

Research Article

Production of Bacterial β -Galactosidase Using Wastes of Dairy Industry

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Abstract | UF-Cheese permeate is a dairy byproduct characterized by high lactose content, generated through the ultrafiltration process that separates proteins and fats from milk. The aim of this study was to use UF-Cheese permeate as a medium for promoting bacterial growth to producing the β -galactosidase (lactase) enzyme, which is beneficial for individuals with lactose intolerance in manufactured dairy products. Lactic acid bacteria, also known as LAB are recognized as effective producers of the lactase enzyme. A total of eleven LAB isolates were evaluated for their ability to produce intracellular lactase in a submerged fermentation setup under static conditions. Among the isolates that demonstrated positive results, the most prolific lactase producer was identified as *Lactiplantibacillus plantarum* strain *S1* through 16S rDNA sequencing. The assay of β -galactosidase production was performed using O-nitrophenyl β -D-galactopyranoside (ONPG) as a hydrolytic substrate. Improvement of β -galactosidase synthesis from the selected *Lactiplantibacillus plantarum* strain *S1*, utilizing permeate as a growth medium, was achieved through the application of response surface methodology. A peak β -galactosidase activity of 5.523 U/ml was recorded at a pH 7, a lactose concentration of 4.6 %, UF-cheese permeate serving as a carbon source, 2 % potassium nitrate (KNO₃) as a nitrogen source, an inoculum size of 5%, and incubated at 37 °C for 24 h. In this study, the UF-cheese permeate as a dairy waste, considered an environmental pollution, was used as a fermentation substrate for functional microorganisms such as LAB, offering a promising alternative for waste management and sustainable production of high-quality metabolites, including enzymes, mainly proteases and β -galactosidase, organic acids, exopolysaccharides (EPSs), and bacteriocins. This is in addition to isolating a novel lactic acid bacterial strain identified as *Lactiplantibacillus plantarum* strain *S1*, which displayed the potential to generate β -galactosidase when cultivated on UV-cheese permeate as a fermentation medium. The obtained β -galactosidase enzyme had the capacity to facilitate the substantial manufacturing of low-lactose dairy products tailored for individuals with lactose intolerance. This enzyme has diverse applications in food, pharmaceuticals, agriculture, and biofuels. This suggests that a bacterial strain may not only have a capacity for enzyme production but also operates efficiently, representing a promising candidate for further studies and potential applications in the biotechnological processes.

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Introduction

UF-cheese permeate is recognized as a significant byproduct of the cheese manufacture sector, derived through an ultrafiltration (UF) process. This permeate primarily consists of lactose, along with vitamins that are soluble in water and various milk minerals (Gantumur *et al.*, 2025). It typically contains at least 76 % lactose, 14 % ash, and generally 2-7 % proteins. UF-cheese permeate serves as a valuable resource of essential vitamins and minerals that are beneficial to the human health. Nevertheless, its disposal poses challenges for the dairy industry, contributing to environmental pollution. As a result, significant investments have been made to explore the utilization of permeate in the development of advantageous products (Premović *et al.*, 2024). Whey permeate can be considered as a carrier of prebiotics and various bioactive compounds, including peptides, poly- and oligosaccharides, and organic acid that may play a functional role in skin care (Augustyniak *et al.*, 2023).

The dairy sector is commonly regarded as a carbon-intensive industry with a significant global environmental impact, contributing to considerable greenhouse gas (GHG) emissions and aggravating global warming. Previous studies concluded that without comprehensive actions to attain near “net-zero” emissions, global climate changes will be intensified, leading to several climatic disasters and serious problems to food security and even human existence (Lam *et al.*, 2021; Cheng *et al.*, 2023).

A carbon or greenhouse gas emissions frequently has a close correlation with energy use. Thus, given that dairy is a significant energy user, carbon emissions at each stage of dairy supply chain may be estimated based on energy consumption levels (Malliaroudaki *et al.*, 2022). Since climate change is the most pressing global concern currently, significant efforts have been devoted to mitigating carbon emissions in the dairy industry, which can be divided into two facets: (i) at the component level, novel techniques and strategies are being used that can reduce emissions at individual stages of the dairy supply chain, and (ii) at the system level, focusing on holistic improvements throughout the entire dairy supply chain (Chen *et al.*, 2021).

Lactose intolerance is a common medical condition that impacts individuals across various ethnicities

globally. β -galactosidase is important for numerous applications; particularly within the food industry linked to healthcare, as it facilitates the creation of lactose-free dairy products for individuals with lactose intolerance due to insufficient levels of this enzyme (Chengolova *et al.*, 2024). Deficiency in lactase may lead to a range of medical symptoms, such as gastrointestinal disturbances, bloating, abdominal discomfort, and diarrhea (Khalid *et al.*, 2024; Borralho and Marcos, 2025).

β -galactosidase, commonly referred to as lactase (β -D-galactoside galactohydrolase; EC 3.2.1.23) is an intracellular enzyme that facilitates the hydrolysis of lactose, a disaccharide present in milk and dairy products. This enzymatic process breaks down lactose into its monosaccharide components, galactose and glucose, which are readily absorbable by the majority of organisms, including human (Chengolova *et al.*, 2024).

β -galactosidase is used in the food, pharmaceutical, and dairy industries for producing low-lactose-containing food products, sweeteners, and galacto-oligosaccharides (GOS), in addition to being utilized for dairy waste treatment, and biosensor and ethanol production that is a challenging process in this century (Santra and Banerjee, 2021; Movahedpour *et al.*, 2022). Additionally, consumption of GOS has been linked to various health benefits for humans, including potential anti-carcinogenic properties within the colon, enhanced absorption of minerals, improved lipid metabolism, and anti-inflammatory and immune-boosting effects (Nadeem *et al.*, 2024).

Immobilization of β -galactosidase by nanobiocatalysts has evolved as a novel field for enhancing enzyme stability in biotechnology applications. The immobilized enzyme demonstrated a high degree of hydrolysis, which may be successfully used to hydrolyze lactose in milk and whey. Utilizing β -galactosidase offers a cost-effective alternative for biotechnology and waste management (Alshanberi *et al.*, 2021). Immobilization has proven to be an excellent alternative to the previously described techniques for enzyme stabilization, owing to its lower cost, simplicity, and increased chances of reusing and recovering the enzyme. It is the process by which enzymes are attached or incorporated into a support material. An insoluble, reusable, and more resistant version of the enzyme is generated, capable of participating in chemical reactions under a variety

of processing conditions, ideally without considerable loss of stability or activity (Robescu and Bavaro, 2025). Immobilized enzymes (IE) are used in several fields, such as the food and pharmaceutical industries, medicine, for the production of biofuels, detergents, and cleaning products, and in bioremediation and wastewater treatment. There are several previous studies on this topic (Bié *et al.*, 2022; Maghraby *et al.*, 2023).

Microbial sources are considered the most efficient ones for enzyme production due to several reasons, including ethical issues concerning plants and animals, microbial fast growth, and high yield of the enzyme. Out of the producer microorganisms, LAB were found to be the best choice for enzyme production; especially for food applications (Kumar *et al.*, 2024). LAB; especially species in the *Lactobacillus* genus, have become a prominent focus of research among the different microbial producers of this enzyme because they are safe for both humans and animals; thus, their relevance is continually expanding. They are generally recognized as safe (GRAS), (Kittibunchakul *et al.*, 2020). LAB are frequently employed as probiotics, which offer health benefits to the host when taken in appropriate doses, where they display several health benefits. LAB play a significant role in biopreservation as they produce various antimicrobial metabolites during their development and fermentation processes. Among the most common antimicrobials are bacteriocins, which are peptides that can kill or inhibit undesirable bacterial strains, regardless of their relatedness, without harming the LAB themselves (Ahansaz *et al.*, 2023). The utilization of LAB strains, namely *Enterococcus* spp., *Lactococcus* spp., and *Lactobacillus* spp., possessing antibacterial capabilities, has been employed in biocontrol approaches aimed at diminishing mycotoxins and augmenting bioavailability. Formation of organic acids, carbon dioxide, ethanol, hydrogen peroxide, antifungal chemicals, and antibiotics protects foods (Saleem *et al.*, 2024). Additionally, the utilization of probiotic strains as biofactories for the production of enzymes has gained substantial attention recently, as their application in the food and pharmaceutical industries typically requires minimal purification procedures (Carevic *et al.*, 2018). *Lactiplantibacillus plantarum* represents a significant component in the LAB species. Classified as a facultative anaerobic heterofermentative bacterium, *L. plantarum* falls under the Group B *Lactobacillus* classification that is predominantly originated from vegetable-derived

food items (Liu *et al.*, 2024). This bacterium plays a dual role, as a starting culture for different fermented foods and a probiotic agent (Ma *et al.*, 2021).

Numerous variables affect the synthesis of lactase, including temperature, pH levels, duration of incubation, and composition of the production medium. The refinement of the fermentation medium is vital for effective generation of β -galactosidase, as highlighted by Afolabi *et al.* (2022). Availability of carbon and nitrogen inputs is crucial for promoting enzyme synthesis by the LAB (Ayad *et al.*, 2020). Various studies have examined these variables that impact β -galactosidase fabrication, as discussed by Karlapudi *et al.* (2018).

The objectives of this investigation were to isolate native LAB capable of generating β -galactosidase, identify the specific bacterial strains responsible for its production, and optimize the conditions for enzyme synthesis; utilizing permeate as a substrate *via* a statistical optimization methodology.

Materials and Methods

Isolation of β -galactosidase -producing bacteria

Eighteen dairy products samples were collected from local markets in Giza governorate, Egypt, including 6 yoghurt, 3 raw milk, 5 Rayeb milk, and 4 types of cheese (*i.e.*, 2 Kareish, 1 Romy, and 1 Cheddar). For bacterial isolation, two selective media were used, mainly MRS (De Man Rogosa-Sharpe) and M17 agar media (Biolife Italiana S.R.L. Milan, Italy). The MRS agar medium was employed for the isolation of lactobacilli bacteria, as elaborated by Chandel *et al.* (2020), while isolation of lactococci and streptococci bacteria was conducted utilizing the M17 agar medium in reference to Waiel *et al.* (2020). For bacterial isolation and colony purification, a serial dilution agar plate technique was employed in reference to Ismail *et al.* (2018). 1 ml from dilutions 10^{-5} , 10^{-6} , and 10^{-7} was pipetted into sterile petri plates and then MRS and M17 agar medium were aseptically poured, followed by horizontal rotation for homogenous mixing. The plates were incubated at 37 °C for 48 h under anaerobic conditions using the anaerobic candle jar. After incubation, the developing colonies were purified by successive sub-culturing onto MRS agar and then stored in 20 % glycerol at -80 °C (Naziri *et al.*, 2023).

Screening of bacterial isolates for β -galactosidase production

Qualitative screening: X-gal (5-Bromo-4-chloro-3-indoxyl- β -D-galactopyranoside) (Sigma-Aldrich Chemical Co. located in St. Louis, MO, USA) served as a colorimetric substrate and had been utilized for identifying bacteria that produce β -galactosidase (Vinderola and Reinheimer, 2003). The purified isolates were cultured on MRS and M17 agar petri plates in triplicate with the incorporation of 50 μ l of X-gal (20 mg/ml in DMSO), followed by incubation at 37°C for 24 to 72 h under anaerobic conditions. After incubation, the bacterial strains exhibiting β -galactosidase activity produced blue colored colonies (Gheyntanchi *et al.*, 2010; Sharma and Singh, 2014). The assay was conducted in triplicate.

Quantitative screening: The bacterial colonies displaying a blue color on X-gal agar medium were quantitatively evaluated for β -galactosidase synthesis to identify the potent isolates achieving the highest enzyme yield. Hundred ml Erlenmeyer flasks, individually containing 50 ml of MRS and M17 broth were enriched with 1% lactose and 0.05% L-cysteine, followed by sterilization. Under aseptic conditions, the flasks were inoculated with 2 % (v/v) of the bacterial inoculum (5×10^7 cfu/ ml) followed by incubation at 37 °C for 24h under anaerobic conditions using an anaerobic jar (Shrushti *et al.*, 2017). Post-incubation, the bacterial cells were harvested *via* centrifugation at 2.862 g for 20 min at 4 °C. The activity of β -galactosidase enzyme of the isolates was measured according to the procedure described by Hsu *et al.* (2005), with little modifications. The reaction mixture contained 0.5 ml of supernatant containing the extracted enzyme (intracellular enzyme), 0.5 ml 15 mM O-nitrophenyl β -D-galactopyranoside (ONPG) (sigma-Aldrich Chemical Com. MO, USA) in 0.03 M sodium phosphate buffer (pH 6.8). After incubation at 37 °C for 30 min., 2.0 ml of 0.1 M sodium carbonate was added to the mixture to stop the reaction. Absorbance was measured at 420 nm using a spectrophotometer (Shimadzu UV-vis spectrophotometer, Model UV-1201, Japan). One unit of β -galactosidase was defined as the amount of enzyme that produced 1 μ M of O-nitrophenol/min. under the assay condition. All treatments were conducted in triplicate.

Identification of the selected bacterial isolate

The selected bacterial isolate was identified

morphologically and molecularly.

Morphological characterization: Morphological characteristics of the pure bacterial isolate were studied through Gram stain followed by microscopical investigation as described by Goyal *et al.* (2012).

Molecular identification: Identification of the selected isolate was achieved *via* sequencing of the 16S rRNA using polymerase chain reaction (PCR) (Lagacé *et al.*, 2004). Gene sequencing services were provided by Macrogen laboratories, South Korea. The extraction of the DNA from the isolates was performed using a DNA purification kit, specifically the QIAamp DNA Mini Kit (Catalogue no. 51304). The oligonucleotide primer sequences used in the PCR were identified as follows: Forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') and Reverse primer (5'-TACGGYTACCTTGTTACGACTT-3') (Metabion, Germany).

Testing UF-cheese permeate as a growth medium

The UF-cheese permeate was used as a fermentation medium to test the ability of the selected isolates to utilize it as a growth medium. The fresh permeate (pH 6.6-6.8) was obtained from the dairy industry unit at the Animal Production Research Institute, Ministry of Agriculture, Giza, Egypt. According to Johansen *et al.* (2002), UF-Cheese permeate comprises lactose concentration that varies from 4.4 to 5.2 %, proteins that constitute 0.6 to 0.8 %, and minerals found in concentrations of 0.43-0.95 %. Where, the pore size of the UV-cheese permeate membrane exceeded 30 bars under processing conditions, specifically at a temperature of 40-50 °C and a pressure range of 1-10 atm. To de-proteinize the permeate, the pH was adjusted with lactic acid to 4.5. Thereafter, the sample was heated at 90°C for 30 min. The resulting precipitated proteins were subsequently separated through the use of Whatman No. 1 filter paper, and the clarified permeate was preserved at -20 °C to prevent microbial and enzymatic degradation (El-Sawah *et al.*, 2006). After sterilization at 121°C for 20 min., the pure supernatant was utilized as a substrate medium. The process of submerged fermentation (SmF) was executed using 100 ml Erlenmeyer flasks under anaerobic static culture conditions. The permeate was evaluated as a growth medium *via* the One Factor At a Time (OFAT) method to enhance the influence of carbon and nitrogen sources on β -galactosidase

production in the fermentation medium. Three separate carbon sources were individually tested, mainly glucose, sucrose and starch alongside with the naturally occurring permeate lactose. The lactose concentration was assessed through various dilutions of the permeate, in accordance with the previous procedures outlined by [El-Sawah *et al.* \(2006\)](#); [Murad *et al.* \(2011\)](#). Additionally, the impact of 3 nitrogen sources, including yeast extract, beef extract, and potassium nitrate was examined individually, as reported by [Sumit and Priyanka \(2014\)](#); [Carević *et al.* \(2015\)](#). The indigenous lactose concentration within the permeate was assessed colorimetrically at wavelength 490 nm using the phenol sulfuric acid method described by [Mohamed *et al.* \(2012\)](#). Afterward, the production medium flasks were adjusted to pH 6.8, inoculated with 2 % (v/v) from the selected bacterial isolates that were cultured individually for 24 h in MRS and M17 broth, and maintained at the optimal temperature of 37 °C under anaerobic conditions for 24 h. After incubation, the production medium underwent centrifugation at 2.862 g for 20 min. at 4 °C to collect the bacterial cells. After the enzyme was extracted, the supernatant was subjected to enzyme assay using O-nitrophenyl β -D-galactopyranoside (ONPG) method.

Measurement of the lactose concentration

The estimation of lactose concentration was conducted following the methodology outlined by [Mohamed *et al.* \(2012\)](#). Initially, a volume of 2.0 ml of the permeate solution was transferred into a colorimeter tube for analysis, 2.0 ml of 80 % w/v phenol reagent were added (composed of 20 ml water and 80 g phenol dissolved at 50 °C). This reagent remained stable for minimum of 4 min. at room temperature. Subsequently, 5.0 ml of concentrated sulfuric acid were introduced gradually, enabling the acid to descend along the internal wall of the tube. The mixture was then swirled to ensure throughout mixing and allowed to cool for a period of 10 min. at ambient temperature. Absorbance readings were taken at 490 nm utilizing a UV/Vis spectrophotometer, with a blank sample created by substituting the diluted extract with 2 ml of distilled water. L-lactose served as the standard, while the concentration of lactose was evaluated by employing the regression equation derived from the standard curve ($92.804x + 0.6703$, $R^2 = 0.995$). The result was expressed in g/ 100 g of the sample. All treatments were performed in triplicate.

The β -galactosidase enzyme activity assay

Extraction of the β -galactosidase using sodium dodecyl sulfate (SDS) chloroform processing: Following 24 h of incubation, the bacterial cell cultures were collected through centrifugation at 2.862 g for 20 min. at 4 °C. The resulting supernatant was deemed to contain extracellular enzymes. Subsequently, the cell pellet was homogenized and subjected to two washes with 0.03 M sodium phosphate buffer, maintained at pH 6.8. The washed pellets were reconstituted in 5 ml of the identical phosphate buffer. The process of extracting the intracellular enzymes was accomplished through the application of Sodium Dodecyl Sulfate (SDS) and Chloroform treatments, in reference to [Prasad *et al.* \(2013\)](#).

Disruption of the bacterial cell membrane was achieved by vortexing a 5 ml suspension of cells with 250 μ l of chloroform (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 100 μ l of 0.1 % SDS solution (Park Scientific Limited, 24 Low Farm Place, Moulton Park, Northampton, Northamptonshire, NN3 6HY, UK) for 30 min. at ambient temperature. Afterward, centrifugation of the mixture was conducted with a Harrier 18/180 refrigerated centrifuge (model MSE, U.K.) at 2.862 g for 20 min. at 4 °C. The supernatant produced was subsequently kept at -20 °C until the enzyme activity was determined, as indicated by [Prasad *et al.* \(2013\)](#).

Estimation of the β -galactosidase enzyme activity

β -galactosidase activity was assessed following the methodology outlined by [Hsu *et al.* \(2005\)](#), with slight modifications. The reaction mixture consisted of 0.5 ml of the supernatant that contained the extracted intracellular enzyme, 0.5 ml 15 mM O-nitrophenyl β -D-galactopyranoside (ONPG) solution (Sigma-Aldrich, Chemical Co., St. Louis, MO, USA), and dissolved in 0.03 M sodium phosphate buffer at pH 6.8. Following 30 min. incubation at 37 °C, 2.0 mL of 0.1 M sodium carbonate were added to halt the reaction. The value of absorbance was subsequently recorded at 420 nm using a Shimadzu UV-vis spectrophotometer (Model UV-1201, Japan). One unit of β -galactosidase is defined as the amount of enzyme that catalyzes the formation of 1 μ M of O-nitrophenol / min. within the specified test aspects ([Gomes *et al.*, 2021](#)). All tests were performed in triplicate.

Assessment of the specific activity of β -galactosidase

The specific activity of β -galactosidase is defined in

terms of U/ mg protein/ ml, utilizing the following formula reported by [Geiger et al. \(2016\)](#):

$$\text{Specific activity (U/ mg)} = \frac{\text{enzyme activity (U/ ml)}}{\text{protein content (mg/ ml)}}$$

Protein assay

The overall protein concentrations of the bacterial isolates were evaluated utilizing the methodology reported by [Yglesias-Rivera et al. \(2020\)](#), employing Bovine Serum Albumin (BSA) at a concentration of 0.2 mg/ ml as the standard protein reference. Reagent A: 2 % sodium carbonate (Na_2CO_3) in 0.1 N sodium hydroxide (NaOH) containing 2 g anhydrous Na_2CO_3 dissolved in 100 ml of 0.1M NaOH. Reagent B: 1 % sodium potassium tartrate in distilled water, containing 1 g of sodium potassium tartrate dissolved in 100 ml distilled water. Reagent C: 0.5 % $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in distilled water containing 0.5 g of copper sulfate hepta hydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) dissolved in 100 ml distilled water. Reagent D (Lowry solution) containing 98 ml of A +1 ml of B + 1 ml of C, while Reagent E contained 1 part Folin: 1 part distilled water. Reagent D (4.5 ml) were added to 1 ml of each properly diluted crude enzyme filtrate, mixed by vortexing and incubated at room temperature for 10 min, After incubation, 0.5 ml of Reagent E was added to the mixture, mixed well, and incubated at room temperature for 30 min. The absorbance of the resulting colored fluid was measured using a spectrophotometer (Shimadzu UV-vis spectrophotometer, Model UV-1201, Japan) at 660 nm against a control without the sample. All testes were carried out in triplicate.

Optimization of the culture conditions for β -galactosidase biosynthesis

Five process parameters (factors), mainly temperature, pH, inoculum volume, nitrogen resource, and growth time were systematically tailored to enhance the yield of β -galactosidase, using UF-cheese permeate as a growth environment *via* Response Surface Methodology (RSM) ([Deng et al., 2020](#)). The optimization procedures were carried out utilizing the Minitab (18-1) software package.

Application of response surface methodology for bacterial culture optimization using central composite design

Analysis of the data was conducted utilizing Minitab Statistical Software (version 18-1) developed by Minitab®, State College, PA, USA. To ascertain the

optimal levels of the five tested parameters impacting enzyme generation, a response surface methodology (RSM) was employed, based on findings from our one-factor at a time (OFAT) tests and previous studies reported by [Wang et al. \(2004\)](#), [Hsu et al. \(2007\)](#), [Murad et al. \(2011\)](#), [Shrushti et al. \(2017\)](#). The experimental design consisted of a single replicate. Each parameter was assigned five level codes: $-\alpha$, -1 , 0 , $+1$, and $+\alpha$. From the combinations of these five variables at the corresponding levels, a total of 54 experimental designs were created. The study utilized a central composite design (CCD), which is a preferable method compared to other methods such as Box–Behnken design etc., because CCD methodology provides better information within or beyond the limits of the spinning process. The CCD contains a combination of extreme factors, whereas the box Behnken does not examine the boundary area. Therefore, the CCD is considered more accurate and does not require a three-level factorial experiment in the operation of the quadratic model ([Safira and Purbasari, 2023](#)).

The CCD incorporated five unrelated factors, each with five distinct tiers along with 32 star points ($\alpha = 2.366$) with eight repetitions at the center points. This design was implemented to formulate a prototype, which would identify the ideal levels for the parameters influencing the β -galactosidase production. The experiments were conducted in 100 ml Erlenmeyer flasks, where each flask contained 50 ml of the generating medium. The variables tested, along with their coded and non-coded values are presented in [Table 1](#).

Table 1: The various values of the factors utilized in the experimental design.

The variables	The source code	The tested levels					Unit
		-2	2	0	+1	+2	
Nitrogen concentration	N	0.634	2	3	4	5.366	%
pH	pH	3.634	5	6	7	8.366	pH
Inoculum size	IS	1.634	3	4	5	6.366	%
Temperature	T	25.219	30	33.5	37	41.781	° C
Incubation period	IP	7.608	24	36	48	64.392	h

Statistical analysis

The experiments were executed with triplicate to improve the credibility of the findings. The outcomes were articulated as mean $\pm$ standard deviation (SD).

In the context of CCD tests, multiple runs were replicated, and the mean results served as the input for further analysis. Regression analysis, along with the significance testing of the regression coefficient and the identification of significant differences among the assessed variables was conducted by studying the analysis of variance (ANOVA) through Minitab (18-1) Statistical Software.

Results and Discussion

Isolation of β -galactosidase -producing bacteria

Seventeen different bacterial isolates were picked up from the surfaces of M17 and MRS (De Man-Rogosa-Sharpe) agar plates. After purification, the obtained isolates were evaluated qualitatively for their capability to generate the target enzyme.

Screening of isolates for β -galactosidase production

Qualitative screening: Upon qualitative screening of the seventeen bacterial isolates obtained using the X-gal technique, eleven isolates demonstrated positive results for β -galactosidase production, exhibiting blue or white-blue colonies when plated on MRS and M17 agar. These isolates were subsequently selected for further studies.

Quantitative screening: Following the qualitative screening, eleven isolates that showed positive for β -galactosidase production using the X-gal method were quantitatively evaluated for their levels of β -galactosidase production by employing ONPG substrate estimation. The isolates that produced β -galactosidase enzyme gave a yellow color. Upon colorimetric estimation, the best enzyme producers were isolates 3aY, 9bR, 5aY and 8aR1; while upon estimating the enzyme activity, the 3aY isolate displayed the superiority compared to the other isolates, recording 14.154 U/ ml (Figure 2).

Identification of the bacterial isolates

Morphological characterization: All the selected LAB isolates showed the colony morphology of LAB colonies, which was cream-white, round, and smooth surface colonies on MRS agar in agreement with Kouadio *et al.* (2024). Meanwhile, the *Lactococcus* colonies on M17 agar were yellowish in color, small in size, and circular, in accordance with Taye *et al.* (2021). Upon Gram staining, the isolates obtained from MRS plates were Gram (+), characterized by being rod-shaped cells arranged either individually or in short chains.

Molecular characterization

The identification of the selected bacterial isolates at the species level was confirmed and validated through the application of a molecular technique. Four bacterial isolates that revealed higher β -galactosidase activity were identified *via* partial sequencing of their 16S rRNA. According to the alignment results, all the sequenced samples were assigned to 2 distinct species within the genus *Lactiplantibacillus*, as illustrated in Table 2. The potent enzyme producer was identified as *Lactiplantibacillus plantarum* strain S1 and assigned an accession no. of PQ722056. Phylogenetic studies focusing on 16S rRNA amplification were primarily performed, and the acquired partial 16S rRNA sequence was deposited in a Genbank database for verifying the homology alignment. The phylogenetic tree was constructed using MEGA11: Molecular Evolutionary Genetics Analysis version 11, and visualized and annotated using the interactive online tool iTOL v7 (Tamura *et al.*, 2021; Letunic and Bork, 2024). The phylogenetic tree alignment of the *Lactiplantibacillus plantarum* strain S1 is presented in the Figure 1.

Table 2: Identification of the selected bacterial isolates for β -galactosidase production using 16S rRNA sequencing.

Isolate code	Identification	Accession number	Similarity
3aY	<i>Lactiplantibacillus plantarum</i> strain S1	PQ722056	98.26%
9bR	<i>Lactiplantibacillus pentosus</i> strain S2	PQ722057	98.08%
5aY	<i>Lactiplantibacillus plantarum</i> strain S3	PQ722058	89.29%
8aR1	<i>Lactiplantibacillus plantarum</i> strain S4	PQ722059	97.33%

Testing UF-cheese permeate as a growth medium

Under anaerobic static conditions, the submerged fermentation (SmF) process was executed in 100 ml Erlenmeyer flasks. These anaerobic static cultures facilitated greater outputs of biomass and enzymes. The enhanced biomass production is ascribed to the microorganism's ability to effectively utilize different carbon sources; particularly glucose, as documented by Tesfaw (2023). Anaerobic environments usually trigger the activation of the pyruvate-formate lyase system, which *via* the actions of acetyl phosphate and acetyl kinase, promotes a higher ATP release per glucose molecule metabolized (Taggar *et al.*, 2024). The occurrence of this feature has been observed in

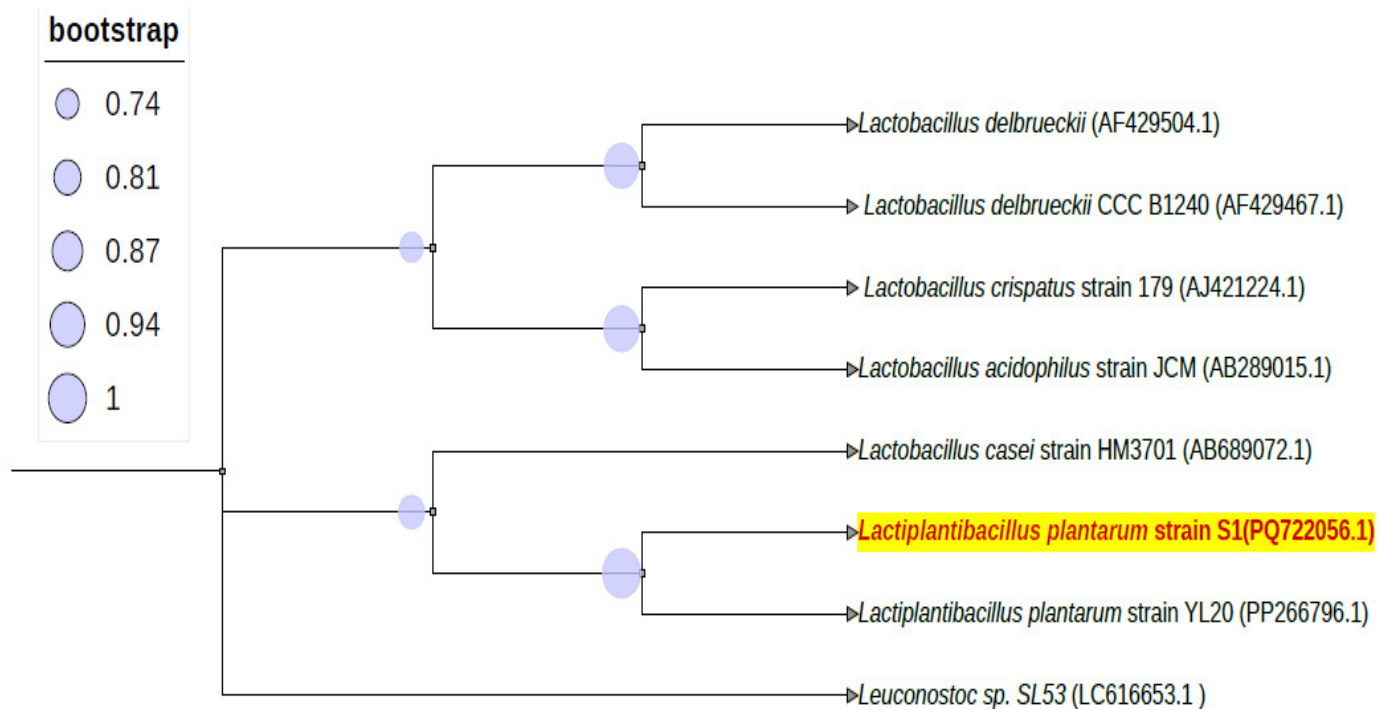


Figure 1: The phylogenetic tree of 16S rRNA sequence of *Lactiplantibacillus plantarum* strain S1. The bootstrap symbols, often represented as percentages or numbers, indicate the level of support for particular node or branch and the confidence in the phylogenetic tree.

various lactobacilli, such as *L. johnsonii* and *L. gasseri*, as reported by Maresca et al. (2018), along with *L. plantarum* as stated by Deng et al. (2020).

Additionally, the role of the carbon resource in enzyme production is dependent on the specific microbial species examined (Ray et al., 2023). The characteristic and concentration of the carbon substrate are recognized as two of the most crucial determinants for β -galactosidase fabrication in lactobacilli in accordance with Mukherjee et al. (2024).

The data obtained demonstrated that the most potent carbon resource for β -galactosidase activity stimulation was lactose. Afterward, glucose and sucrose contributed to lower levels of enzyme activity, while starch inhibited this activity, as shown in Figure 3. Our findings coincide with the previous study conducted by Gomes et al. (2021), who identified lactose as the prime carbon substrate for achieving optimal β -galactosidase production in *Bifidobacteria*.

Measurement of the lactose concentration

The results depicted in Figure 3 demonstrate that the production of β -galactosidase by the selected bacterial strain rose in correlation with the level of cheese permeate, reaching its peak at 12 %. The peak enzyme

activity was achieved at a lactose content of 4.0 % existing in the 12% permeate concentration, and then decreased when the lactose content in 15 % permeate concentration increased more than 4 %. Similarly, *Candida pseudotropicalis* exhibited maximum enzyme production at cheese whey concentrations between 10 % and 12 %, corresponding to lactose levels of 7.3 % to 8.7 % (Carvalho et al., 2021). Furthermore, in another study, the maximal lactase yield from *S. thermophilus* was obtained at a permeate concentration of 10 % according to Chandel and Sharma (2020). Recently, a 4% lactose concentration effectively elicited the optimal levels of β -galactosidase expression within the parameters of the study reported by Hooda et al. (2025). However, further increments in lactose concentration resulted in diminished β -galactosidase activity. This may be ascribed to the fact that elevated levels of glucose generated inhibited the generation of lactase enzyme in the microorganism being tested (catabolite repression). This finding is in harmony with the study performed by Sahoo et al. (2023). Furthermore, Da Silva et al. (2020) reported that the best concentration of lactose for β -galactosidase fabrication by *Enterococcus faecium* was 5.35 %. In contrast, it was reported that 7.0 % lactose is the ideal concentration to produce lactase from *Lactobacillus* spp. (Chandel and Sharma, 2020).

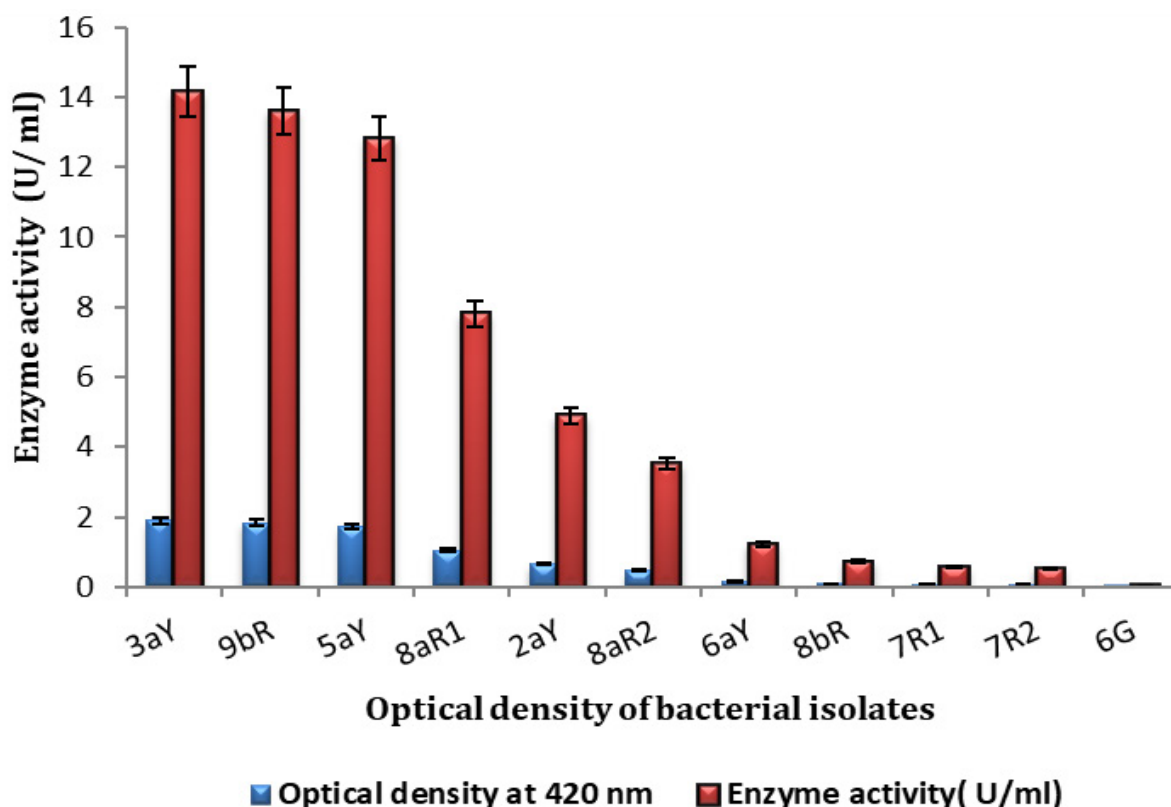


Figure 2: β -galactosidase activity and optical density for isolated bacterial strains. Each run was carried out as three replicate, and the results are represented as the mean $\pm$ standard deviation (SD). Each bacterial isolate demonstrated a statistically significant difference in the optical density and enzyme activity ($p < 0.05$). However, the results of the enzyme activity for each isolate varied significantly.

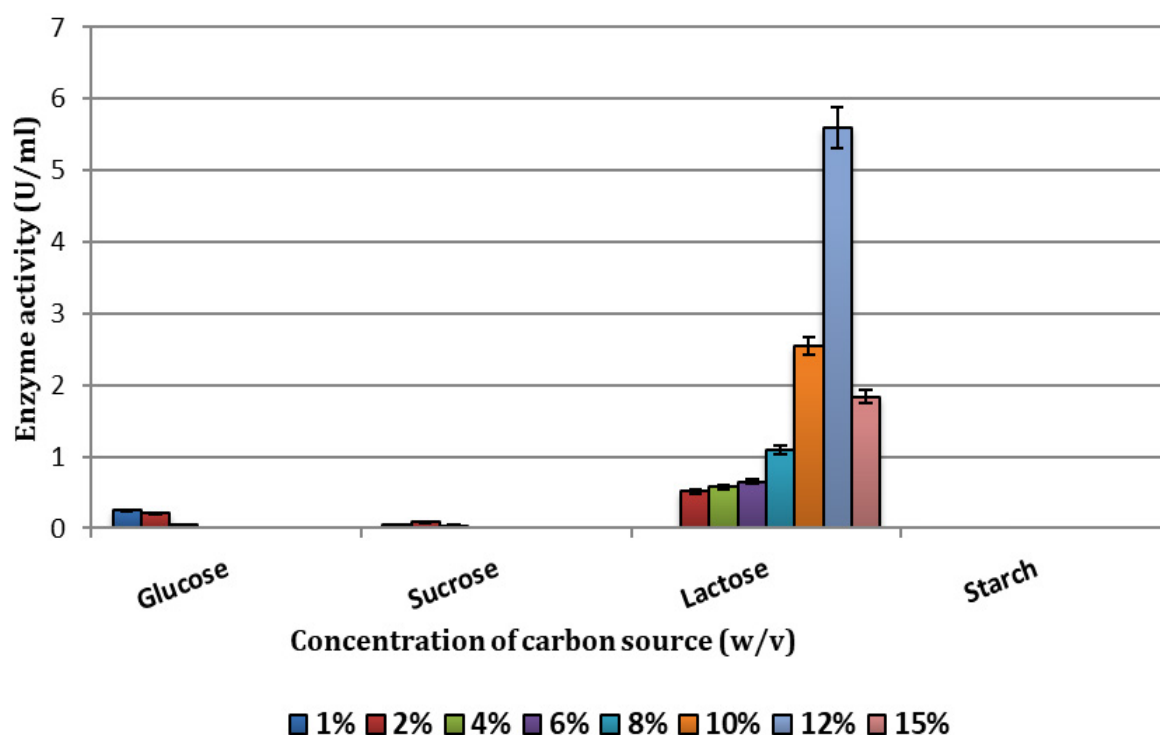


Figure 3: The relationship among various carbon sources and β -galactosidase activity of the isolated *Lactiplantibacillus plantarum* strain S1. Each run was carried out as three replicate, and the results are represented as the mean $\pm$ standard deviation (SD). Each carbon source has a statistically significant effect on the enzyme activity ($p < 0.05$) of the isolate. However, the maximum enzyme activity was achieved using lactose compared to other carbon sources.

Several previous studies reported by [Li et al. \(2023\)](#); [Wahab et al. \(2024\)](#) indicated that the inclusion of lactose as a carbon source in the growth medium performs a significant part in the synthesis of β -galactosidase by bacteria in consistence with our experimental findings. Moreover, the results documented by [Chandel and Sharma \(2020\)](#), [Gomes et al. \(2021\)](#) revealed how lactose sugar facilitates enzyme production in *Bifidobacteria* spp., *Lactobacillus reuteri*, and *Streptococcus thermophilus*. Furthermore, it has been reported that lactose is a substrate that significantly enhances the yield of lactase enzyme in *L. acidophilus* ATCC 4356, *L. plantarum* B110, and various *Lactobacillus* species ([Chandel and Sharma, 2020](#), [Ayivi and Ibrahim, 2022](#)).

The existence of lactose sugar is recognized as the best effective supply of carbon for inducing high levels of β -galactosidase production. This phenomenon is attributed to the fact that lactose substrate particularly in the allolactose isomer configuration, promotes the expression of the *lacZ* gene by attaching the *Lac* repressor protein, which in turn obstructs the activation of an operative zone of the *lac* operon as revealed by [Deng et al. \(2020\)](#). According to [Lappa](#)

[et al. \(2019\)](#), lactose derived from cheese whey serves as the best sugar precursor for the generation of β -galactosidase by *L. acidophilus*.

The majority of microbial species assimilate nitrogen at its organic and inorganic states to facilitate the creation of amino acids, nucleic acids, proteins, and cell wall constituents. The impact of nitrogen sources on β -galactosidase output has been previously assessed utilizing One Variable at a Time (OFAT) methodology ([Vithalani et al., 2024](#); [Malos et al., 2025](#)). The current results indicated that potassium nitrate (KNO_3) emerged as the optimal form of nitrogen for achieving the highest levels of β -galactosidase production by the *Lactiplantibacillus plantarum* strain S1 at a concentration of 2 %, as illustrated in [Figure 4](#). These findings align with those of ([Singh and Sambyal, 2023](#)) who similarly identified $(\text{NH}_4)_2\text{NO}_3$ and KNO_3 as the most effective nitrogen sources for various *L. plantarum* strains. It has been revealed that sodium nitrate is the most suitable nitrogen source for *Aspergillus oryzae*'s β -galactosidase synthesis ([Akcan, 2018](#)). However, excessive nitrogen levels may contribute to microbial cell mortality ([Sun et al., 2024](#)).

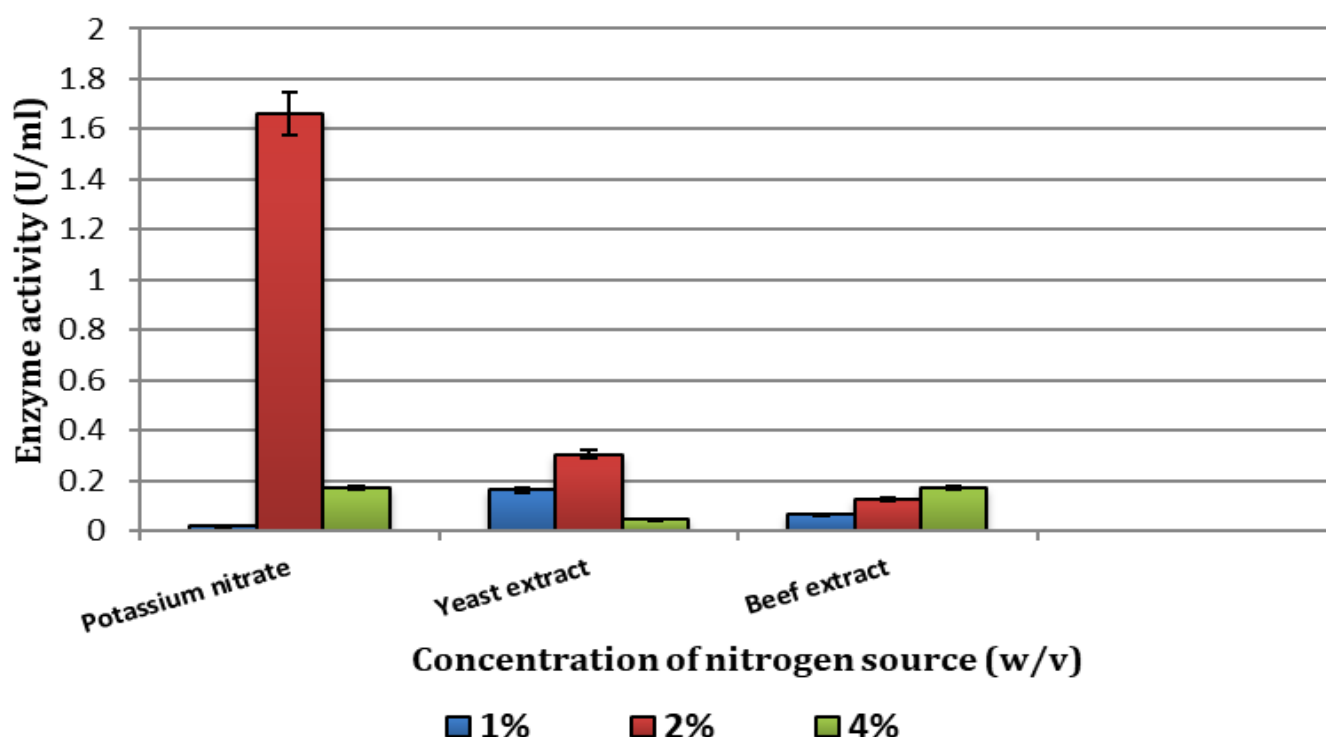


Figure 4: The correlation between the enzymatic activity of β -galactosidase and various nitrogen sources in the isolated *Lactiplantibacillus plantarum* strain S1. Each run was carried out as three replicate, and the results are represented as the mean $\pm$ standard deviation (SD). Each nitrogen source has a statistically significant effect on the enzyme activity ($p < 0.05$) of the bacterial isolate. However, the optimum enzyme activity was obtained using potassium nitrate (KNO_3) compared to other nitrogen sources.

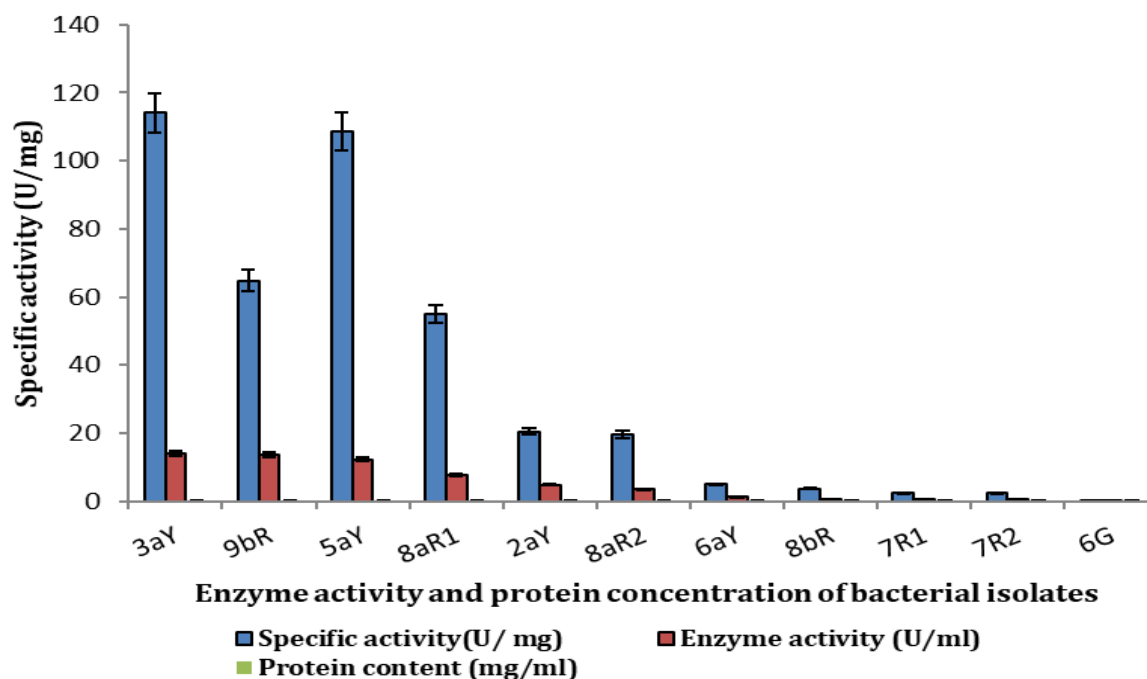


Figure 5: Correlation among enzyme activity, specific activity, and protein concentration of selected bacterial isolates. Each run was carried out as three replicate, and the results are represented as the mean $\pm$ standard deviation (SD). Each bacterial isolate demonstrated a statistically significant difference in specific activity and enzyme activity ($p < 0.05$). However, the results of the protein contents for each isolate varied significantly.

The β -galactosidase enzyme assay

Extraction of β -galactosidase utilizing sodium dodecyl sulfate (SDS)-chloroform processing: During this investigation, β -galactosidase enzyme was extracted utilizing SDS-chloroform treatment. SDS is classified as an inert cleaner that denaturates and alters the proteins' original structure by dissolving the non-covalent links within them. Due to its low reactivity, convenient volatility, and miscibility with various biological solvents, the chloroform solution is a liquid that is widely utilized. As a result, SDS and chloroform may operate in concert to enhance cell wall permeabilization and facilitate the development of enzymes (Mahadevaiah *et al.*, 2020).

Estimation of the enzyme activity: The eleven isolates that showed blue-colored colonies on X-gal agar plates were examined for their β -galactosidase production. The β -galactosidase activity with the corresponding specific activity for the eleven bacterial isolates is depicted in Figure 5. Among the bacterial strains analyzed, strain (3aY) demonstrated the greatest level of lactase activity (14.154 U/ ml) and specific activity (114.15 U/ mg) compared to the other tested isolates, when cultivated on an optimized MRS media. This is in agreement with the results reported by Nawal *et al.* (2018) on the *L. plantarum* P4 strain producing β -galactosidase with a value of 14.84 U/ mL.

Assessment of the protein and specific activity for β -galactosidase

The protein contents and specific activity of the β -galactosidase enzyme for the selected isolates were evaluated as shown in Figure 5. Based on the results obtained, we observed that when the enzyme activity of each bacterial isolate increased and protein content declined, the specific activity increased. There is a direct relationship between enzyme activity and specific activity, and an inverse relationship between them and protein content. Currently, the bacterial isolate (3aY) was the best enzyme producer compared to the other tested isolates, recording enzyme activity (14.154 U/ml), specific activity (114.15 U/mg), and protein content (0.124 mg/ml). The currently obtained results of the isolate (3aY) displayed that β -galactosidase enzyme's activity and specific activity increased with decreasing protein concentration, in accordance with the previous study reported by Nawal *et al.* (2018) on the *L. plantarum* P4 strain recording β -galactosidase activity of 14.10 U/ ml. The increasing bacterial biomass correlated with the specific enzyme activity, which reached a maximum of 114.15 U/ mg after 24 h of growth. This time point corresponds with the early stationary phase of bacterial growth. The fact that β -galactosidase levels were highest in the initial stationary phase is consistent with the findings of a previous study involving lactobacilli (Deng *et al.*, 2020).

Optimization of the culture conditions for β -galactosidase biosynthesis using response surface methodology

Based on the data obtained through the common One Factor at a Time (OFAT) approach, lactose was selected as a carbon supply, while potassium nitrate (KNO_3) used as a nitrogen source for the current experimental setup. A pivotal, widely employed mathematical design that facilitates the development of quadratic response surface models is the response surface methodology (RSM), as reported by [Abdella and Ibrahim \(2024\)](#) within the framework of RSM, the Central Composite Design (CCD) is a frequently utilized technique used to investigate how the experimental parameters affect the synthesis of β -galactosidase ([Sharma et al., 2023](#); [Bella et al., 2024](#)).

Our study aimed to enhance the medium's composition by evaluating the different levels of several factors previously identified as key contributors to β -galactosidase production. The implementation of RSM, enabled efficient improvement more than the conventional one-variable-at-a time approach ([Devi et al., 2022](#); [Bhatia et al., 2023](#)). The CCD was utilized within the scope of the RSM to enhance enzyme production. Afterward, we aimed to optimize growth conditions for the manufacture of β -galactosidase using the *Lactiplantibacillus plantarum* strain S1. This investigation considered five independent variables, mainly nitrogen, pH, inoculum size, incubation time, and temperature, with the corresponding predicted and observed activity of β -galactosidase presented in [Table 3](#). All observed values for β -galactosidase activity spanned from 0 to 5.523 U/ ml. Every parameter was investigated in two specific tiers (-1, +1) in addition to the central point (0), acting as the midway within the range of every component. Assessment of each variable was conducted at five levels, and the interactions affecting the creation of β -galactosidase were evaluated through a total of 54 experiments, as detailed in [Table 3](#).

The currently used independent variables (*i.e.*, pH, incubation temperature, inoculation volume, nitrogen level, and cultivation period), and the corresponding responses of the activity of β -galactosidase are detailed in [Table 3](#). Observed values for β -galactosidase activity varied between 0.007 and 5.523 U/ ml. Notably, a reduction in lactase activity was recorded at pH 6, whereas a peak activity of 5.523 U/ ml was achieved at pH 7 (level 2, run 28). In consistence

Table 3: Central composite design (CCD) values for β -galactosidase activity obtained by *Lactiplantibacillus plantarum* strain S1.

Run order	N	PH	IS	T	IP	E.A	Fit values
1	2	5	3	30	24	0.112	0.06116
2	4	7	3	30	24	0.045	-0.14099
3	4	5	5	30	24	0.030	-0.26927
4	2	7	5	30	24	0.648	0.88516
5	4	5	3	37	24	0.171	0.53146
6	2	7	3	37	24	2.996	2.83038
7	2	5	5	37	24	1.752	2.06861
8	4	7	5	37	24	4.673	4.36696
9	4	5	3	30	48	1.185	1.69924
10	2	7	3	30	48	0.060	0.06666
11	2	5	5	30	48	0.097	0.12088
12	4	7	5	30	48	0.030	-0.45426
13	2	5	3	37	48	0.119	0.03461
14	4	7	3	37	48	0.067	-0.02954
15	4	5	5	37	48	0.052	-0.11732
16	2	7	5	37	48	0.827	1.09361
17	3	6	4	33.5	36	0.097	0.12616
18	3	6	4	33.5	36	0.097	0.12616
19	3	6	4	33.5	36	0.097	0.12616
20	3	6	4	33.5	36	0.097	0.12616
21	4	5	3	30	24	0.030	-0.10646
22	2	7	3	30	24	0.134	0.19846
23	2	5	5	30	24	0.000	-0.17557
24	4	7	5	30	24	0.231	0.61704
25	2	5	3	37	24	0.760	0.94191
26	4	7	3	37	24	1.998	2.24551
27	4	5	5	37	24	1.632	1.72948
28	2	7	5	37	24	5.523	4.87791
29	2	5	3	30	48	1.588	1.58068
30	4	7	3	30	48	0.067	0.01079
31	4	5	5	30	48	0.052	0.31076
32	2	7	5	30	48	0.030	-0.47232
33	4	5	3	37	48	0.082	-0.09226
34	2	7	3	37	48	0.142	0.26916
35	2	5	5	37	48	0.089	-0.06437
36	4	7	5	37	48	0.581	0.86624
37	3	6	4	33.5	36	0.075	0.12551
38	3	6	4	33.5	36	0.075	0.12551
39	3	6	4	33.5	36	0.075	0.12551
40	3	6	4	33.5	36	0.075	0.12551
41	0.634	6	4	33.5	36	0.157	0.35484
42	5.366	6	4	33.5	36	0.030	-0.1109
43	3	3.634	4	33.5	36	0.015	-0.23629

Table continues on next column.....

Run order	N	PH	IS	T	IP	E.A	Fit values
44	3	8.366	4	33.5	36	0.783	1.09122
45	3	6	1.634	33.5	36	0.030	-0.23913
46	3	6	6.366	33.5	36	0.216	0.54206
47	3	6	4	25.219	36	0.015	0.14816
48	3	6	4	41.781	36	2.830	2.75378
49	3	6	4	33.5	7.608	2.460	2.45208
50	3	6	4	33.5	64.39	0.045	0.10986
51	3	6	4	33.5	36	0.007	-0.06417
52	3	6	4	33.5	36	0.007	-0.06417
53	3	6	4	33.5	36	0.007	-0.06417
54	3	6	4	33.5	36	0.007	-0.06417

Where N: Nitrogen concentration, IS: Inoculum size, IP: Incubation period, T: Temperature degree, EA: Enzyme activity

with Boukezzoula *et al.* (2019), the peak enzyme output for β -galactosidase reached 6.80 U/ ml for *Bifidobacterium animalis* ssp. *lactis* Bb12 and 7.77 U/ ml for *L. delbrueckii* ssp. *bulgaricus* ATCC 11842. Both strains were grown in cheese whey at a pH of 6.8 for 18 h at 37 °C. The peak yield of β -galactosidase generated by *S. thermophilus* cultivated on a permeate-based environment reached 7.85 U/ ml. This was achieved under specific culture conditions of 10 % lactose concentration within the permeate medium, a pH range of 6.0-6.5, utilizing ammonium phosphate as the nitrogen supply, and incubation at 35 °C for 24 h (Chandel and Sharma, 2020). Furthermore, the productivity of lactase created by *Bifidobacteria* sp. increased when the cultivation temperature rose above 22 °C–37 °C (Gomes *et al.*, 2021). Elevated cultivation temperatures led to declined enzyme synthesis. The results of the study conducted by Boukezzoula *et al.* (2019) on the β -galactosidase production from *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Lactobacillus delbrueckii* spp. *bulgaricus* ATCC 11842 that were grown in cheese whey are consistent with our results for β -galactosidase biosynthesis by the selected isolate of *Lactiplantibacillus plantarum* strain S1; yielding 5.523 U/ ml, under culture conditions of pH 7, temperature 37 °C, and incubation for 24 h, when cultivated in UV-cheese permeate medium. Where, lactose in cheese permeate served as a carbon source and KNO₃ used as a nitrogen source. This is also compatible with Chandel and Sharma (2020) investigation on β -galactosidase production by *Streptococcus thermophilus* in a permeate-based environment.

Several previous studies emphasized the significance

of cultural conditions, including the starting pH and the amounts of lactose and nitrogen suppliers for the enzyme synthesis process (Amin *et al.*, 2023; Zuhri and Wilda, 2024; Kazemi *et al.*, 2025). Investigations focused on the production of β -galactosidase from lactic acid-producing bacteria identified the optimal pH values for culture media ranging between 6.0 and 7.5 (Ohaegbu *et al.*, 2023; Valenzuela *et al.*, 2024). The pH levels recorded in our study varied from 3.6 to 8.4, with an optimal enzymatic activity noted within the range of 5-7. Given that β -galactosidase can be induced, it is essential for the growing environment to contain lactose as a carbon source, in addition to the amino acids necessary for the synthesis process (Mukherjee *et al.*, 2023; Nawaz *et al.*, 2024). ANOVA revealed that both the response data and the interaction among the five variables were statistically important ($p < 0.05$). The method used for assessing the quadratic model's significance is detailed in Table 4. The coefficient of determination (R^2) was found to be 96.09 %, while the adjusted coefficient of determination (R^2 adj) was 93.31 %. These outcomes are in alignment with those reported by Da Silva *et al.* (2020).

According to Danbaba *et al.* (2019), a system with a coefficient of determination (R^2) value greater than 75 % is considered acceptable, indicating its effectiveness. This suggests that RSM is efficient for enhancing the culture environment composition required for β -galactosidase expression from the *Lactiplantibacillus plantarum* strain S1. This model accounts for 96.09 % of the diversity in the final result. Additionally, the model displays statistical importance at the 95 % confidence interval ($p \leq 0.05$). A higher R^2 value, approaching 1, indicates a more accurate prediction of the output. The adjusted coefficient of determination of 0.9331 demonstrated a strong correlation with the R^2 , underscoring the model's substantial significance. The fit value (F) for the regression analysis reached its significance at the 5 % threshold ($p < 0.05$), which confirms that the model is well-suited to account for the variability in enzyme synthesis as influenced by the five identified factors. Thus, the substantial F-value of 34.62 and the corresponding small predicted value (P) suggest that the system is indeed relevant.

The overall linear, quadratic, and the two ways interaction effects of all the investigated factors were significant. The obtained results are fitted with the following:

Regression equation depicted by [Mehiaoui et al. \(2023\)](#):

$$E.A = 38.74 + 0.260 N - 4.513 PH - 3.922 IS - 1.890 T + 0.6136 IP + 0.0333 N*N + 0.0878 PH*PH + 0.0385 IS*IS + 0.02209 T*T + 0.001669 IP*IP - 0.0433 N*PH + 0.0182 N*IS - 0.0174 N*T + 0.00593 N*IP + 0.2305 PH*IS + 0.1250 PH*T - 0.03443 PH*IP + 0.0973 IS*T - 0.02551 IS*IP - 0.01445 T*IP$$

Where; E.A= β -galactosidase activity (U/ ml), N= Nitrogen concentration (g/ ml), IS= Inoculum size (v/ ml), T= Temperature ($^{\circ}$ C), and IP= incubation duration (h).

Table 4: Statistical analysis of variation for the experimental results of the quadratic model obtained by CCD for β -galactosidase activity produced by *Lactiplantibacillus plantarum* strain S1.

Source	DF	Adj SS	Adj MS	F-value	p-value
Blocks	2	0.3744	0.1872	2.07	0.143
N	1	0.4184	0.4184	4.63	0.039
pH	1	3.3996	3.3996	37.58	0.000
IS	1	1.1772	1.1772	13.01	0.001
T	1	13.0971	13.0971	144.79	0.000
IP	1	10.5830	10.5830	117.00	0.000
N*N	1	0.0659	0.0659	0.73	0.400
PH*PH	1	0.4599	0.4599	5.08	0.031
IS*IS	1	0.0885	0.0885	0.98	0.330
T*T	1	4.3678	4.3678	48.29	0.000
IP*IP	1	3.4426	3.4426	38.06	0.000
N*PH	1	0.0599	0.0599	0.66	0.422
N*IS	1	0.0105	0.0105	0.12	0.735
N*T	1	0.1192	0.1192	1.32	0.260
N*IP	1	0.1623	0.1623	1.79	0.190
PH*IS	1	1.7006	1.7006	18.80	0.000
PH*T	1	6.1241	6.1241	67.70	0.000
PH*IP	1	5.4623	5.4623	60.39	0.000
IS*T	1	3.7108	3.7108	41.02	0.000
IS*IP	1	2.9982	2.9982	33.15	0.000
T*IP	1	11.7916	11.7916	130.36	0.000
Error	31	2.8041	0.0905		
Lack of fit	22	2.8041	0.1275	-	-
Pure error	9	0.0000	0.0000		
Total	53	71.7019			

Where DF: Degrees of freedom, AdjSS: Adjusted Sum of Squares, Adj MS: Adjusted Mean Square, F-value: actual response, p-value: Predicted response.

Results of regression analysis concerning the enzymatic activity indicated that the tested factors of pH, nitrogen concentration, inoculum size, incubation duration, and temperature had substantial positive impacts on the enzyme synthesis ($p < 0.05$) as shown in [Table 5](#).

Table 5: Estimated regression coefficients for enzymatic activity of *Lactiplantibacillus plantarum* strain S1.

Term	Coefficient	SE coefficient	T value	p value	VIF
Constant	0.0625	0.0867	0.72	0.476	
Blocks					
1	0.0637	0.0568	1.12	0.271	1.19
2	0.0630	0.0568	1.11	0.276	1.19
N	-0.0984	0.0458	-2.15	0.039	1.00
PH	0.2805	0.0458	6.13	0.000	1.00
IS	0.1651	0.0458	3.61	0.001	1.00
T	0.5506	0.0458	12.03	0.000	1.00
IP	-0.4950	0.0458	-10.82	0.000	1.00
N*N	0.0333	0.0389	0.85	0.400	1.01
PH*PH	0.0878	0.0389	2.25	0.031	1.01
IS*IS	0.0385	0.0389	0.99	0.330	1.01
T*T	0.2707	0.0389	6.95	0.000	1.01
IP*IP	0.2403	0.0389	6.17	0.000	1.01
N*PH	-0.0433	0.0532	-0.81	0.422	1.00
N*IS	0.0182	0.0532	0.34	0.735	1.00
N*T	-0.0610	0.0532	-1.15	0.260	1.00
N*IP	0.0712	0.0532	1.34	0.190	1.00
PH*IS	0.2305	0.0532	4.34	0.000	1.00
PH*IP	-0.4132	0.0532	-7.77	0.000	1.00
IS*T	0.3405	0.0532	6.40	0.000	1.00
IS*IP	-0.3061	0.0532	-5.76	0.000	1.00
T*IP	-0.6070	0.0532	-11.42	0.000	1.00

Where, SE Coefficient: standard error coefficient, T-value: observed response value, p-value: predicted response value, VIF: variance inflation factor.

The Pareto chart serves as a tool for illustrating the impact of the independent variables under investigation; along with their interactions, on the response variables ([Duan et al., 2021](#)). Moreover, its analysis demonstrated that all variables (A-E) investigated had a statistically substantial influence on the β -galactosidase enzyme's output and specific activity as indicated in [Table 4](#) and [Figure 6](#). This is evidenced by their p -values being below 0.05 and their corresponding F-values being notably high, as detailed in [Table 4](#) for ANOVA results.

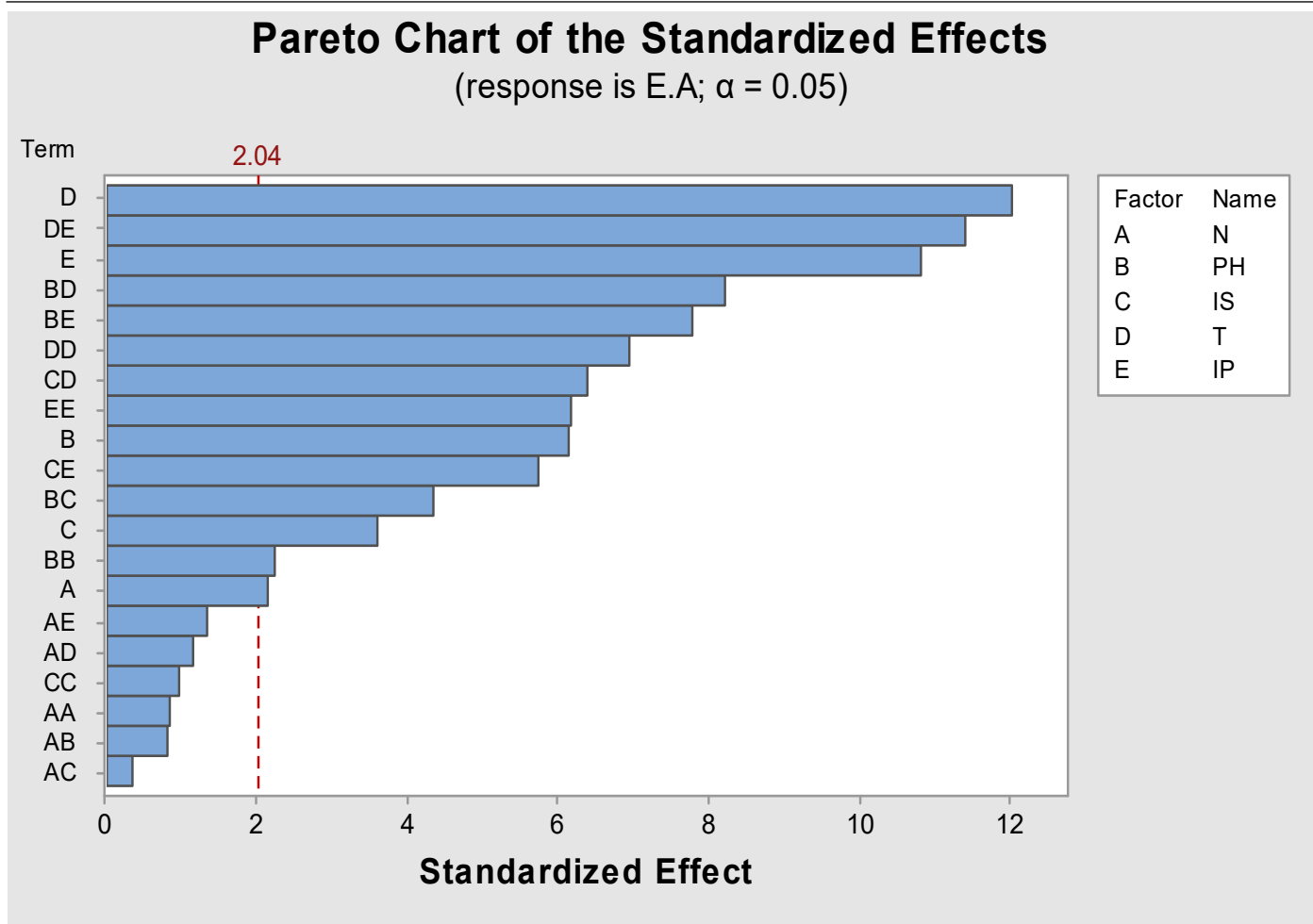


Figure 6: A pareto chart exhibiting typical influences of various parameters on β -galactosidase production. Where each symbol indicates; A = N, B = pH, C = IS, D = T, and E = IP. E.A: Enzyme activity, N: Nitrogen concentration, pH: pH, IS: Inoculum volume, T: Temperature, IP: Incubation duration, α : confidence interval.

The residuals plotted against the predicted responses demonstrate the lack of any identifiable patterns or trends, indicating that the disparity in the initial data analysis is consistent. Furthermore, the fact that the majority of residuals were below 0.5 % suggests that the design is appropriate for characterizing the β -galactosidase synthesis within the parameters being investigated.

Figure 7a-j, represents the response surface charts, developed to investigate the best values of each variable and their interactions concerning enzyme production, based on the regression model. Notably, the optimal yield of β -galactosidase obtained within a pH range of 6 to 8 with a nitrogen concentration maintained at 2 % as illustrated in Figure 7a. Among the variables examined, pH emerged as a highly critical component affecting enzyme function and nutrients uptake by the cell, which in turn impacts RNA and protein synthesis. This study indicated that lower nitrogen concentrations correlated with

increased β -galactosidase production, whereas higher nitrogen levels resulted in a decreased enzyme activity. Meanwhile, elevated levels of nitrogen can result in cellular apoptosis (Yi *et al.*, 2025). It was indicated that a higher inoculum size of 4 % was effective for biosynthesis of β -galactosidase using *Enterobacter cloacae* B5 (Chandel and Sharma, 2020), as depicted in Figure 7b.

Temperature emerged as the most critical factor influencing β -galactosidase production, with activity levels that increased within the ideal temperature level of 25-40 °C on a chart, and peaked at 37° C (Figure 7c). The study conducted by Singh and Sambyal (2023) demonstrated that β -galactosidase production in *Bifidobacterium animalis* had been maximized at 37°C. The current optimal temperature for β -galactosidase synthesis aligns with the findings of Niamah *et al.* (2023) on their study of *Bacillus* spp. The levels of β -galactosidase production and the final population size significantly rose as the

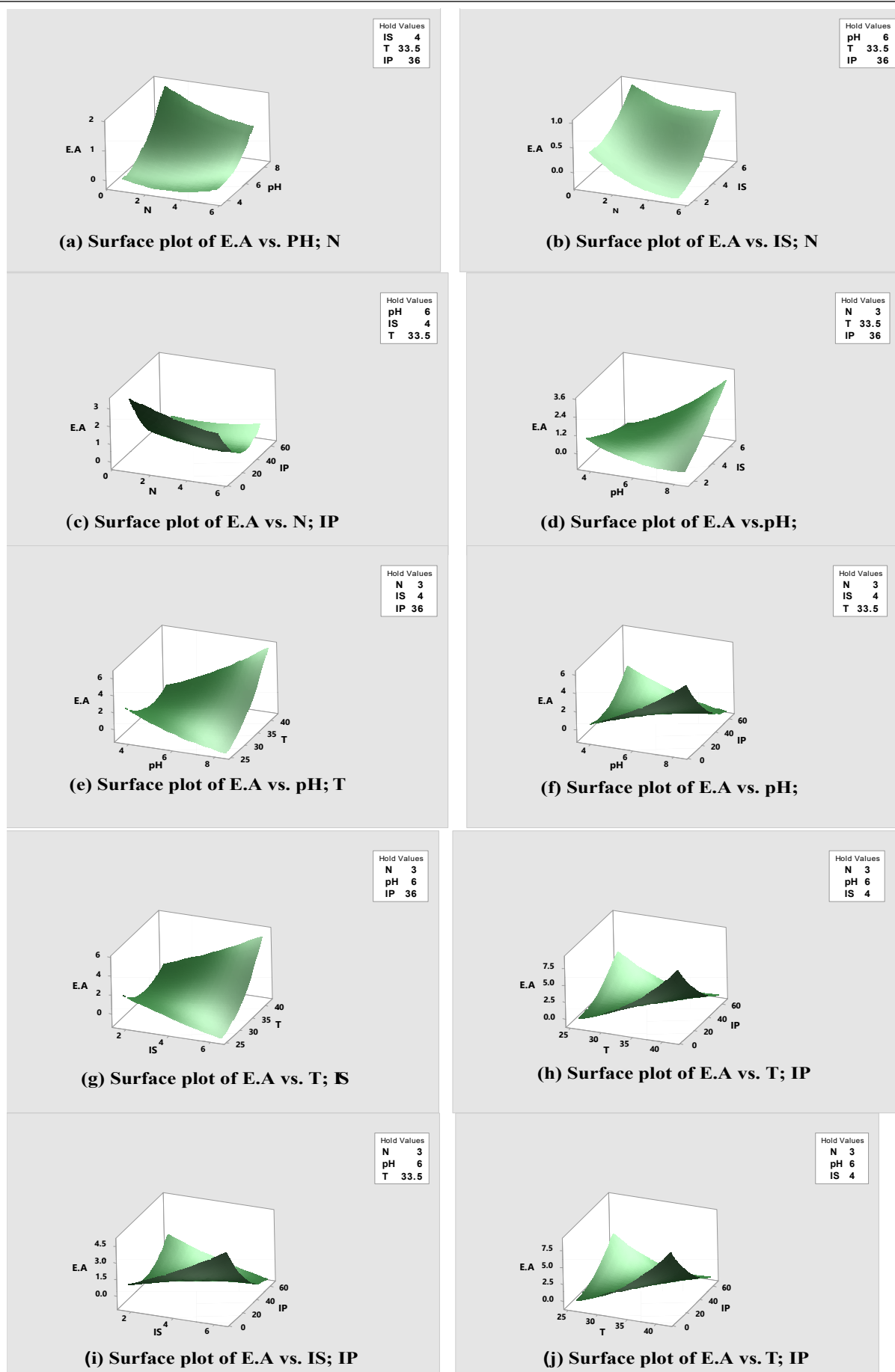


Figure 7: Response surface plots illustrating the interactions among various factors affecting β -galactosidase production by the isolated *Lactiplantibacillus plantarum* strain S1. Where; E.A: Enzyme activity, N: Nitrogen concentration, pH: pH, IS: Inoculum volume, T: Temperature, IP: Incubation duration.

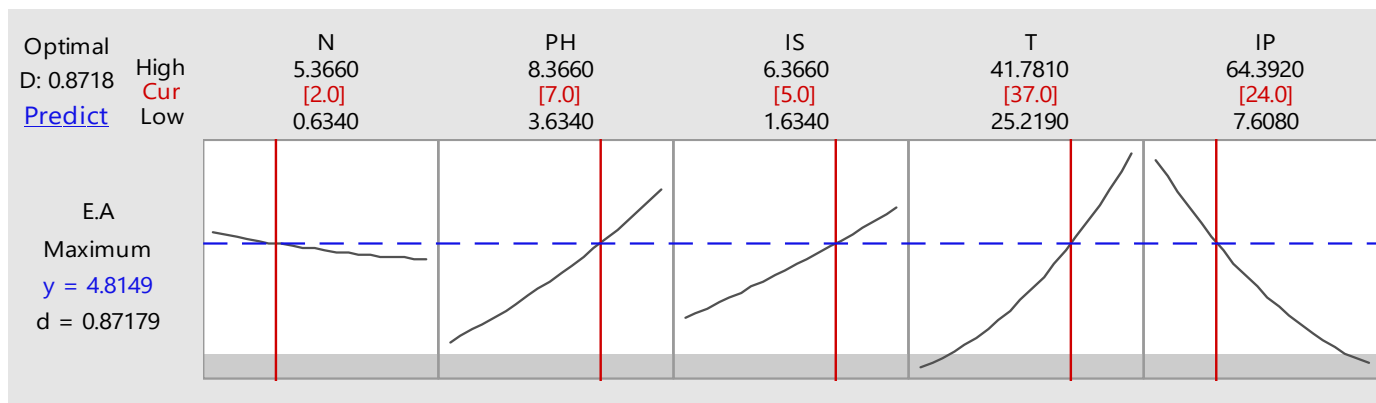


Figure 8: Diagrams illustrating the optimization of the various responses in relation to the optimal values of the variables under investigation. Where; E.A: Enzyme activity, N: Nitrogen concentration, pH: pH, IS: Inoculum volume, T: Temperature, IP: Incubation duration.

incubation time increased, peaking at 24 h (Figure 7d). This observation corroborates with the previous results presented by Oparaji (2022) on their study on *Bifidobacteria* sp. Moreover, these results are in harmony with the previous conclusions drawn by Singh and Sambyal (2023) regarding *L. plantarum*.

Figure 7a, e, f, g, indicate that the perfect pH for the *Lactiplantibacillus plantarum* strain S1 to release β -galactosidase lied between pH 6.8 and 7; with pH 7 identified as the most suitable. These outcomes are in consistence with those of Choi *et al.* (2021), who found that the highest growth of *L. plantarum* 200655 was observed at pH 7 and that low pH levels (< 4.4) may influence bacterial growth and diminish it. Furthermore, similar neutral pH optima have been documented for various LAB species, including *L. acidophilus* (Abdaltef *et al.*, 2023).

In Figures 7h, i, j, it is evident that the yield of lactase enzyme reached its peak at 37 °C. This finding aligns with the previous studies conducted by Chandel and Sharma (2020), Gomes *et al.* (2021) which showed that temperatures under 40 °C were optimal for the synthesis of β -galactosidase by *L. reuteri*, *S. thermophilus*, and *B. longum*. In contrast, enzyme production was enhanced with larger inoculum sizes; particularly between 3 % and 5 %, but declined as the duration of incubation increased.

Following the statistical analysis results, additional tests were conducted as detailed in Table 6 and Figure 8. The ideal parameters for β -galactosidase fabrication were found to be 37 °C, pH 7.0, 2 % nitrogen concentration, 5 % inoculum size, 4.6 % lactose as the medium's carbon source. In the context of the given

conditions, the recorded level of β -galactosidase yield was 5.523 U/ ml, which marginally deviated from the anticipated range of 4.8149 U/ ml. This indicates that the system was established is both a precise and dependable manner for forecasting the perfect culture conditions for enhancing β -galactosidase biosynthesis.

Table 6: Multiple response prediction of β -galactosidase enzyme activity for *Lactiplantibacillus plantarum* strain S1.

Variable	Setting			
N	2			
PH	7			
IS	5			
T	37			
IP	24			
Response	Fit	SE Fit	95% CI	95% PI
E.A	4.815	0.202	(4.403; 5.22)	(4.076; 5.554)

Where SE Fit: Standard error fit, 95%CI:95 % Confidence interval, 95 % PI: Predicted interval.

The integration of the Plackett–Burman designs with RSM is prevalent in the field of experimental analysis; particularly for enhancing enzyme production from the microorganisms (Abdella and Ibrahim, 2024; Karamchandani *et al.*, 2025).

Conclusions and Recommendations

The production of bacterial β -galactosidases has significant applications owing to their activity across a broad spectrum of pH levels and temperatures. The selection of LAB as an appropriate β -galactosidase resource is attributed to their recognized safety for

use in food industry applications. Consequently, it has been deduced that cheese permeate; an industrial dairy byproduct, can be effectively utilized for the biosynthesis of β -galactosidase by the *Lactiplantibacillus plantarum* strain *S1*, yielding 5.523 U/ ml, when grown at initial pH 7, lactose as carbon source in the medium (4.6 %), potassium nitrate as a nitrogen source (2.0 %), and an incubation temperature of 37 °C for 24 h. RSM is a highly effective approach used for the optimization of the fermentation media aimed at enhancing β -galactosidase production from the *Lactiplantibacillus plantarum* strain *S1*.

In response to global climate change and other environmental issues, new emission reduction strategies and development models for the dairy industry (a significant energy-intensive food sector) must be established in order to meet net-zero objectives. The dairy sector currently lacks worldwide standards and hence requires further efforts to thoroughly address sustainability challenges. β -galactosidase enzyme is a crucial product for the dairy sector and expensive to get. It is therefore recommended that more efforts should be undertaken to pursue further exploration and evaluation of superior β -galactosidase-generating microbial species. Other β -galactosidase-producing microorganisms should also be explored for deployment and application in our home-developed dairy industries.

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Novelty Statement

This study is the first to isolate and characterize a novel lactic acid bacterium such as *Lactiplantibacillus plantarum* strain *S1*, which produced β -galactosidase enzyme by using a dairy waste cheese permeate as a growth medium.

Author's Contribution

Safaa Salah El-Din Taha: Methodology, investigations, and writing the original draft.

Osama Abd El-Hamid Ibrahim: Supervision,

Research design and editing the data.

Einas Hamed El-Shatoury and Amany Mohamed El-Deeb: Supervision, research design, and revision.

Hayam Abd El-Naby Sayed: Supervision, statistical study design and analysis, molecular data analysis, revision and editing the manuscript.

All authors approved the final version the manuscript.

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Ethical approval

Ethical approval is not required for this study as it is purely computational and does not involve human subjects and/or animals.

Conflict of interests

The authors have declared no conflicts of interest.

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