and the reaction was then stopped by adding 1 mL of a 100 g L^{-1} sodium carbonate solution. The yellow colour of ONP obtained as a result of ONPG hydrolysis was measured at 420 nm (Spectronic Genesys 5 UV-VIS spectrophotometer, Thermo Electron Corp, USA) using ONP as the standard.

The amount of ONP released min^{-1} by the cell-free supernatant was directly proportional to the quantity of enzyme released. One unit of enzyme was defined as μmoles of ONP released per min per gram DCW using ONPG as the substrate under the reaction conditions specified.

Protein estimation

The total soluble protein (mg $gDCW^{-1}$) estimation was carried out by the modified Folin Lowry method with bovine serum albumin (BSA) as a standard.[23]

Measurement of dry cell weight

Dry cell weight was determined by centrifuging 1 mL of the cell suspension used for lysis in pre-weighed eppendorfs and further drying the pellet at 70 °C for 24 h.[24]

All experiments were carried out in triplicate and the results shown represent their average.

Results and discussion

Optimization of release of β-galactosidase by lysozyme using RSM

In order to optimize the release of β-galactosidase by enzymatic permeabilization using lysozyme, the central composite rotatable design (CCRD), which is generally the best design for response surface optimization, was selected. The design consisting of $2^3 = 8$ plus 6 centre points and $(2 \cdot 3 = 6)$ star points leading to a total of 20 experiments (Table 2) was performed to examine the combined effect of three variables (lysozyme concentration, cell density and incubation time) on β-galactosidase recovery. The results were analyzed via multiple regression analysis using Design Expert Software Version 6.0.10 trial version (State Ease, Minneapolis, MN). An effective correlation between the process variables and the response was determined from the second-order polynomial equation obtained through multiple regressions. The experimental yields obtained from each experimental run and their respective predicted yield obtained from the polynomial equation along with the entire design of experiments are listed in Table 2. Analysis of variance (ANOVA) of the experimental data was carried out as an important step for determination of adequacy and significance of the quadratic model. These results are presented in Table 3. The significance of each of the coefficients was evaluated by using the *P* and *F* values as the statistical tool. These values were also necessary to understand the pattern of the mutual interactions between the factors used in our study. Significance of the factors or coefficients of the model was based on the magnitude of the *P* and *F* values, so that the smaller the *P* value the more significant was the corresponding coefficient, while it was completely opposite for *F* value. The model *F-value* of 26.55 implied that the model was significant and there was only an 0.01 % chance of the *F-value* that could be due to noise. The *P* values of the model coefficients, listed in Table 3, suggested that, among the test variables used in the study, A, C, A^2, B^2, C^2, (where A = lysozyme concentration, B = cell density and C = incubation time) were significant model terms.

Table 3 – *Analysis of variance and regression for release of β-galactosidase [ANOVA for Response Surface Quadratic Model]*

Source	DF	Mean square	F value	Prob > F	Remarks
Model	9	15944.20	26.55	< 0.0001	Significant
A	1	64711.31	107.74	< 0.0001	Significant
B	1	696.67	1.16	0.3608	
C	1	37541.50	62.50	< 0.0001	Significant
A^2	1	8052.72	13.41	0.0044	Significant
B^2	1	13650.71	22.73	0.0008	Significant
C^2	1	25117.54	41.82	< 0.0001	Significant
AB	1	839.73	1.260E-004	0.9913	
AC	1	6703.25	1.04	0.3329	
BC	1	19635.02	0.33	0.5755	
Residual	10	538.12	600.64		
Std. Dev.	24.51				
Mean	465.08				
C.V.	5.27				
PRESS	42743.56				
R-squared	0.9598				
Adj R-squared	0.9237				
Pred R-squared	0.7141				
Adeq precision	16.006				

Certain other statistical parameters, like *Coefficient of Variation* (*C.V*), *R-squared* (R^2), *Adjusted R-squared* and *Adequate Precision*, presented in Table 3, were necessary to evaluate the Model fitting efficiency. Each of the aforementioned parameters is explained below. The low *C.V* value of 5.27 %, as obtained in our study, was nothing but the standard deviation expressed as a percentage of the mean suggesting a higher degree of precision of the model with a good deal of reliability of the experimental data. Such reliability was expressed in terms of closeness of the R^2 value, as explained by the model, and *adjusted*-R^2 value, as explained by the model adjusted for the number of terms in the model. The values indicate the amount of variation around the mean and R^2 value and *adjusted* R^2 value, of 0.9598 and 0.9237 respectively, suggesting that the model was reliable and significant, as well as able to explain 95.98 % of the total variations. To achieve better model prediction, it is necessary to have signal-to-noise ratio that is high enough to compare

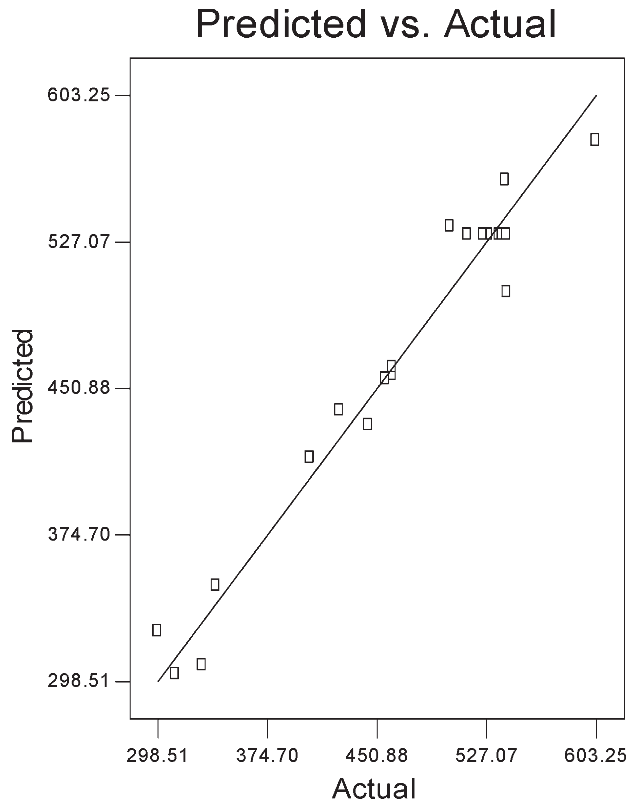

Fig. 1 – *Parity plot for release of β-galactosidase*

the range of predicted values to the average prediction error. *Adequate Precision* value of 16.006 indicated an adequate signal confirming that the model was robust.

A parity plot of the predicted enzyme release and the experimental values of the released enzyme for each run are shown in Figure 1. In the present study, there are no experimental run values with large deviation, and so it could be concluded that the model is able to predict the response values with adequate precision. The response and contour curves for β-galactosidase yield as a function of concentrations of two independent variables with the third variable being at a fixed level were plotted using the software. These plots for lysozyme concentration, cell density and incubation time are presented in Figures 2a-2c.

Application of the response surface method resulted in the following empirical relationship between β-galactosidase yield and enzymatic cell disruption variables in coded value:

$$\begin{aligned} Activity = {} & 530.74 + 68.84 \times A + 7.14 \times B + 52.43 \times C - \\ & - 23.64 \times A^2 - 30.78 \times B^2 - 41.75 \times C^2 + \\ & + 0.097 \times A \times B - 8.82 \times A \times C + 5.02 \times B \times C \end{aligned} \quad (6)$$

The above model was used for numerical optimization predicting a release of 591.1 U gDCW^{-1} under the following optimized conditions: lysozyme concentration 33.63 $\cdot$ 10^4 U mL^{-1}, cell density

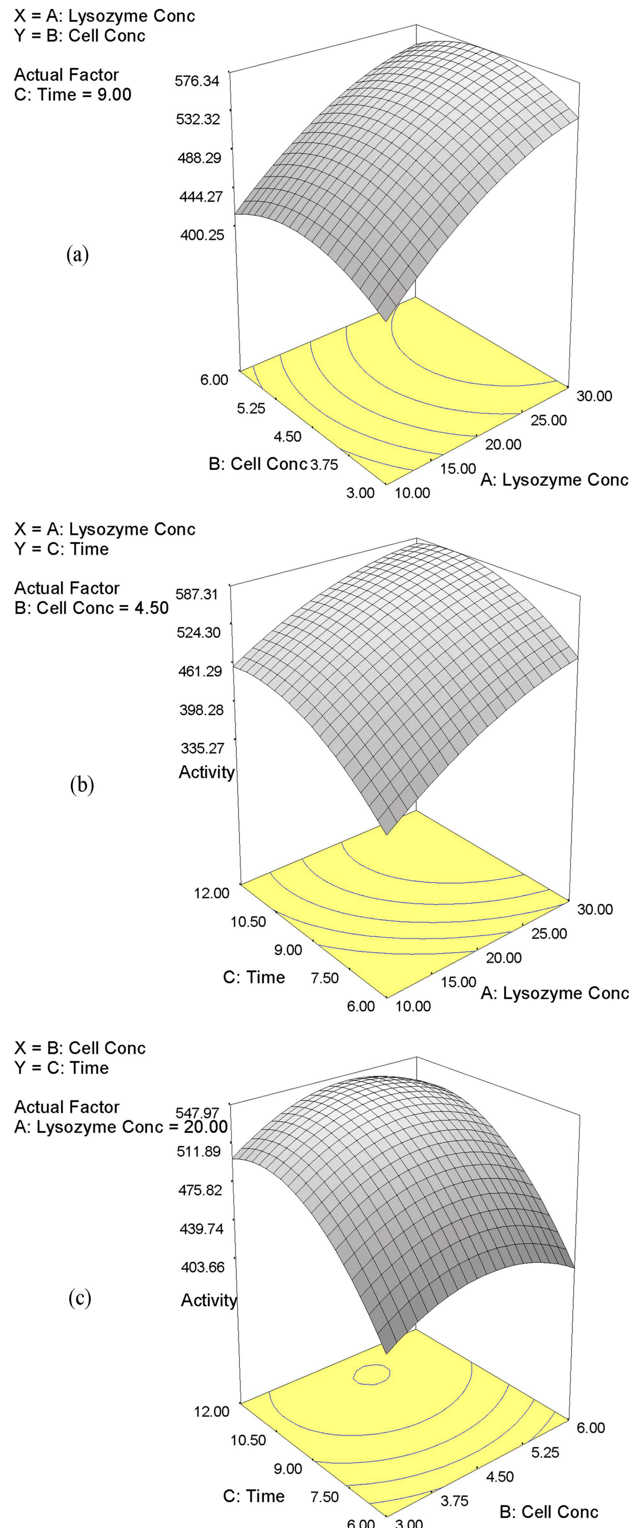

Fig. 2 – *Response surface plots showing:*
a – interaction between lysozyme and cell concentration;
b – interaction between lysozyme concentration and time;
c - interaction between cell concentration and time

4.7 % w/v on wet basis, and incubation time 10 h 30 minutes. The experimental results showed that at the optimized conditions predicted by the model, there was a release of 587.69±6.01 U gDCW^{-1}, which was sufficiently close to the predicted value

confirming the precision and robustness of the model.

In our earlier report, we showed optimization of β-galactosidase release from indigenous *L. acidophilus* by RSM.[24] The process was optimized with cell density, sample volume and ultrasonication time as the variables, at a constant acoustic power (50 W) and 70 % duty cycle. The RSM optimized variables (cell density 2.1 % weight on wet basis, suspension volume 11 mL and ultrasonication time of 15 minutes) resulted in a maximum release of 483 U gDCW^{-1} of β-galactosidase. Thus, optimization of enzymatic permeabilization process using the response surface methodology resulted in 1.2 times increase in the enzyme released as compared to ultrasonication. A comparison of release of intracellular β-galactosidase from this indigenous isolate using ultrasonication and enzymatic permeabilization is presented in Table 4. This study, focused on the application of the response surface methodology for optimization of β-galactosidase release by permeabilization of *L. acidophilus* by lysozyme, may provide useful information regarding the development of economic and efficient processes using enzyme-catalyzed reaction systems.

Table 4 – *Comparison of β-galactosidase release from L.acidophilus using ultrasonication and enzymatic lysis by lysozyme*

Lysis method	β-galactosidase release (U gDCW^{-1})	References
Ultrasonication (unoptimized)	320.30±2.4	24
Ultrasonication (optimized)	483±1.9	
Lysozyme (optimized)	587.69±6.01	Present work

Kinetics of β-galactosidase release

Enzymatic cell disruption for release of β-galactosidase can be highly influenced by the temperature of incubation of the enzyme and the cell mass. The enzymatic lysis of *L. acidophilus* to release β-galactosidase and total proteins may be explained by considering the lytic process to follow first-order kinetics. An incubation time of 10 h 30 min. resulted in maximum release of β-galactosidase (585.97 U gDCW^{-1}) at a temperature of 37 ºC with a protein release of 5.82 mg gDCW^{-1}. The rate constants for release of β-galactosidase (K_a) and protein (K_p) were determined from the slope of a plot of $\ln[1-(R_a/R_{am})]$ versus t and $\ln[1-(R_p/R_{pm})]$ versus t, respectively (Figures 3 and 4). At a lysozyme concentration of $33.63 \cdot 10^4$ U mL^{-1} at 37 ºC, K_a and K_p were found to be 0.229 h^{-1} and 0.233 h^{-1}, respectively. Table 5 showing the location factor of

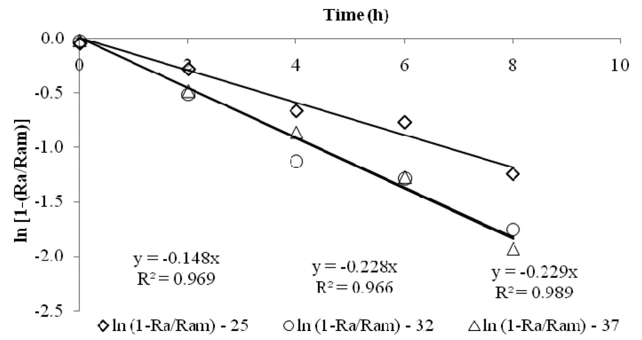

Fig. 3 – *Kinetics of β-galactosidase release using lysozyme at varying temperatures*
Where, ◊ depicts the plot of $\ln[1-(R_a/R_{am})]$ versus t at 25 °C, ○ depicts the plot of $\ln[1-(R_a/R_{am})]$ versus t at 32 °C and Δ depicts the plot of $\ln[1-(R_a/R_{am})]$ versus t at 37 °C, when the reaction was carried out at 4.7 % wv^{-1} (wet basis) Cell density with $33.63 \cdot 10^4$ UmL^{-1} of lysozyme for a maximum of 10h 30 min

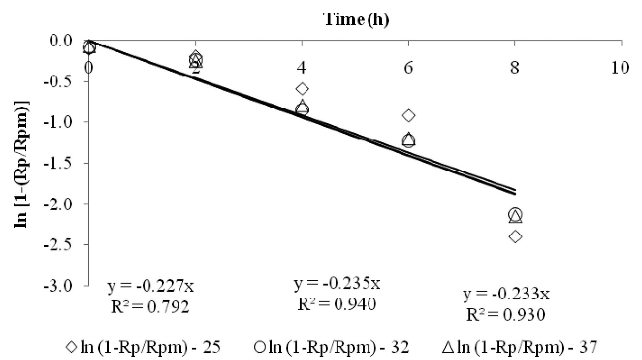

Fig. 4 – *Kinetics of protein release using lysozyme at varying temperatures*
Where, ◊ depicts the plot of $\ln[1-(R_p/R_{pm})]$ versus t at 25 °C, ○ depicts the plot of ln[1-(Rp/Rpm)] versus t at 32 °C and Δ depicts the plot of $\ln[1-(R_p/R_{pm})]$ versus t at 37 °C when the reaction was carried out at 4.7 % wv^{-1} (wet basis) Cell density with $33.63 \cdot 10^4$ UmL^{-1} of lysozyme for a maximum of 10h 30 min

Table 5 – *Location factor of β-galactosidase at varying temperatures*

Temperature	K_a (h^{-1})	K_p (h^{-1})	Location factor (LF)
25	0.148	0.227	0.652
32	0.228	0.225	0.972
37	0.229	0.233	0.983

β-galactosidase at varying temperatures indicates that at all experimental temperatures LF values were found to be less than 1.0, suggesting cytoplasmic location of the enzyme. This is the first report that calculates the location factor of an enzyme using lysozyme as the method of cell permeabilization. All other previous reports have used mechanical methods of disruption, such as ultrasonication or high-pressure homogenization[4,20–21] for determination of the location factor of the enzyme under study.

Conclusions

Enzymatic treatment of the indigenous *L. acidophilus* strain using lysozyme was found to be an efficient method for release of intracellular β-galactosidase. The enzymatic release process, developed using RSM, with lysozyme concentration of 33.63 $\cdot 10^4$ U mL^{-1}, cell density of 4.7 % wv^{-1} on wet basis, and incubation time of 10 h 30 min. at 37 ºC, resulted in maximum release of β-galactosidase (587.69 U $gDCW^{-1}$) as against 483 U $gDCW^{-1}$ obtained by an optimized ultrasonication process. The kinetics of release of β-galactosidase using the optimized release conditions was carried out at varying temperatures, with maximum release being achieved at 37 ºC, with rate constants of 0.229 h^{-1} and 0.233 h^{-1} for the enzyme and total protein, respectively. The kinetic results obtained by enzymatic permeabilization were used for the first time to calculate its location factor within the bacterial cell.

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List of abbreviations:

MRS medium – de Man, Rogosa and Sharpe medium for cultivation of *Lactobacillus* species
x_i – dimensionless value of independent variable
X_i – real value of a independent variable
X_{cp} – real value of independent variable at center point
ΔX_i – step change of real value of variable *i* corresponding to a variation of a unit for the dimensionless value of variable *i*
Y – predicted response
β_0 – constant
β_i – linear coefficient
β_{ii} – squared coefficient
β_{ij} – cross-product coefficient
k – number of factors
R_{am} – maximum amount of enzyme released, U $gDCW^{-1}$
R_{pm} – maximum amount of protein released, mg $gDCW^{-1}$
R_a – amount of enzyme released in time t, h U $gDCW^{-1}$
R_p – amount of protein released in time t, h mg $gDCW^{-1}$
K_a – enzyme disruption rate constant, h^{-1}
K_p – protein disruption rate constant, h^{-1}
LF – enzyme location factor
CCRD – central composite rotatable design
ONPG – o-nitrophenyl-β-galactopyranoside
ONP – o-nitrophenol
ANOVA – analysis of variance
C.V – coefficient of variation
R^2 – determination coefficient

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