Original Article

An Experimental Study Using the Statistical Tool D-optimal Design for the Process Optimization to Enhance the Yield of β-galactosidase from *Lactobacillus plantarum*

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ABSTRACT

Context: The Novelty of the research is to formulate the probiotic product with low-cost submerged fermentation for the mitigation of lactose intolerance. The utilization of substrate enhances the production of β-galactosidase from the potent strain *Lactobacillus plantarum* by optimizing the media using the response surface methodology tool. This research is expected to result in innovative scientific outcomes leading to the development of lactose-free products cheaply. **Aim:** The current research is focused on the optimization of cultural conditions using a D-optimal experimental design to enhance the yield of β-galactosidase. **Materials and Methods:** Interaction of two physical and chemical factors such as lactose 0.5–2%, beef extract 0.5–2%, pH 6–8, and temperature 30–40°C, were studied in 3D interaction. β-galactosidase production was estimated using the hydrolysis of o-Nitrophenyl β-D-galactopyranoside as the substrate. Further, the enzyme was purified by 70% precipitation, fractionated with 50-kDa ultra-filtration, and separated in gel permeation chromatography with the matrix Sephadex-G100. The extracted β-galactosidase from *Lactobacillus plantarum* has been immobilized in alginate and hardened with calcium chloride for lactose hydrolysis using Glucose oxidase-Peroxidase assay. **Results:** The molecular sequencing of VITDM15 was confirmed as *Lactobacillus plantarum* in 16srRNA sequencing. Using the optimized conditions there was a six-fold increase in the yield of β-galactosidase. The purified β-galactosidase from *Lactobacillus plantarum* KX838907 yielded 31.75% with 67.73 fold and specific activity of 1705IU/mg. The molecular weight of the enzyme was found to be 112kDa at denaturing conditions in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. In GOD-POD assay the glucose dosage increased up to a maximum level of 4.891mg/l in the 4th cycle. **Conclusion:** Based on the results obtained we claim that this might be the first report on the enhanced yield of β-galactosidase and lactose hydrolysis. Thus novel strain *Lactobacillus plantarum* KX838907 can be used in the commercial production of β-**galactosidase** as it is considered safe to use as a probiotic microorganism.

Key words: β-galactosidase, D-Optimal, SDS-PAGE, Sodium alginate, Lactose hydrolysis.

INTRODUCTION

This research discusses one of the most appropriate problems in abdominal clinical practice: LACTOSE INTOLERANCE- when there is a lack of a β-galactosidase enzyme in the digestive tract. It is a clinical disorder and the widespread condition differs from one individual to another with one or more symptoms such as vomiting sensation, abdominal pain, diarrhea, nausea, gas/ flatulence, emergency to go for the toilet, abnormal stomach, etc., People sensitive to lactose are not able to take dairy products, which deliver a major source of calcium. If dairy products are eliminated from the human body, they can be the lack calcium and finally leads to bone diseases called osteoporosis. To absorb calcium, our body needs Vitamin D which is again rich in

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milk, yogurt, curd, butter, etc., In this case, lactose intolerance people can go with lactose-free products. The intestinal lining of the digestive tract produces a catalyst called lactase that hydrolyzes the dietary lactose from dairy items to beat lactose narrow-mindedness inadequacy.[1,2] About 60% of people in the world having difficulty digesting lactose and that leads to a syndrome called lactose intolerance and reduces their quality of life.[3] Lactose intolerance can be treated by utilizing extra probiotic microbes like *Lactobacilli* or β-galactosidase catalysts that can hydrolyze the lactose sugar present in dairy items. Yet, the utilization of lactic acid bacteria as a probiotic is confined to treat the lactose narrow mindedness because it fails to frame spores and persevere through the acidic condition of the stomach.[4]

β-galactosidase is also called lactase enzyme plays a major role in lactose intolerance. Enzymes are proteins that help us to cause chemical changes in the body. The small intestine breaks down food and absorbs nutrients from the food. As the same principle, the small intestine produces an enzyme called β-galactosidase break down to lactose and is absorbed into the bloodstream for the conversion of glucose and galactose. If the small intestine doesn't make enough or absence of the β-galactosidase enzyme the dairy products will not digest and leads to lactose intolerance. β-galactosidase is most commonly used in dairy products for proper digestion to improve sweetness, solubility, and flavor in food industries.[5] For the utilization of dairy items with no extreme side effects for lactose-intolerance individuals, β-galactosidase enzymatic hydrolysis is one of the most standard procedures for lactose-free product development.[6-9]

The enzyme is also found in animal organs like in the digestive tract, cerebrum, placenta and testis of mutts, hares, snails, calves, sheep, goats, rodents.[10] Moreover, β-galactosidase is also found in human salivation, in hatchlings of primates and domesticated animals, in tissues of rodents and mice, and the plasma serum and pee of mutts.[11] β-galactosidase is produced by some microorganisms. They are produced from yeasts such as *Kluyveromyces lactis*, from fungi such as *Neurosporacroussa*, *Aspergillus foetidus*,[12] from bacterial cultures like *Bacillus megaterium, Escherichia coli, Thermus aquaticus, Streptococcus thermophilus, Lactobacillus bulgaricus.*[13] Furthermore, lactose intolerance is not milk hypersensitivity; People regularly confuse milk sensitivity and lactose intolerance due to the similarity of manifestation. Be that as it may, lactose intolerance contrasts extraordinarily with milk sensitivity.[14,15] Lactose intolerance includes the stomach-related system through milk sensitivity includes the immune system.

Hence the objective of the study is focused on the optimization of β-galactosidase production from *Lactobacillus plantarum* VITDM15 using the statistical tool D-optimal experimental design Response Surface Methodology. Further, the enzyme purified for the maximum yield and its application in lactose hydrolysis. The novelty of the research is to enhance the yield of β-galactosidase for lactose-free product development using the probiotic bacteria which is considered to be Generally Recognized as Safe (GRAS).

MATERIALS AND METHODS

Chemicals

Mann Ragosa Sharpe broth or agar (MRS), lactose, sodium alginate, yeast extract, beef extract, Ortho-Nitrophenyl-β-galactoside (ONPG) (Himedia, Mumbai, India) chloroform, toluene, acetone, high range protein marker (SRL chemicals, Chennai, Tamil Nadu, India) were purchased.

Probiotic bacteria strain VITDM15 and culture condition

Lactic Acid Bacteria (LAB) isolated from donkey milk, curd and yogurt were screened for β-galactosidase using X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in our previous paper.[16] Out of this, the potent strain VITDM15 was taken for further study. The stock culture was maintained at −20°C in Mann Ragosa Sharpee broth (MRS) with 20% (v/v) glycerol. It was retrieved and multiplied twice in MRS broth before the conduct of experiments. The strain VITDM15 will be identified based on the biochemical and 16S rRNA sequencing.

β-galactosidase production of the cell-free extract by ONPG method

The capacity of β-galactosidase production was estimated using the hydrolysis of Ortho-Nitrophenyl β-D-galactoside (ONPG) as the substrate. The positive strain VITDM15 from the X-Gal assay was grown in MRS broth and the overnight culture was centrifuged at 12,000rpm for 20min at 4°C. Obtained culture pellet (100µl) mixed with 900µl of Z-Buffer (Na_2HPO_4, NaH_2PO_4, KCl, $MgSO_4$, β-Mercaptoethanol) with pH 7.0, 100µl of chloroform, 50µl of 1:9 ratio of toluene: acetone and disrupted using probe sonicator (pH meter-Systronics). To the disrupted cells 200µl of ONPG (4mg/ml of Z-Buffer) was added and reacted for 15min at 37°C. The yellow color was observed which determines the presence of β-galactosidase activity. The β-galactosidase catalysis reaction was stopped by adding 200µl of 1M Na_2CO_3 and the optical density value noted

at 420 and 550nm for each isolate. The β-galactosidase value was calculated by the standard formula. The one isolate VITDM15 exhibited maximum enzyme activity was characterized for 16S rRNA sequencing, optimized by RSM followed with purification and immobilized for lactose hydrolysis.[17,18]

$$\beta = \text{galactosidase} = 100 \times \frac{\text{OD420 nm}}{T \times V \times \text{OD600 nm}}$$

Molecular characterization of *Lactobacillus*

The species-level identification for the potent strain VITDM15 was obtained by 16S rRNA sequencing and followed for the construction of a phylogenetic tree using MEGA 5.05 software. Genomic DNA was extracted from bacterial isolate (Yazzh genomics) according to the manufactures instruction. The universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') are used for the species-level identification. The acquired sequences were processed in BLASTn for sequence homology and submitted in the NCBI database to get the Genbank accession number. The partial sequence obtained from 16S rRNA was constructed for the phylogenetic tree using MEGA software by the neighbor join method.[19,20]

Scanning Electron Microscope (SEM)

The aliquots of fresh *L. plantarum* culture was incubated for 48 ± 2 hr at 37°C and was harvested, fixed, dehydrated, and essentially embedded respectively according to McDougall *et al.*[21] The overnight culture was smeared on the coverslip and fixed for 1h in a solution containing 2.5% glutaraldehyde. A Further coverslip was dehydrated using 70% ethanol and final immersion in 100%. Subsequently, the coverslip was subjected to 2.5% glutaraldehyde and allowed to dry overnight. Microscopic analysis was carried out with a scanning electron microscope (Leo Electron Microscopy Ltd. Cambridge, UK) operated at 10keV.

Primary optimization stage

The production media was inoculated with *L. plantarum* VITDM15. After the incubation, cells were harvested by centrifugation and disrupted using ultra-sonication to analyze the activity of β-galactosidase. To optimize the physical and chemical parameters using the one factor-at-a-time method (OFAT), the effect of carbon and the nitrogen source was replaced in the MRS medium and tested for the enhanced yield of β-galactosidase. Various carbon and nitrogen sources were selected for one factor-at-a-time method (OFAT) study using *L. plantarum*. The carbon source (maltose, lactose, fructose, and starch) were added at 1% (w/v) and the influence of nitrogen sources (beef extract, yeast extract, peptone, and ammonium nitrate) in a modified MRS medium was replaced by protease peptone with 1% w/v and tested for the enhance yield of β-galactosidase. The same way effect of temperature was determined by incubating the potential strain at a different temperature such as 30°C, 40°C, and 50°C and pH 3, 5, 7, 9, and 11 respectively. For each parameter, a blank sample was maintained. The culture was allowed to grow for 48h at 37°C and the activity of β-galactosidase was determined by ONPG analysis in triplicates and reports as mean value.

Experimental design by RSM (D-optimal design)

Based on the results obtained in the classical method (OFAT), lactose as a carbon source and beef extract as a nitrogen source are selected as chemical variables. Along with these conditions temperature and pH were selected as additional physical variables. The production of β-galactosidase from *L. plantarum* VITDM15 was analyzed by statistical tool D-optimal experimental design using RSM. The experimental design summary was predicted with 26 experimental trials with D-optimal initial design by the quadratic model (Design-Expert 7.0.0, StatEase, Inc., Minneapolis, USA). An experimental design was formulated with different concentrations of lactose and beef extract at different temperatures and pH with 1% inoculums in a 500ml erlenmeyer flask. To determine the dependent variable response in β-galactosidase production and to establish the specific interactions of different factors and also to evaluate the conditions to optimize the responses 3D contour plots were plotted. To evaluate the effect of four variables 26 sets of experiments were carried out with lactose (0.2-1%), beef extract (0.5-1%), pH (6-8), and temperature (25-45°C) concentration with 2 coded levels (-1, +1 level) used for their combined influence the production of β-galactosidase (gL−1).

β-galactosidase production and purification

The production and purification of β-galactosidase from *L. plantarum* VITDM15 were grown in the production media under optimized conditions. Cells were removed by centrifugation at 8000rpm at 4°C for 20 min. The pellet was disrupted using a probe sonicator and ammonium sulfate was added slowly to achieve 70% saturation. The precipitated protein was collected and solubilized in Z-buffer (0.1M, pH 7.2).[22] The protein was then ultra-filtered with 50Kda cut off the membrane (Amicon, Millipore) and the collected retentate was

collected loaded onto a sephadex G-100 column. To find out the elution of the protein the absorbance of the protein was recorded at 280nm.[23] The eluted protein was estimated by lowry's method using Bovine Serum Albumin (BSA) as a standard.[24] Purified β-galactosidase was subjected to molecular weight determination using 12% SDS-PAGE (Bio Rad) with a standard high range protein marker (20-206kDa, SRL). All the purification steps were analyzed for both β-galactosidase activities by ONPG assay and protein estimation by lowry's method to construct the purification table. The specific activity of an enzyme, purification folds, and yield percentage were calculated.

Application study on immobilized β-galactosidase used to make lactose-reduced milk

In a 2% sodium alginate solution the purified enzyme was mixed properly. The alginate enzyme slurry was taken in the syringe and dropped onto the 1.5% calcium chloride solution and beads were collected (2.5g). The immobilized enzyme-containing was allowed to harden for an hour. Immobilized matrix packed in 20ml syringe with a height of 1.5cm. The syringe was connected with a short-length tube to the tip of the syringe barrel and nylon gauze was placed inside the barrel to prevent the beads from blocking the syringe outlet. The fresh milk sample was treated with an immobilized enzyme in a syringe and again returns the treated milk in the syringe to reach the desired concentration of glucose. A total of 4 cycles were carried out and all four cycles were analyzed for glucose content by GOD-POD assay, therefore glucose (0.4mg/mL) was used as standard and fresh milk as reference. The glucose content was measured in units before and after treating milk with enzyme and calculated with a standard formula. The percentage value was also calculated for the improvement of glucose (mg/l) dosage in each cycle. The diagrammatic flow chart for the study is shown in Figure 1.

$$C(D-Glucose) = \frac{\text{Sample } \Delta A}{\text{Glucose standard } \Delta A} \times 1000$$

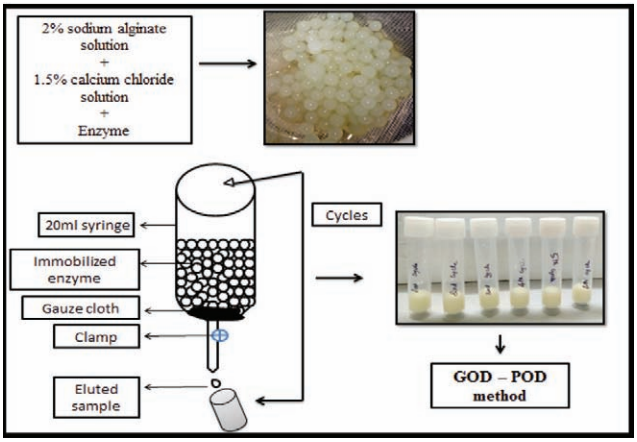

Figure 1: Diagrammatic flow chart for application study on immobilized β-galactosidase.

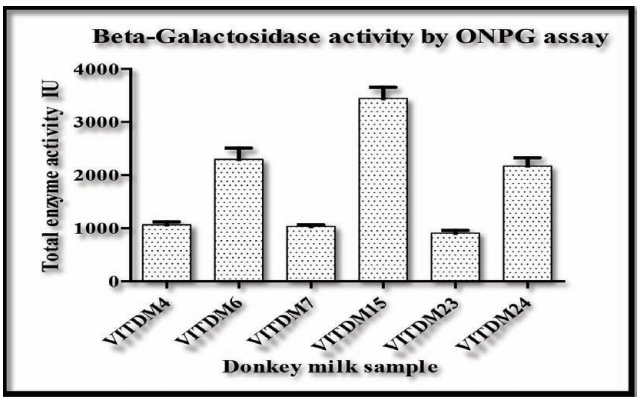

Figure 2: Estimation of β-Galactosidase activity by ONPG assay for crude (Donkey milk).

RESULTS AND DISCUSSION

Isolation of β-galactosidase producing *Lactobacilli*

The current study was carried out to investigate the efficiency of β-galactosidase production from different bacterial isolates. The maximum β-galactosidase production was noted in one strain isolated from donkey milk with 3222IU and named VITDM15. The estimation of β-galactosidase activity by ONPG assay for crude (donkey milk) is represented in Figure 2. The one potent isolate VITDM15 was identified for the biochemical characterization with rod shape gram-positive bacteria and negative for catalase test. The biochemical and probiotic property specifies that the strain belongs to lactic acid bacteria. From the results obtained, it is evident that the β-galactosidase produced by the strain VITDM15 was comparatively more active than the results reported DIY11 335U/min/cell yeast earlier by Al-jazairi *et al.*[18] Thus the VITDM15 potent strain *Lactobacilli* was subjected to 16s rRNA sequencing and phylogenetic tree construction. Similarly, it is also carried out for extraction, optimization, purification, and immobilization of the enzyme for lactose hydrolysis.

Molecular sequencing of potent isolate *Lactobacilli*

The partial 16s rRNA sequence obtained was exported to the database and checked for homology alignment. The alignment results of VITDM15 exhibited 99% similarity with *L. plantarum*. The sequence of the strain

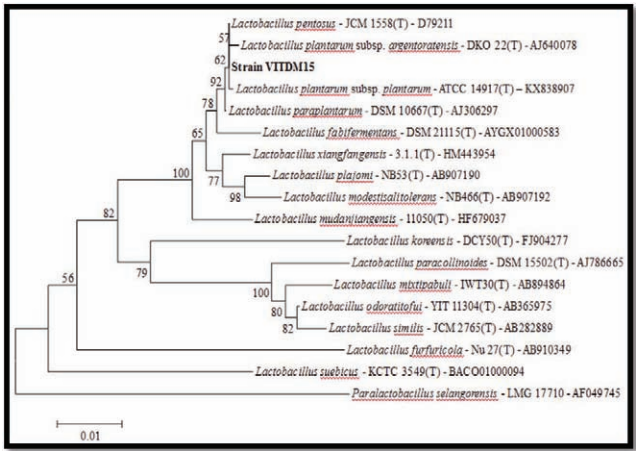

Figure 3: The neighbor-joining method cladogram showing a phylogenetic relationship between *Lactobacillus plantarum* VITDM15 and other related *Lactobacillus plantarum* based on the 16s rRNA gene sequence analysis.

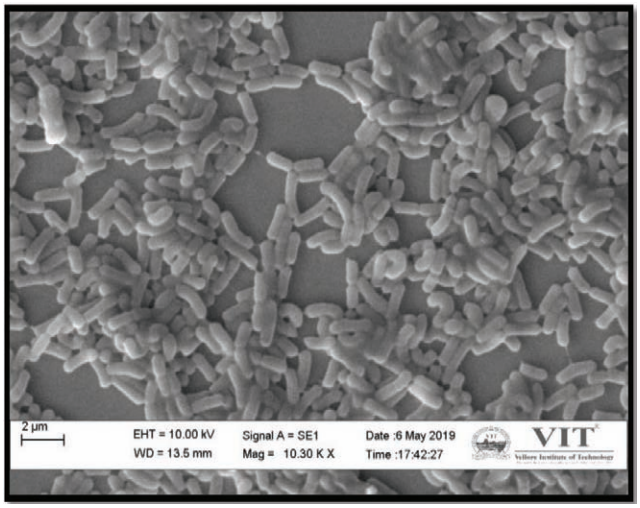

Figure 4: SEM analysis of *Lactobacillus plantarum* at 10.30 KX magnifications.

has been submitted to NCBI and the gene bank accession number was obtained as KX838907 for *L. plantarum*. Thus the study reveals the identification of *L. plantarum*-VITDM15 from donkey milk with the efficient production of β-galactosidase. Murua *et al.* also reported the isolation of *L. plantarum* from donkey milk samples.[25] The phylogenetic tree was constructed between *L. plantarum* and other related *Lactobacillus* species based on 16s rRNA gene sequencing using MEGA 5 in Figure 3.

Scanning Electron Microscope (SEM)

The SEM images revealed the bacterial cell size, shape, length, diameter, and physical morphology of the bacteria. The cells were found to be attached to the surface of the coverslip which was immersed in the MRS broth for 48h. The SEM analysis showed that the cells were rod in shape at 10.30KX magnification observed in Figure 4. The length of the cell was observed to be 2µm and 13.5mm in diameter.

Optimization study on β-galactosidase production from *L. plantarum*

Carbon and nitrogen source are essential nutrients for the effective production of β-galactosidase. The most effective carbon source to obtain maximum β-galactosidase production was found to be lactose (1170.00U/mL) followed by maltose (965.00U/mL), fructose (744.66U/mL), and starch (345.00U/mL). Therefore lactose was identified to be the best carbon source to enhance the maximum enzyme activity when compared to other carbon sources. Among the different nitrogen sources, the beef extract was observed with the maximum production of β-galactosidase with 1356.67U/mL. Yeast extract revealed 864.66U/mL followed by that peptone and ammonium nitrate were found to be 544.66U/mL respectively. A similar result was reported by Siham *et al.* in the case of *B.circulans*.[26] The consequence of pH and temperature on *L. plantarum* KX838907 was analyzed for β-galactosidase activity. The enzyme was more active in temperature ranging from 30°C to 40°C, with an optimum value around 40°C (1419.86U/mL) and slowly decreased from 40°C to 50°C. Therefore the isolate was able to survive and produce β-galactosidase with least to maximum temperature. This optimum temperature for β-galactosidase production was similar to the results reported by Desire *et al.* in *Bacillus* sp.[27] The stability of the pH for enzyme production was investigated from acidic to basic condition. The enzyme fairly can be extracted and stable at pH 7 with 1149.11U/mL and it rapidly decreased from pH 8 to pH 11. This study showed the ability to produce β-galactosidase from *L. plantarum* in different carbon and nitrogen sources followed by temperature and pH in Figure 5 (A).

Statistical analysis RSM

The results obtained in the OFAT approach revealed the optimum levels of significant factors. The effect of their interactions on β-galactosidase production was further confirmed with enhanced productivity using RSM the statistical tool called D-optimal experimental design with varying parameters. The design expert of the D-optimal model was enhanced to optimize the β-galactosidase production by varying the parameters (A) temperature, (B) pH, (C) lactose (D) beef extract. Parameters were selected based on the result of the primary optimization method. The experimental design determines the parameter factors ranges together with

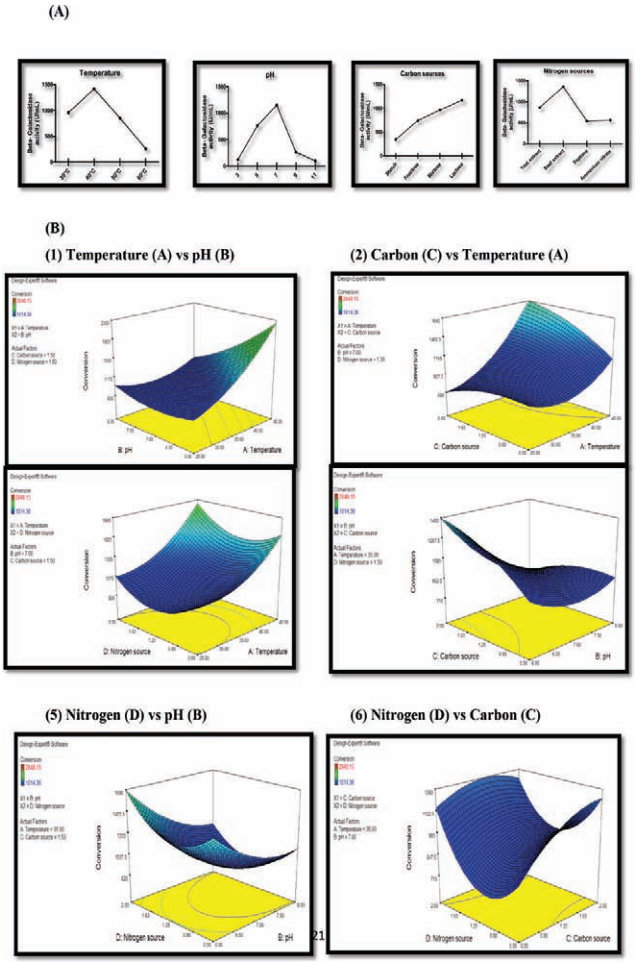

Figure 5: (A) Effect of β-galactosidase on temperature, pH, carbon and nitrogen sources (B) Response surface plot in relation to different parameters for β-galactosidase production.

the coded and actual values from lower to upper-level variables for β-galactosidase production. The results of the 26 runs from D-optimal experiments study the effects of 4 independent variables on β-galactosidase production. The maximum experimental value for β-galactosidase production was 2848.15IU/mL based on RSM. Parameters were optimized by D-optimal design with three central points and the response of β-galactosidase yield was studied. Design expert 7.0.0 was used to predict the values, regression analysis, and experimental design with obtained data. The enzyme activity expressed in the design expert was computed by analysis of variance (ANOVA) to check the quality of fit of the polynomial expression to predict the responses.

3D interaction between the different parameters of the media was optimized to enhance the production of β-galactosidase by *L. plantarum* VITDM15 by RSM design expert tool, where (1) represents temperature (A) vs pH (B), (2) carbon (C) vs temperature (A), (3) nitrogen (D) vs temperature (A), (4) carbon (C) vs pH (B), (5) nitrogen (D) vs pH (B), (6) nitrogen (D) vs carbon (C) is shown in Figure 5 (B). The statistical significance and the analysis of variance (ANOVA) for the quadratic model – response for β-galactosidase production tabulated in Table 1.

The regression analysis data were fitted to a quadratic model and the second-order regression equation obtained was a full actual model on β-galactosidase production as shown in equation 1.

β-galactosidase production = +917.20 +277.19*A -188.27*B +98.59*C +67.64*D
-305.23*A*B +178.53*A*C +48.15*A*D -173.68*B*C
-49.03*B*D -71.19*C*D +205.00*A^2 +133.08*B^2
-83.62*C^2 +268.64*D^2 (1)

Where A is temperature, B is pH, C is a carbon (g/L) and D is nitrogen source (g/L)

From ANOVA, the model *F*-value determines 11.54 for β-galactosidase production and it reveals that the model is significant. This is likewise obvious from the model F esteem and the probability value at *P* > F was about 0.0500. There is just a 0.02% possibility that a "Model F-value" this huge could happen because of noise. The adequacy of the model can be analyzed by a coefficient (R^2) and the connection coefficient (R).[28,29] The R^2 estimation of 0.9417 suggests 94.17% fluctuation in β-galactosidase extraction. In this case, A, B, AB, AC, BC, D2 are huge model terms. The "Lack of Fit *F*-value" of 3500.29 includes the lack of fit is significant.

Purification of β-galactosidase from the optimized media for maximum yield

Purification of β-galactosidase was done in three steps, includes ammonium sulfate precipitation, ultracentrifugation, and gel permeation chromatography with sepahdex G100. In ammonium sulfate precipitation assay, at 70% of saturation, the precipitated protein showed the highest β-galactosidase activity. Further, from ultracentrifugation, the retentate was collected and eluted in gel permeation chromatography at 0.5mL/min of flow rate. A total of 22 fractions were collected and maximum activity was determined between 5th and 6th fractions at 280nm. Enzyme activity, protein estimation, specific activity, and yield percentage were calculated in purification Table 2.

Summary of purified β-galactosidase from *L. plantarum* KX838907 was yielded 31.75% with 67.73 fold and specific activity of 1705IU/mg. The purity of the protein yielded with 0.6mg with 1235IU enzyme acitivity. To the best of our knowledge, this is the first report on β-galactosidase with maximum yield and analyzed for lactose hydrolysis. There are reports which showed

Table 1: ANOVA for the quadratic model – response for β-galactosidase production and R-squared, Adj R-squared, Pred R-squared, and Adeq precision value of the model.

Source	Sum of squares	d_f	Mean square	*F* value	*p*-value Prob>F	
Block	59013.29	1	59013.29			
Model	6.42E+06	14	4.59E+05	11.54	0.0002	Significant
A-Temperature	1.18E+06	1	1.18E+06	29.68	3.00E-04	
B-pH	5.68E+05	1	5.681E_005	14.29	3.60E-03	
C-Carbon source	1.68E+05	1	1.68E+05	4.23	0.0667	
D-Nitrogen source	7.49E+04	1	7.49E+04	1.88	2.00E-01	
AB	1.23E+09	1	1.23E+06	30.97	2.00E-04	
AC	3.79E+05	1	3.79E+05	9.54	1.15E-02	
AD	2.84E+04	1	2.84E+04	0.71	4.18E-01	
BC	3.72E+05	1	3.72E+05	9.36	1.21E-02	
BD	2.79E+04	1	2.79E+04	0.7	4.22E-01	
CD	6.13E+04	1	6.13E+04	1.54	2.43E-01	
A2	1.42E+05	1	1.42E+05	3.58	8.78E-02	
B2	5.39E+04	1	5.39E+04	1.36	2.71E-01	
C2	2.24E+04	1	2.24E+04	0.56	4.70E-01	
D2	2.80E+05	1	2.80E+05	7.05	2.41E-02	
Residual	3.98E+05	10	3.98E+04			
Lack of Fit	3.97E+05	5	7.95E+04	3500.29	<0.0001	Significant
Pure Error	1.14E+02	5	2.27E+01			
Cor Total	6.88E+09	25				

Std. Dev.	199.38	**R-Squared**	0.9417
Mean	1268.86	**Adj R-Squared**	0.8601
C.V.%	15.71	**Pred R-Squared**	0.0181
PRESS	6.699E+006	**Adeq Precision**	12.842

Table 2: Summary of purification of β-galactosidase from *L. plantarum*. Purification fraction

	Total volume (mL)	Total enzyme activity(IU)	Total protein (mg)	Specific activity ($IUmg^1$)	Purification fold	Yield percentage
Crude extract	50	3222	128	25.17	1	100
70% Ammonium sulphate precipitation	10	1235	37	33.37	1.3	38.33
Ultrafiltration	5	1213	1.9	638.42	25.36	37.64
Gel permeation chromatography	1	1023	0.6	1705	67.73	31.75

low specific activity with 32.68U/mg proteins for β-galactosidase by HebaEzz and Beltagey.[30] Comparatively our results were found to be having increased in a specific activity with the results of Mozumder *et al.* for β-galactosidase purified by the earlier standard procedure with the specific activity of 42.67U/mg. [22]

Molecular weight determination by SDS-PAGE

The SDS-PAGE analysis of the purified enzyme revealed the molecular weight of the enzyme was 112kDa which was confirmed the presence of a unique band with β-galactosidase activity is showed in Figure 6. Elmira Gheytanchi *et al.* reported the molecular weight of β-galactosidase with a single band of 116kDa.[31] Thus in

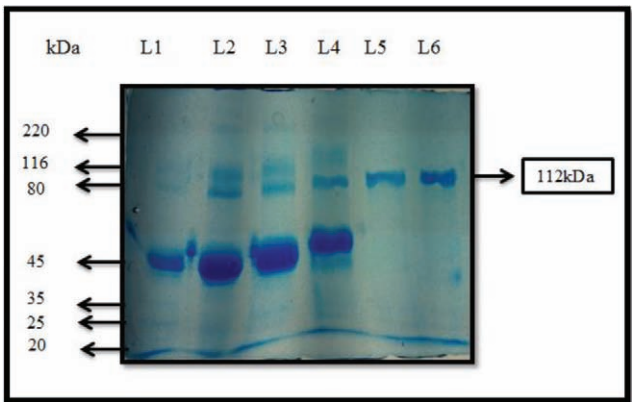

Figure 6: Purified β-Galactosidase from the potent strain Lactobacillus plantarum VITDM15. Lane 1- Protein marker (High molecular weight 20kDa to 220kDa, SRL) Lane 2- Crude enzyme; Lane 3-Ammonium sulphate precipitation; Lane 4-Retentate from ultra-filtration; Lane 5 and Lane 6- Gel permeation chromatography.

the present research work, SDS-PAGE yielded a single band, which confirms the homogeneity of the purified β-galactosidase from *L. plantarum* KX838907. Partially purified enzyme immobilized with sodium alginate and cross-linked with calcium chloride for the lactose hydrolysis.

Storage stability of immobilized β-galactosidase

Immobilized β-galactosidase was stored at 4°C and the enzyme activity was analyzed. The soluble enzyme revealed 80% of enzyme activity comparatively the immobilized enzyme retained with 95% enzyme activity after 15 days. The cross-linker can increase the stability of an enzyme that can withstand conformational changes.[32]

Application study of immobilized β-galactosidase for lactose hydrolysis

The milk sample was treated with an immobilized enzyme and analyzed for glucose content by the Glucose oxidase-peroxidase (GOD-POD) method. In the present study, the glucose standard was found to be 1.472mg/ml and fresh milk 2.109mg/ml of glucose. Hence the fresh milk was treated with immobilized β-galactosidase for the 1st cycle and observed for the glucose dosage. The 1st cycle of the treated milk was revealed with 2.771mg/ml in the GOD-POD method. It is predicted that the level of glucose was increased in treated milk in comparison to fresh milk. The percentage of glucose production after the quantitative test for the 1st cycle was recorded as 31.38%. The eluted milk from the 1st cycle was followed by the 2nd cycle and analyzed for the glucose dosage. It is estimated the glucose dosage was about 2.965mg/ml. The concentration of glucose was moderately improved from the 1st cycle (31.38%) to the 2nd cycle (40.58%). Moreover, the obtained study revealed that the packed immobilized β-galactosidase started to release the enzyme frequently with its stability. Comparatively, the milk eluted from the 2nd cycle subjected to the 3rd and 4th cycle and analyzed for the increase or decrease of glucose content like the previous procedure. The glucose content was increased steeply between the 2nd and 3rd cycles with 4.831mg/ml (129.06%). The production of glucose can be significantly influenced by the extreme release of enzymes to bind with lactose present in the milk. This may be due to the sustainable release of the enzyme from the immobilized beads and also it is time-dependent. A maximum enzyme may be released during the third cycle hence the enzyme-substrate concentration might be more in this cycle. Because of the adequate substrate concentration with more enzymes released in this cycle, the enzyme might be acting at its maximal velocity for the conversion of lactose to glucose and galactose in this cycle. Similarly, from the 3rd to 4th cycles, the glucose content was further slightly increased up to 4.891mg/ml with an increase in the percentage of 131.91. This indicates, there was a maximum glucose level rise in the 4th cycle with an immobilized enzyme. However, the concentration of glucose seems to be high between the 2nd and 3rd cycles. This can be standardized in lactose hydrolysis for lactose-free product development for large-scale production in an industrial approach. This may be due to stability, fast release of an enzyme, and also eliminating the contamination of the protein. Cycles of treated milk samples with immobilized enzymes were given with an increase in the percentage value of glucose was observed in Figure 7. The concentration of glucose standard, fresh milk, and different cycles of treated milk samples was also examined in Figure 8.

CONCLUSION

This study shows its novelty by highlighting the β-galactosidase activity from potential non-pathogenic lactose fermenting *L. plantarum* which is GRAS. The maximum β-galactosidase production was achieved under optimized conditions with a maximum yield of 31.75%. Enzyme immobilization was carried out for lactose-free milk as the application study by enhancing the constancy and minimizes the chance of product contamination. Also, using immobilized enzyme improve recovery and reuse of expensive catalyst. The milk sample was treated with enzyme and analyzed for the glucose content by the GOD-POD method. After the treatment, the glucose dosage increased up to a

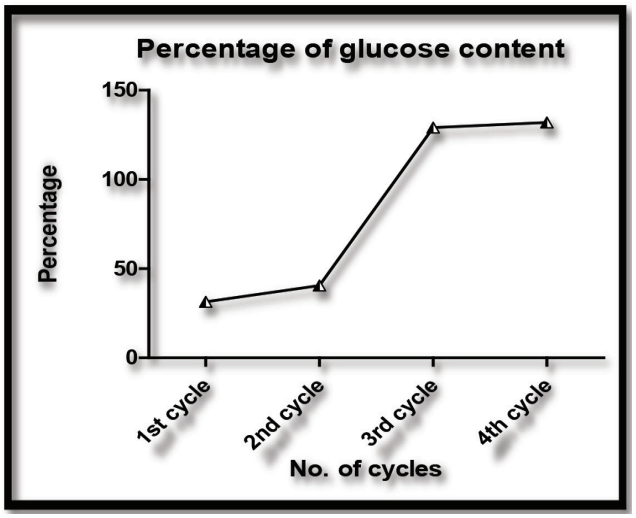

Figure 7: Percentage value of glucose content for each cycle of the treated sample.

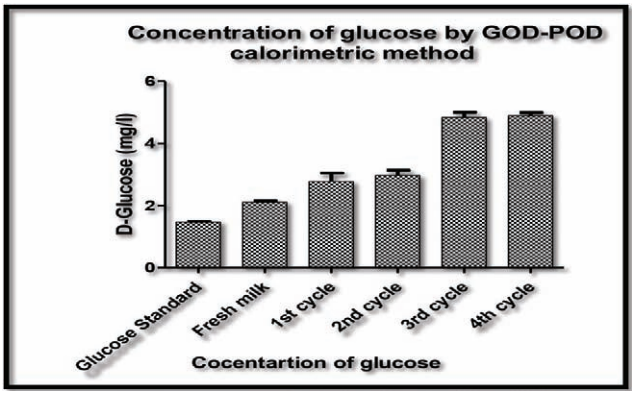

Figure 8: Concentration of glucose for each cycle by GOD – POD method.

maximum level of 4.891mg/l in the 4th cycle. The milk treated with enzyme was found to have less lactose and can be used by lactose-intolerant individuals in the hope to overcome health issues such as nutrient deficiency, osteoporosis disease. The problem is that there is no domestic production of lactose-free milk reported in India, but every day the consumption of lactose-free milk is getting increased. Thus novel strain *L. plantarum* KX838907 can be used in the commercial production of β-galactosidase as it is considered safe to use as a probiotic microorganism.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

ABBREVIATIONS

MRS: de Man, Rogosa and Sharpe; **X-Gal:** 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; **ONPG:** o-Nitrophenyl β-D-galactopyranoside; **Na_2HPO_4:** Disodium phosphate; **NaH_2PO_4:** Monosodium phosphate; **KCl:** Potassium chloride; **$MgSO_4$:** Magnesium sulfate; **Na_2CO_3:** Sodium Carbonate; **BLAST:** Basic Local Alignment Search Tool; **MEGA:** Molecular Evolutionary Genetics Analysis; **NCBI:** National Center for Biotechnology Infomation; **SEM:** Scanning Electron Microscope; **OFAT:** One Factor at a Time; **RSM:** Response Surface Methodology; **SDS-PAGE:** Sodium dodecyl sulphate polyacrylamide gel electrophoresis; **BSA:** Bovine Serum Albumin; **GOD-POD:** Glucose Oxidase-Peroxidase; **ANOVA:** Analysis of Variance.

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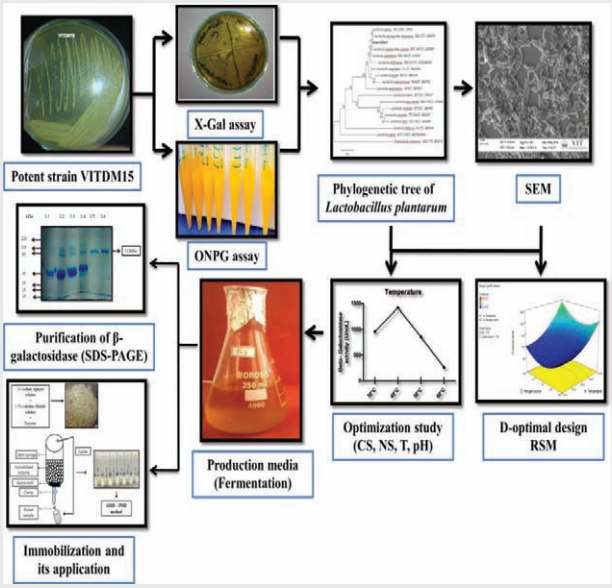
SUMMARY

The main aim of the research is to solve the most appropriate problem in abdominal clinical practice LACTOSE INTOLERANCE which causes a major issue in the digestive system when there is a lack of a β-galactosidase enzyme. The present study mainly focuses on the production, optimization, and purification of the β-galactosidase enzyme from *Lactobacillus plantarum* VITDM15. Lactic Acid Bacteria (LAB) isolated from donkey milk, curd, and yogurt in MRS media. Further, the isolates screened for β-galactosidase using an X-gal assay.

The one potent isolate VITDM15 isolated from donkey milk revealed maximum β-galactosidase production in the hydrolysis of o-Nitrophenyl β-D-galactopyranoside (ONPG) as the substrate. The partial sequence obtained from 16S rRNA was constructed for the phylogenetic tree using MEGA software by the neighbor join method. VITDM15 exhibited 99% similarity with *L. plantarum* and gene sequence submitted in the NCBI to obtain accession number. Optimized media enhanced the maximum β-galactosidase activity in lactose as a carbon source followed by beef extract as a nitrogen source. The enzyme was more active in temperatures ranging from 30°C to 40°C. The Stability of the pH for enzyme production was 7. The effect of their interactions on β-galactosidase production was further confirmed using RSM the statistical tool with a D-optimal experimental design in 3D interaction. The β-galactosidase enzyme was precipitated using 70% ammonium sulfate precipitation followed by ultracentrifugation and eluted in gel permeation chromatography. The molecular weight of the protein was 112Kda in SDS-PAGE. Purified β-galactosidase from *L. plantarum* KX838907 was yielded 31.75% with 67.73 fold and specific activity of 1705IU/mg. Further, the enzyme was immobilized by 2% sodium alginate to make lactose-free milk. In the milk treated with purified β-galactosidase, the glucose dosage increased up to a maximum level of 4.891mg/l in the 4th cycle in GOD-POD assay. From the results obtained in the study, it was summarized that the novel strain *L. plantarum* VITDM15 can efficiently produce the β-galactosidase enzyme. Thus *L. plantarum* can be used in the commercial production of β-galactosidase as it is considered safe to use as a probiotic microorganism and it also gives high enzyme yield.

PICTORIAL ABSTRACT



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