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Enhancing the Nutritional and Functional Quality of Bovine and Bubaline Milk Via Enzymatic Lactose Hydrolysis: Reaction Kinetics, Methodological Limitations, and Physicochemical Implications

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***Abstract*-- The global prevalence of primary adult-type hypolactasia (lactose intolerance), affecting an estimated 65–70% of the world population, necessitates the development of nutritionally complete lactose-free or low-lactose dairy alternatives. Enzymatic hydrolysis of lactose using exogenous β-galactosidase (EC 3.2.1.23) represents the current industrial gold standard for this purpose. The present study systematically evaluated the kinetic efficacy of a commercial, food-grade microbial β-galactosidase preparation applied to both bovine (*Bos taurus*) and bubaline (*Bubalus bubalis*) milk matrices at two enzyme concentrations (0.2% and 0.6% w/v) across a three-hour incubation period at 37°C. The generation of reducing monosaccharides was quantified via the 3,5-dinitrosalicylic acid (DNS) colorimetric assay. Raw spectrophotometric data indicated substantially elevated absorbance readings and calculated glucose concentrations in the bubaline milk matrix compared t.o the bovine matrix. However, a rigorous critical evaluation reveals that this apparent disparity is primarily a severe methodological artifact attributable to the non-specific reactivity of the DNS reagent with the considerably higher protein and lipid content of the bubaline matrix, compounded by turbidity-induced light scattering during spectrophotometry. Compositional data from peer-reviewed literature confirms that the absolute lactose content of bovine and bubaline milk is statistically comparable (approximately 4.7–5.2% vs. 4.7–4.98%, respectively), refuting substrate availability as an explanation for the apparent kinetic divergence. A critical analysis further identifies a significant unaddressed variable in the experimental protocol: the thermal enzyme inactivation step (80°C, 5–10 min) applied to lactose-hydrolyzed milk — now enriched with highly reactive free glucose and galactose — initiates an accelerated Maillard reaction cascade. This non-enzymatic browning generates advanced glycation end-products (AGEs), including furosine and 5-hydroxymethylfurfural (HMF), with demonstrable implications for product safety, sensory quality, and amino acid bioavailability. Analytical, toxicological, and industrial scale-up perspectives are synthesized, and evidence-based recommendations for superior methodologies — including HPLC, HPAEC-PAD, and GOD-POD assays, alongside immobilized enzyme bioreactor processing — are presented.**

***Keywords*-- β-Galactosidase; lactose hydrolysis; lactose intolerance; bovine milk; bubaline milk; DNS assay; Maillard reaction; 5-hydroxymethylfurfural (HMF); immobilized enzyme bioreactor; HPLC; enzyme kinetics**

I. INTRODUCTION

Mammalian milk is one of the most biologically complex and nutritionally complete dietary fluids in nature, providing an indispensable matrix of high-quality proteins (micellar caseins and soluble whey proteins), essential lipids, bioavailable calcium and phosphorus, and critical micronutrients including Vitamin D, Vitamin B_{12}, and riboflavin. These constituents support skeletal mineralization, neurological transmission, muscular function, and immunological maintenance across multiple stages of human development [18]. Despite this exceptional nutritional density, global dairy consumption is severely constrained by the high prevalence of primary adult-type hypolactasia.

Lactose — chemically designated as β-D-galactopyranosyl-(1→4)-D-glucose — constitutes approximately 4.5% to 5.2% of the total solids content of both bovine and bubaline milk matrices. In individuals exhibiting lactase persistence, this disaccharide is efficiently cleaved by lactase-phlorizin hydrolase (LPH), expressed in the brush-border microvilli of the small intestinal epithelium, into the constituent monosaccharides D-glucose and D-galactose, which are rapidly absorbed into systemic circulation. In lactase-deficient individuals — an estimated 65–70% of the global adult population, with prevalences exceeding 90% in parts of East Asia, Sub-Saharan Africa, and Latin America — unhydrolyzed lactose transits intact to the large intestine [12, 2]. There it serves as a fermentable substrate for the colonic microbiome, generating short-chain fatty acids (SCFAs) and substantial volumes of intestinal gases (H_2, CO_2, CH_4) [1].

This fermentative process precipitates a well-characterized cascade of gastrointestinal symptoms collectively termed lactose intolerance: bloating, flatulence, abdominal cramping, nausea, and osmotic diarrhea. Chronic dairy avoidance in symptomatic populations carries significant nutritional risk, including calcium deficiency, osteopenia, and elevated susceptibility to osteoporosis [11].

To address this public health challenge, the dairy industry faces a technological imperative to manufacture lactose-free and low-lactose products that faithfully replicate the sensory, viscosity, and nutritional properties of conventional milk [4, 5]. Regulatory bodies impose stringent compositional thresholds: the Food Safety and Standards Authority of India (FSSAI) mandates residual lactose below 1.0% for "low-lactose" designation and below 0.1% for "lactose-free" classification. Traditional lactose-reduction methodologies — including membrane ultrafiltration, chromatographic separation, and lactic acid bacterial fermentation — are encumbered by high operational costs, suboptimal yields, and detrimental alterations to milk's organoleptic properties [17]. Consequently, enzymatic hydrolysis using exogenous β-galactosidase (EC 3.2.1.23) has become the accepted industrial standard, offering high selectivity, nutritional integrity, and scalability [3, 16].

Industrial β-galactosidases are principally sourced from filamentous fungi — chiefly *Aspergillus oryzae* and *Aspergillus niger* (optimal activity at pH 4.5–5.5) — or from yeast strains such as *Kluyveromyces lactis* and *Kluyveromyces marxianus* (optimal activity at pH 6.5–7.0). The latter are preferred for direct in-process hydrolysis of sweet milk. Mechanistically, the enzyme's active site binds the β-galactosidic linkage of lactose and facilitates a nucleophilic attack cleaving the β-(1→4) bond, releasing equimolar quantities of glucose and galactose [8, 9]. Because these monosaccharides possess a higher relative sweetness index than the intact disaccharide, the resulting hydrolyzed milk exhibits enhanced natural sweetness without caloric sweetener addition [7 , 11].

The physicochemical disparities between bovine and bubaline milk are a highly relevant variable in enzymatic processing kinetics [6, 10]. Bubaline milk accounts for approximately 15% of global milk production and holds considerable economic and dietary importance in South Asia. Relative to bovine milk, bubaline milk contains significantly higher total solids, lipids, total protein, and divalent mineral cations — differences that materially influence the thermodynamic behavior and catalytic efficiency of exogenous microbial enzymes [14].

The present study systematically evaluated enzymatic lactose hydrolysis in both milk matrices, and critically integrates the empirical findings with contemporary literature on assay limitations, thermal degradation chemistry, toxicological safety, and industrial bioprocess engineering [15].

II. Materials And Methods

2.1 Sample Procurement And Pasteurization

Fresh raw bovine (*Bos taurus*) and bubaline (*Bubalus bubalis*) milk were procured directly from local dairy vendors under strict sanitary handling conditions to minimize initial microbial loads. Raw milk harbors indigenous lactic acid bacteria (LAB) with endogenous lactase activity that would confound the exogenous enzyme assay. To eliminate this competitive variable, samples were subjected to High-Temperature Short-Time (HTST) pasteurization at 72°C for 15 seconds, sufficient to denature native enzymes and substantially reduce the vegetative microbial population without initiating extensive whey protein denaturation or non-enzymatic browning. Following pasteurization, samples were rapidly cooled to ambient temperature. Equal volumetric aliquots of 100 mL of each milk type were transferred to sterilized borosilicate glass beakers for enzymatic processing.

2.2 Reagents And Commercial Enzyme Preparation

A commercial, food-grade microbial-origin β-galactosidase (lactase) preparation was employed as the primary biocatalyst. The preparation was maintained within its recommended shelf-life parameters and stored appropriately prior to use. Analytical-grade reagents prepared for spectrophotometric quantification included 3,5-dinitrosalicylic acid (DNS), sodium potassium tartrate (color stabilizer), sodium hydroxide (NaOH), and hydrochloric acid (HCl). For qualitative validation, Benedict's reagent, Fehling's solutions (A and B), Barfoed's reagent, Tollen's reagent, and the synthetic chromogenic substrate *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) were utilized.

2.3 Enzymatic Hydrolysis Protocol

To establish a dose-response relationship, two enzyme concentrations were evaluated: 0.2 g and 0.6 g per 100 mL of milk (0.2% and 0.6% w/v, respectively). Inoculated milk samples were incubated in a thermostatically controlled water bath at 37°C ± 0.5°C — the kinetic optimum for the commercial yeast-derived lactase — for a total of three hours, with analytical sampling at 1-hour, 2-hour, and 3-hour intervals.

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At each time point, 10 mL aliquots were carefully extracted from each beaker and transferred to borosilicate test tubes. To arrest further enzymatic hydrolysis and fix the carbohydrate profile for downstream analysis, samples were thermally inactivated by heating to 80°C for 5–10 minutes on a controlled hot plate.

2.4 Spectrophotometric Quantification: Dns Assay

Reducing sugar concentrations were quantified using the 3,5-dinitrosalicylic acid (DNS) colorimetric method. Under highly alkaline conditions and thermal activation, the free carbonyl groups of reducing monosaccharides (glucose and galactose) are oxidized while DNS is concurrently reduced to the intensely colored 3-amino-5-nitrosalicylic acid chromophore. Briefly, 3 mL of freshly prepared DNS reagent was added to each heat-inactivated 10 mL milk aliquot and the mixture was submerged in a vigorously boiling water bath for exactly 10 minutes. Test tubes were then cooled rapidly to ambient temperature and optical absorbance was measured at 540 nm using a calibrated UV-Vis spectrophotometer. A glucose standard calibration curve was generated using known concentrations of pure anhydrous glucose (0.0–1.4 mg/mL), and absorbance values were converted to glucose concentrations (g/L) using this regression.

2.5 Qualitative Carbohydrate Validation Assays

Orthogonal qualitative confirmation of reducing sugar generation was obtained using four classical biochemical assays: Benedict's test (cupric sulfate in alkaline sodium citrate), Fehling's test (Fehling's A + B, precipitating Cu_2O from reducing sugars), Barfoed's test (copper(II) acetate in acetic acid, differentiating monosaccharides from disaccharides based on reduction kinetics), and Tollen's test (ammoniacal silver nitrate, detecting free aliphatic aldehydes).

2.6 Onpg Assay For Enzyme Activity Validation

An independent confirmation of the intrinsic catalytic activity of the commercial lactase preparation was conducted using the synthetic chromogenic substrate ONPG. Commercial lactase tablets were crushed and dissolved in a pH-optimized phosphate buffer, filtered, and centrifuged at 5,000 rpm for 10 minutes to obtain a clarified enzyme supernatant. This extract was incubated with ONPG, and the release of *ortho*-nitrophenol (ONP) — producing an intense yellow chromophore in alkaline conditions — was quantified spectrophotometrically at 420 nm, confirming enzyme structural integrity and specific activity prior to milk matrix applications.

III. RESULTS

3.1 Enzymatic Hydrolysis Kinetics In Bovine Milk

DNS spectrophotometric analysis of the bovine milk samples demonstrated a clear time- and concentration-dependent increase in reducing sugar generation over the three-hour incubation period (Table 1). At the 1-hour interval, the 0.2% (w/v) enzyme concentration produced an absorbance of 0.182 (calculated glucose: 0.0629 g/L), while the 0.6% concentration approximately doubled hydrolytic output, yielding an absorbance of 0.301 (0.1195 g/L). This dose-dependent enhancement continued at the 2-hour mark (0.2%: 0.1200 g/L; 0.6%: 0.1352 g/L). By 3 hours, the 0.2% and 0.6% concentrations reached 0.1914 g/L and 0.1943 g/L, respectively, representing a notable convergence. This diminishing marginal difference at the terminal time point is consistent with substrate saturation kinetics or competitive end-product inhibition by accumulating free galactose within the closed incubation system.

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Table 1.
DNS Spectrophotometric Readings and Calculated Glucose Concentrations for Bovine (Bos taurus) Milk Following β-Galactosidase-Mediated Lactose Hydrolysis at 37°C.

Enzyme Concentration (% w/v)	**Incubation Time**	**Absorbance at 540 nm**	**Calculated Glucose Concentration (g/L)***
0.2%	1 h	0.182	0.0629
0.6%	1 h	0.301	0.1195
0.2%	2 h	0.302	0.1200
0.6%	2 h	0.334	0.1352
0.2%	3 h	0.452	0.1914
0.6%	3 h	0.458	0.1943

** Glucose concentrations calculated from a purified anhydrous glucose standard calibration curve ($r^2 \geq 0.99$). Values subject to DNS matrix interference (see Section 4.2). All readings are single-replicate determinations.*

3.2 Enzymatic Hydrolysis Kinetics In Bubaline Milk

Application of the identical enzymatic hydrolysis protocol and DNS analytical procedure to the bubaline milk matrix yielded substantially and consistently elevated spectrophotometric readings at all time points (Table 2). Control samples (0.0% enzyme) registered a stable absorbance of 0.000 across all intervals, confirming baseline stability. Introduction of 0.2% enzyme produced an absorbance of 1.129 (0.5136 g/L) at 1 hour, while the 0.6% concentration generated an absorbance of 1.853 (0.8582 g/L) at the same interval — a value exceeding the linear range of the glucose standard curve and suggestive of methodological interference. Over the 3-hour course, absorbance values exhibited a slight non-monotonic pattern (peaking at 1 hour for the 0.6% concentration and declining slightly at 2 and 3 hours), which is inconsistent with standard enzymatic kinetic models and further implicates matrix-related analytical artifacts.

Table 2.
DNS Spectrophotometric Readings and Calculated Glucose Concentrations for Bubaline (Bubalus bubalis) Milk Following β-Galactosidase-Mediated Lactose Hydrolysis at 37°C.

Enzyme Concentration (% w/v)	**Incubation Time**	**Absorbance at 540 nm**	**Calculated Glucose Concentration (g/L)***
0.0% (Control)	1, 2, 3 h	0.000	0.000
0.2%	1 h	1.129	0.5136†
0.6%	1 h	1.853	0.8582†
0.2%	2 h	1.268	0.5798†
0.6%	2 h	1.693	0.7820†
0.2%	3 h	1.332	0.6102†
0.6%	3 h	1.721	0.7954†

** Glucose concentrations calculated from a purified anhydrous glucose standard calibration curve. † Values flagged as probable analytical artifacts attributable to non-specific DNS reactivity with the dense bubaline protein-lipid matrix (see Section 4.2). Values exceeding 0.7 g/L exceed the linear range of the standard curve and should be treated with extreme caution.*

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3.3 Comparative Milk Composition

Published compositional data for bovine and bubaline milk, cross-referenced from peer-reviewed analyses, are summarized in Table 3. Critically, the lactose content of both milk types is statistically comparable (bovine: 4.68–5.20%; bubaline: 4.66–4.98%), while bubaline milk exhibits markedly higher total protein (up to 5.26% vs. 3.36%), total fat (up to 8.68% vs. 4.07%), and total solids (up to 19.82% vs. 13.28%). Calcium and magnesium concentrations are also substantially elevated in bubaline milk, a finding with direct implications for metalloenzyme kinetics discussed in Section 4.1.

Table 3.
Comparative Macro-Compositional and Mineral Profile of Bovine (Bos taurus) and Bubaline (Bubalus bubalis) Milk (values represent published mean ranges from peer-reviewed compositional analyses).

Compositional Parameter	Bovine Milk (Bos taurus)	Bubaline Milk (Bubalus bubalis)
Total Protein (%)	3.25 – 3.36	4.34 – 5.26
Total Fat (%)	3.93 – 4.07	7.56 – 8.68
Total Solids (%)	12.75 – 13.28	18.88 – 19.82
Solids-Not-Fat / SNF (%)	8.65 – 8.67	9.79 – 10.55
Lactose (%)	4.68 – 5.20	4.66 – 4.98
Calcium (mg/100 mL)	111.99 – 129.67	167.98 – 185.66
Magnesium (mg/100 mL)	9.73 – 16.54	14.59 – 21.40

3.4 Qualitative Assay And Onpg Validation Results

The qualitative biochemical assay panel and ONPG validation produced a complex set of results summarized in Table 4. The most anomalous finding was the false-negative Benedict's test result (pure blue colouration) in the presence of samples that the DNS assay reported as containing substantial reducing sugar concentrations. This internal inconsistency is a critical indicator of assay-matrix interference and is discussed fully in Section 4.2.

Fehling's test produced the expected positive brick-red cuprous oxide precipitate, confirming the biochemical presence of reducing monosaccharides. Barfoed's and Tollen's tests yielded inconclusive or negative results, attributable to the complex organic matrix. The ONPG assay produced a vivid yellow colouration upon enzyme incubation, unequivocally confirming the structural integrity and high specific activity of the commercial β-galactosidase preparation prior to milk matrix application.

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Table 4.
Summary of Qualitative Biochemical Assay Results for β-Galactosidase-Treated Milk Samples.

Assay	Reagent / Principle	Observed Result	Interpretation
Benedict's Test	Cu^{2+} / alkaline sodium citrate	Blue (false negative)	Severe interference from milk proteins and turbidity
Fehling's Test	Cu^{2+} sulfate (A) + tartrate (B)	Brick-red Cu_2O precipitate	Positive; confirms reducing monosaccharides present
Barfoed's Test	Cu^{2+} acetate / acetic acid	Delayed / absent precipitate	Inconclusive; likely interference from disaccharides and matrix
Tollen's Test	Ammonical $AgNO_3$	No silver mirror formed	Negative; no free aliphatic aldehydes detected
ONPG Assay	ortho-Nitrophenyl-β-D-galactopyranoside	Vivid yellow (ONP release)	Confirms potent β-galactosidase catalytic activity

IV. Discussion

4.1 Compositional Determinants Of Apparent Kinetic Divergence

The most striking observation in the primary data is the approximately 400–700% difference in DNS-calculated glucose concentrations between bubaline and bovine samples under identical processing conditions. Two hypotheses were considered to account for this disparity: (a) substantially higher initial lactose substrate availability in bubaline milk, or (b) enhanced catalytic efficiency of the lactase enzyme within the bubaline biochemical environment. Hypothesis (a) is definitively refuted by the compositional data in Table 3. The lactose content of both milk types is statistically comparable, and multiple global analyses consistently report equivalent or marginally lower lactose in bubaline milk relative to bovine milk. A difference of this magnitude in substrate concentration cannot account for a 400–700% divergence in measured product yield. Hypothesis (b) has partial biochemical validity. Commercial β-galactosidases, particularly those derived from *Kluyveromyces lactis*, are well-characterized metalloenzymes whose catalytic rate and structural stability are significantly potentiated by free divalent metal cations, primarily Mg^{2+} and, in specific kinetic contexts, Ca^{2+}.

As shown in Table 3, bubaline milk contains substantially higher concentrations of both calcium (up to 185.66 mg/100 mL vs. 129.67 mg/100 mL) and magnesium (up to 21.40 mg/100 mL vs. 16.54 mg/100 mL). It is plausible that the elevated divalent cation concentration in the bubaline matrix induced allosteric activation of the enzyme, stabilizing its active site conformation and marginally increasing V_{max}. However, this mechanism alone cannot account for the observed magnitude of divergence, and the dominant explanation lies in methodological artifacts, as detailed in Section 4.2.

4.2 Critical Evaluation: The Dns Assay In Complex Dairy Matrices

A rigorous analytical evaluation of the foundational methodology reveals a severe and well-documented limitation that almost certainly accounts for the majority of the disparity between the bovine and bubaline DNS readings. The DNS assay is a robust and valid quantitative method for reducing sugar estimation in purified, simple aqueous solutions. When applied to unclarified, protein-dense, complex biological matrices such as whole mammalian milk, however, it is subject to catastrophic analytical interference. The primary source of interference is the non-specific reactivity of the DNS reagent.

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While DNS selectively oxidizes the free carbonyl (aldehyde and ketone) groups of reducing monosaccharides under alkaline conditions, it will also react indiscriminately with free sulfhydryl (–SH) groups present on cysteine residues of milk proteins — most notably β-lactoglobulin and α-lactalbumin in the whey fraction. These protein-bound functional groups possess sufficient reducing potential to generate the 3-amino-5-nitrosalicylic acid chromophore in the complete absence of reducing sugars, directly inflating absorbance readings. This well-documented interference has been the subject of multiple critical peer-reviewed evaluations and is a known limitation explicitly acknowledged in the DNS methodology literature. Compounding this chemical interference is a severe physical artifact. The DNS protocol requires both strongly alkaline conditions and prolonged boiling (10 minutes in a water bath at 100°C). These extreme conditions cause extensive protein coagulation, irreversible thermal denaturation, and precipitation of the milk protein matrix — particularly the casein micelles — generating substantial turbidity within the sample tube. Standard UV-Vis spectrophotometers measure total light extinction at the target wavelength; they cannot discriminate between true chromophore absorbance and non-specific light scattering caused by particulate matter. Consequently, the pronounced turbidity generated from the denser bubaline protein matrix (total protein up to 5.26% vs. 3.36% in bovine milk; larger and more numerous casein micelles; higher lipid globule volume) would generate proportionally greater light scattering, erroneously registered by the instrument as elevated absorbance and interpreted as an inflated glucose concentration. These combined effects — non-specific chemical reduction by milk protein sulfhydryl groups and protein coagulation-induced turbidity scattering — provide the most scientifically sound explanation for the extreme and anomalous bubaline DNS readings. The discordant Benedict's test result (false negative despite high DNS readings) further substantiates this conclusion, as Benedict's reagent, applied at lower concentrations and shorter reaction times, is less susceptible to these specific interferences and likely reflects a more accurate, though qualitative, assessment of the reducing sugar pool. For quantitative, peer-reviewed dairy research, the DNS assay must be replaced with methodologically specific, non-interfering analytical techniques: High-Performance Liquid Chromatography (HPLC) and High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) provide gold-standard separation and quantification of individual carbohydrates; the Glucose Oxidase-Peroxidase (GOD-POD) coupled enzymatic assay selectively measures free D-glucose without interference from structural milk components; and the validated K-LOLAC enzymatic kit is specifically engineered to quantify trace residual lactose in complex protein-containing dairy matrices, meeting the analytical standard required for regulatory "lactose-free" labeling claims.

4.3 Thermal Enzyme Inactivation And The Maillard Reaction Cascade

A critical and unaddressed variable in the original experimental design is the physicochemical consequence of the thermal enzyme inactivation step. The protocol dictated heating enzyme-treated milk samples to 80°C for 5–10 minutes to permanently denature the β-galactosidase. While functionally effective for halting enzymatic activity, this step fails to account for a predictable and serious chemistry: the Maillard reaction. The Maillard reaction is a non-enzymatic browning process involving the condensation of free reducing carbonyl groups (from glucose or galactose) with free amino groups — principally the ε-amino group of lysine residues in milk proteins — to form unstable Schiff bases. These intermediates rearrange to relatively stable Amadori products (e.g., fructosyllysine), which upon further thermal degradation yield advanced glycation end-products (AGEs), including furosine and the heterocyclic compound 5-hydroxymethylfurfural (HMF). Critically, lactose-hydrolyzed milk undergoes the Maillard reaction substantially faster, at lower activation energies, and to a greater final extent than unhydrolyzed milk, because free monosaccharides — particularly galactose — are substantially more reactive in condensation reactions than the intact lactose disaccharide. The application of 80°C for up to 10 minutes to a milk matrix already enriched with free glucose and galactose therefore guarantees the aggressive initiation of this cascade, with multiple peer-reviewed studies confirming measurable browning (negative shifts in colorimeter L* values, positive shifts in b*), cooked off-flavors, and significantly elevated furosine and HMF in heat-treated lactose-hydrolyzed milk relative to untreated controls. Furthermore, because bubaline milk inherently contains a higher total protein concentration and greater density of reactive lysine residues than bovine milk, the extent, rate, and severity of Maillard browning and HMF accumulation are statistically greater in heat-treated hydrolyzed buffalo milk. The failure of the original study to monitor, quantify, or implement mitigation strategies for this reaction pathway represents a fundamental gap in assessing the true product quality of the processed milk samples.

4.4 Toxicological Profile Of Thermal Degradation Products And Enzyme Safety

The unmitigated generation of HMF during the thermal inactivation step introduces a significant toxicological concern that warrants explicit discussion. HMF is a primary, readily measurable product of the thermal degradation of reducing sugars, and its long-term systemic safety profile remains under active regulatory scrutiny. At elevated chronic dietary exposures, HMF undergoes hepatic metabolic activation by sulfotransferases to the highly reactive and mutagenic metabolite 5-sulphoxymethylfurfural (SMF). Extensive in vivo murine studies have documented SMF-mediated oxidative stress, depletion of intracellular glutathione (GSH) reserves, and targeted hepatotoxicity and nephrotoxicity. While HMF levels in conventionally pasteurized whole milk are well within accepted safety limits, the exponential accumulation of HMF in heavily heat-treated lactose-hydrolyzed milk — particularly in sensitive applications such as infant formula and clinical nutrition products — constitutes a non-trivial public health risk. The European Food Safety Authority (EFSA) actively monitors HMF as a process contaminant in heat-processed foods. This regulatory attention underscores the critical imperative to eliminate crude thermal enzyme inactivation from lactose-free milk production protocols and replace it with non-thermal or low-temperature alternatives, including advanced microfiltration, chromatographic enzyme removal, or ultra-high-pressure processing (UHP). In direct contrast to the hazards of thermal processing, the commercial β-galactosidase enzyme itself presents an exceptionally well-characterized and robust safety profile. Industrial lactase preparations derived from *A. oryzae* and *K. lactis* hold official Qualified Presumption of Safety (QPS) status under the EFSA framework and carry Generally Recognized As Safe (GRAS) designation from the US FDA and the Joint FAO/WHO Expert Committee on Food Additives (JECFA). Rigorous 90-day repeated-dose oral toxicity studies in murine models have established extremely high no-observed-adverse-effect levels (NOAELs) for these preparations, with comprehensive bioinformatic analyses confirming no structural homology to known human allergens or toxins. The primary safety concern in lactose-free milk production therefore lies not with the enzyme but with the thermochemical management of its highly reactive monosaccharide products.

4.5 Industrial Scale-Up Considerations And Bioprocess Engineering

The bench-scale batch methodology employed in this study — free enzyme added directly to glass beakers — is economically impractical at industrial volumes. The prohibitive cost of single-use commercial enzyme preparations, compounded by multi-hour incubation requirements that limit factory throughput, render simple batch dosing non-viable at scale. Modern dairy biotechnology has extensively transitioned to continuous-flow processing using immobilized enzyme bioreactors. In these systems, purified β-galactosidase is covalently cross-linked or physically entrapped within inert polymeric support matrices (e.g., packed-bed columns or fluidized-bed reactors), allowing pasteurized milk to be continuously pumped through the enzyme bed for near-instantaneous, high-volume hydrolysis. The ability to reuse the enzyme preparation over extended operational periods (weeks to months) dramatically reduces cost-per-liter metrics.

However, the distinctive physicochemical properties of bubaline milk pose significant and largely unresolved engineering challenges for immobilized systems. The markedly higher viscosity of bubaline milk — attributable to its elevated protein concentration, larger and more numerous casein micelles, and substantially higher lipid globule volume — causes rapid fouling of packed-bed reactor matrices, generating catastrophic pressure drops and severely reducing enzymatic operational half-life. Dense protein and lipid deposition physically occludes substrate access to the enzyme's active sites. Overcoming these rheological barriers will likely require integration of membrane pre-filtration, development of anti-fouling nanostructured enzyme carrier materials, or modification of the bubaline milk matrix prior to reactor entry. These engineering challenges represent a defined and active frontier in the industrial optimization of lactose-free bubaline dairy products.

V. Conclusion

Enzymatic hydrolysis of lactose using microbial β-galactosidase remains the most nutritionally sound, biochemically selective, and industrially viable strategy for producing lactose-free and low-lactose dairy products that meet modern regulatory and consumer quality standards.

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The primary empirical data of this study confirm the foundational dose-dependent and time-dependent kinetics of the biocatalytic process in both bovine and bubaline milk matrices, validating the principle that increasing enzyme concentration and extended incubation effectively cleave lactose into its constituent monosaccharides, glucose and galactose, thereby enhancing the natural sweetness and gastrointestinal digestibility of the final product. However, the integration of these empirical findings with the contemporary scientific literature reveals three critical areas requiring immediate methodological revision. First, the apparent 400–700% superiority of bubaline milk as a hydrolysis substrate is identified as a severe analytical artifact. The combination of non-specific DNS reactivity with milk protein sulfhydryl groups and protein coagulation-induced turbidity scattering in the denser bubaline matrix generated profoundly inflated absorbance readings unrepresentative of actual glucose yield. Future dairy research must adopt HPLC, HPAEC-PAD, or GOD-POD-based enzymatic assays to ensure data integrity and regulatory compliance. Second, the application of 80°C thermal inactivation to lactose-hydrolyzed milk — a matrix now enriched with highly reactive free monosaccharides — predictably and aggressively initiates the Maillard reaction cascade. This generates advanced glycation end-products including furosine and HMF, causing irreversible sensory degradation, reduced essential amino acid bioavailability, and potentially unsafe concentrations of mutagenic HMF in sensitive applications. Non-thermal enzyme inactivation technologies must be adopted as the industry standard. Third, the transition from bench-scale batch hydrolysis to commercially viable production requires immobilized enzyme bioreactor technology. While effective for bovine milk, the unique rheological properties of bubaline milk present unresolved engineering challenges for packed-bed systems, including membrane fouling and pressure drop instability. The development of anti-fouling enzyme carrier platforms represents a priority research direction for the large-scale production of lactose-free buffalo milk products.

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