β-Galactosidase-Mediated Lactose Hydrolysis in Milk by *Bacillus aryabhattai*-A Marine Bacterium

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Abstract
β-Galactosidase hydrolyses lactose into glucose and galactose and has potential applications in dairy processing. In this study, 24 marine bacterial isolates were screened for β-galactosidase production. Isolate MBC1 showed the highest activity in quantitative screening and was identified as *Bacillus aryabhattai* using cultural, morphological, biochemical, VITEK 2, and 16S rRNA gene analyses; its sequence was deposited in GenBank (MG831954). Enzyme production was evaluated under different nutritional and physicochemical conditions. The enzyme was purified by ammonium sulfate precipitation, dialysis, DEAE-cellulose chromatography, and Sephadex G-200 gel filtration. The purified preparation showed an activity of 1326 U/mL, a specific activity of 491.10 U/mg, 6.20-fold purification, and 60% recovery; its apparent molecular mass was approximately 45 kDa. Maximum catalytic activity was observed at pH 7.0 and 35 °C. During buffalo-milk treatment, reducing sugars increased from 20.87 ± 1.47 µg/mL at 1 h to 307.09 ± 4.01 µg/mL at 6 h, corresponding to 4.17 ± 0.29% and 61.42 ± 0.80% apparent hydrolysis, respectively. In a separate 5% lactose-solution assay, the recalculated 6-h reducing-sugar concentration was 407.39 ± 4.80 µg/mL in the 100-fold-diluted assay sample, corresponding to 81.48 ± 0.96% apparent hydrolysis when compared with the equivalent diluted substrate concentration. Sensory scores were generally higher for enzyme-treated milk, although the small panel and zero standard deviations reported for some attributes require careful interpretation. Overall, MBC1 is a potential source of β-galactosidase for further study. Since DNS estimates reducing sugars rather than residual lactose directly, the hydrolysis estimates should be confirmed using a validated lactose-specific method.

Keywords: β-galactosidase, *Bacillus aryabhattai,* marine bacteria, lactose hydrolysis, buffalo milk, enzyme purification

1. Introduction
Lactose is the main carbohydrate in milk. It is a disaccharide made up of glucose and galactose linked by a β-1,4-glycosidic bond. β-Galactosidase (EC 3.2.1.23), also called lactase, breaks lactose into glucose and galactose. Low intestinal lactase activity can cause lactose malabsorption and gastrointestinal symptoms after consumption of lactose-containing foods. Therefore, enzymatic lactose hydrolysis is useful for producing lactose-reduced and lactose-hydrolyzed dairy products (Schulz & Rizvi, 2023; Zhou et al., 2024).

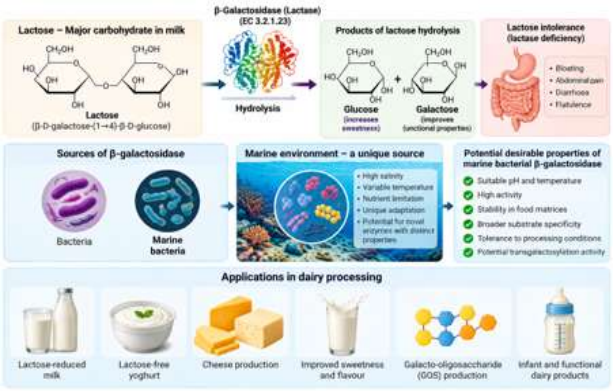


Figure 1. Lactose hydrolysis by marine bacterial β-galactosidase and its applications in the dairy industry

β-Galactosidase is widely used in dairy processing. Hydrolysis of lactose reduces the lactose content and produces glucose and galactose, which can increase sweetness and affect other properties of the product. Some β-galactosidase can also produce galacto-oligosaccharides through transgalactosylation. Therefore, enzyme source, pH, temperature, stability, and substrate specificity are important when selecting an enzyme for food processing (Ruiz-Ramírez & Jiménez-Flores, 2023; Zhou et al., 2024).

Microorganisms are important sources of β-galactosidase because enzymes from different organisms can have different optimum pH, temperature, substrate specificity, activity, and stability. These properties affect enzyme performance in dairy products. Recent reviews also emphasize the importance of enzyme source and stabilization when developing β-galactosidase for industrial lactose hydrolysis (Schulz & Rizvi, 2023; Zhou et al., 2024).

Marine microorganisms remain a less extensively studied source of enzymes with potential biotechnological applications. Their growth in environments with varying salinity, temperature, nutrient availability, and other ecological conditions may be associated with enzymes that have distinct biochemical properties. Marine bacteria may therefore provide useful candidates for enzyme discovery, including enzymes relevant to food processing.

Bacillus species are known to produce a range of hydrolytic enzymes. Luan and Duan (2022) described a β-galactosidase from *Bacillus aryabhattai* GEL-09 with an apparent molecular mass of approximately 75 kDa and optimum activity at 45 °C and pH 6.0. The enzyme reportedly hydrolysed lactose in milk within 4 h under their experimental conditions. These findings provide a useful point of comparison for the enzyme produced by *B. aryabhattai* MBC1 in the present study. Differences between the reported properties may reflect strain-level variation, enzyme structure, production conditions, and assay conditions.

β-Galactosidase is relevant to dairy processing because it converts lactose into glucose and galactose, which can affect product sweetness and other sensory properties. Whey is also a lactose-rich substrate for enzymatic processing. However, enzyme performance depends on the enzyme source, substrate composition, enzyme dose, pH, temperature, and reaction time. Recent reviews have discussed the use of soluble and immobilized β-galactosidase to improve lactose hydrolysis and process efficiency (Schulz & Rizvi, 2023; Zhou et al., 2024). The present study evaluated a marine bacterial enzyme for lactose hydrolysis in buffalo milk and a model lactose solution.

2. Materials and Methods

2.1 Isolation and screening of marine bacteria

Marine bacterial isolates were obtained from seawater and sediment collected from Manginapudi Beach, Machilipatnam, Andhra Pradesh, India. The samples were serially diluted and plated on Marine Agar 2216 supplemented with 1% (w/v) lactose and incubated at 30 °C for 48 h. β-

Galactosidase-producing isolates were first screened using an X-Gal/IPTG chromogenic assay. Positive isolates were then tested using the ONPG assay. Enzyme activity was measured spectrophotometrically at 420 nm and expressed as enzyme activity units under the assay conditions used.

2.2 Identification of the potent isolate

The potent isolate MBC1 was identified using cultural, morphological, biochemical, and VITEK 2 methods, followed by 16S rRNA gene sequencing. The sequence was analyzed using BLASTN, and a phylogenetic tree was constructed in MEGA 11 using the Neighbor-Joining method with 1,000 bootstrap replicates (Altschul et al., 1990; Tamura et al., 2021). The isolate was identified as *Bacillus aryabhattai* MBC1, and the sequence was deposited in GenBank under accession number MG831954.

2.3 Optimization of β-galactosidase production

β-Galactosidase production was optimized by testing carbon and nitrogen sources, lactose concentration, metal ions, inoculum size, pH, temperature, incubation time, agitation, and salinity. One-factor-at-a-time experiments were followed by response surface methodology as stated in the submitted study. The reported optimum conditions were glucose as the carbon source, 1.5% (w/v) lactose, yeast extract as the nitrogen source, 1 mM $MgCl_2$, pH 7.5, 37 °C, 36 h incubation, 5% (v/v) inoculum, 180 rpm agitation, and 3% (w/v) NaCl. Corn steep liquor at 2% was also tested as an alternative substrate.

2.4 Purification and characterization of β-galactosidase

2.4.1 Purification of β-galactosidase

The enzyme was purified by ammonium sulfate precipitation, dialysis, DEAE-cellulose ion-exchange chromatography, and Sephadex G-200 gel filtration. Protein concentration was determined by the Lowry method (Lowry et al., 1951), and the molecular mass of the purified enzyme was estimated by SDS-PAGE according to Laemmli (1970). Purification fold and enzyme recovery were calculated from the purification data.

2.4.2 Characterization of β-galactosidase

The purified β-galactosidase was characterized to determine its physicochemical and catalytic properties. The enzyme was tested for optimum temperature, optimum pH, metal-ion effects, and thermal stability. These properties are important when considering the use of β-galactosidase for lactose hydrolysis and dairy applications (Husain, 2010; Ruiz-Ramírez & Jiménez-Flores, 2023; Zhou et al., 2024).

2.4.3 Effect of temperature on β-galactosidase enzyme activity

The optimum temperature of the purified β-galactosidase was determined by performing the ONPG assay at 20, 30, 40, 50, 60, and 70 °C. The temperature giving the highest enzyme activity was taken as the optimum temperature (Husain, 2010).

2.4.4 Effect of pH on β-galactosidase stability

To determine pH stability, the purified enzyme was incubated in buffers with different pH values (4.0–10.0) at 4 °C for 24 h. Residual enzyme activity was then measured using the standard ONPG assay. The activity remaining after incubation was expressed as a percentage of the initial activity. The pH range in which the enzyme retained high activity was considered the stable range (Husain, 2010).

2.4.5 Thermal stability of β-galactosidase enzyme

Thermal stability was determined by pre-incubating the purified enzyme at 30, 40, 50, 60, and 70 °C for 30–120 min. Residual enzyme activity was then measured under the standard assay conditions. The activity of the untreated enzyme was taken as 100%, and residual activity was expressed as a percentage (Husain, 2010).

2.4.6 Effect of metal ions on enzyme activity

The effects of Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, and Cu^{2+} were evaluated at a final concentration of

An appropriate volume of each metal-ion stock solution was added to the reaction mixture to give a final concentration of 1 mM. The enzyme-metal ion mixture was incubated for 30 min, and residual enzyme activity was measured using the standard ONPG assay. Enzyme activity without added metal ions was taken as 100%, and the results were expressed as relative activity (%). (Husain, 2010).

2.5 Lactose hydrolysis by β-galactosidase

2.5.1 Lactose hydrolysis in buffalo milk by β-galactosidase

Pasteurized buffalo milk was used as the substrate. Purified β-galactosidase from *B. aryabhattai* MBC1 (1326 U/mL) was added to the milk and incubated at 35 °C under static conditions for 1–6 h. Samples were collected at 0, 1, 2, 3, 4, 5, and 6 h. Two controls were maintained: an enzyme-free milk control to account for background reducing sugars and a substrate control without enzyme to confirm that the increase in reducing sugars was due to enzyme treatment. Enzyme activity was stopped by heating the samples at 90 °C for 5 min, followed by cooling. Reducing sugars were estimated by the 3,5-dinitrosalicylic acid (DNS) method using glucose as the standard, with absorbance measured at 540 nm.

2.5.2. Lactose hydrolysis in 5% lactose substrate by β-galactosidase

Lactose hydrolysis was evaluated using a 5% (w/v) lactose solution. For this assay, 20 mL of lactose solution was mixed with 1 mL of purified β-galactosidase (1326 U/mL), adjusted to pH 7.5, and incubated at 37 °C under static conditions. Aliquots were collected at 0, 1, 2, 3, 4, and 6 h. Substrate and enzyme controls were maintained under the same incubation and heat-inactivation conditions. Samples were heat-inactivated at 90 °C for 5 min. Reducing sugars were estimated by the DNS method at 540 nm. The glucose concentration was calculated from the standard curve ($y = 0.0052x + 0.0098$; $R^2 = 0.9967$), and the dilution factor was applied to obtain the original reducing-sugar concentration. Apparent lactose hydrolysis was calculated relative to the initial lactose concentration.

2.6 Estimation of reducing sugars by the DNS method and glucose standard curve

A glucose stock solution (1000 µg/mL) was prepared by dissolving 100 mg of D-glucose in distilled water and making the volume up to 100 mL. Working standards ranging from 0 to 400 µg/mL were prepared by dilution and analyzed by the DNS method at 540 nm. The calibration curve was expressed as $Y = 0.0052X + 0.0098$ ($R^2 = 0.9967$), where Y is OD_{540} and X is glucose concentration (µg/mL). Glucose-equivalent reducing sugar concentration was calculated from the calibration equation and corrected for the dilution factor. Apparent lactose hydrolysis was calculated relative to the initial lactose concentration.

2.7 Sensory valuation

The sensory properties of β-galactosidase-treated buffalo milk were assessed against untreated milk by a semi-trained panel of 10–15 members. The samples were heat-inactivated at 90 °C for 5 min, cooled, and kept at 4 °C until assessment. Coded portions (20–30 mL) were presented randomly at 8–10 °C, with distilled water supplied for palate cleansing. Appearance, colour, aroma, taste, sweetness, mouthfeel, aftertaste, and overall acceptability were rated using a nine-point hedonic scale from 1 (dislike extremely) to 9 (like extremely) (Lawless and Heymann, 2010).

2.8 Statistical study

Experiments were conducted in replicates, and the results were reported as mean ± standard deviation (SD). The effect of incubation time on reducing sugar release during lactose hydrolysis in buffalo milk was evaluated using one-way analysis of variance (ANOVA). Differences were considered statistically significant at $p < 0.05.3$.

Results

3.1 Isolation, screening, and identification of the potent isolate

A total of twenty-four morphologically different bacterial isolates were recovered from marine seawater and sediment samples collected at Manginapudi Beach, Machilipatnam, Andhra Pradesh,

India. Of these isolates, MBC1 and MBC3 produced positive results in the X-Gal/IPTG assay. These two isolates were chosen for further quantitative screening using the ONPG assay. MBC1 exhibited the highest β-galactosidase activity, with 29.0 U/mL intracellular and 8.0 U/mL extracellular activity. Based on cultural, morphological, biochemical, VITEK 2, and 16S rRNA gene analyses, MBC1 was identified as *Bacillus aryabhattai* MBC1. The 16S rRNA gene sequence of the isolate was deposited in GenBank under accession number MG831954.

3.2 Optimization of β-Galactosidase Production

β-Galactosidase production by *B. aryabhattai* MBC1 was optimized by testing different nutritional and physical conditions. Among the carbon sources tested, glucose gave the highest enzyme production of 398 U/mL. The highest production with lactose was obtained at 1.5% (w/v), with an enzyme activity of 216 U/mL. Among the nitrogen sources tested, yeast extract gave the highest activity of 425 U/mL. Among the metal ions tested, $MgCl_2$ at 1 mM resulted in the highest enzyme activity of 299 U/mL.

The optimum physicochemical conditions were pH 7.5 and 37 °C, 36 h incubation, 5% (v/v) inoculum, 180 rpm agitation, and 3% (w/v) NaCl, with enzyme activities of 399, 487, 319, 269, 219, and 148 U/mL, respectively. Corn steep liquor at 2% was also tested as an alternative substrate and gave an enzyme activity of 269 U/mL.

Table 1. Optimization of β-galactosidase production by the potent strain *Bacillus aryabhattai* MBC1.

Parameter	Optimum condition	β-Galactosidase activity (U/mL)
Carbon source	Glucose	398**(U/mL**
Lactose concentration	1.5% (w/v)	216**(U/mL**
Nitrogen source	Yeast extract	425**(U/mL**
Metal ion	$MgCl_2$, 1 mM	299**(U/mL**
pH	7.5	399**(U/mL**
Temperature	37 °C	487**(U/mL**
Incubation time	36 h	319**(U/mL**
Inoculum size	5% (v/v)	269**(U/mL**
Agitation	180 rpm	219**(U/mL**
NaCl concentration	3% (w/v)	148**(U/mL**
Alternative substrate	Corn steep liquor, 2%	269**(U/mL**

(Source: Authors)

3.3 Purification of β-Galactosidase

The β-galactosidase produced by *B. aryabhattai* MBC1 was purified through a series of purification steps. The final purified enzyme showed an activity of 1326 U/mL and a specific activity of 491.10 U/mg. The purification process resulted in a 6.20-fold purification and a 60% enzyme recovery. The apparent molecular mass was approximately 45 kDa.

3.4 Characterization of β-galactosidase

3.4.1 Effect of pH on β-galactosidase activity

The effect of pH on the activity and stability of β-galactosidase produced by *Bacillus aryabhattai* MBC1 was evaluated over a pH range of 4.0–10.0. The enzyme showed maximum activity at pH 7.0, with an activity of 629 U/mL. Activity decreased at pH values below and above the optimum.

3.4.2 Effect of temperature on β-galactosidase activity

The effect of temperature on β-galactosidase activity and stability was evaluated over the range of 20–70 °C. The enzyme showed maximum activity at 35 °C. Activity decreased at temperatures below and above 35 °C.

3.4.3 Thermal stability

The thermal stability of purified β-galactosidase from *Bacillus aryabhattai* MBC1 was evaluated at different temperatures. Enzyme activity increased from 175 U/mL at 20 °C to 368, 477, 589, and 697 U/mL as the temperature increased, reaching the highest activity at approximately 37 °C. Thereafter, activity decreased to 465 U/mL at 40 °C, 325 U/mL at 45 °C, and 125 U/mL at 50 °C.

3.4.4 Effect of metal ions on enzyme activity

The effect of different metal ions on purified β-galactosidase activity was evaluated at a final concentration of 1 mM. Mg^{2+} showed the highest activity (851 U/mL), followed by Ca^{2+} (759 U/mL) and Mn^{2+} (542 U/mL). Zn^{2+} resulted in 256 U/mL, whereas Fe^{2+} and Cu^{2+} showed activities of 112 and 110 U/mL, respectively.

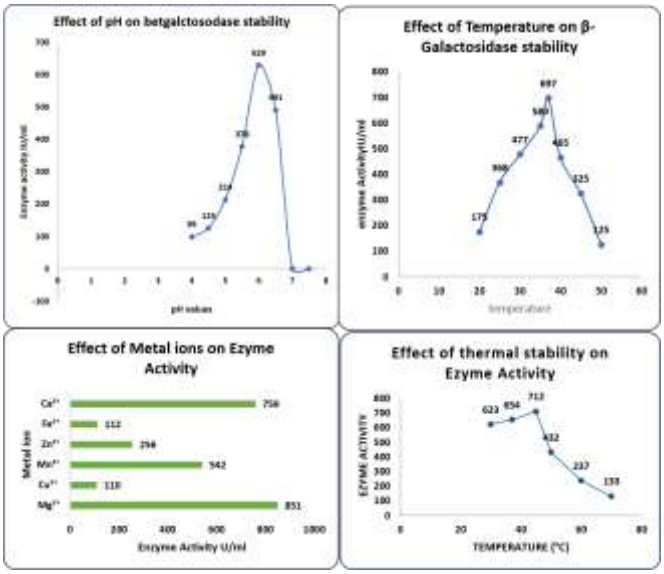


Figure 2. Effects of pH, temperature, metal ions, and thermal treatment on the activity and stability of purified β-galactosidase from *Bacillus aryabhattai* MBC1.

3.5 Lactose hydrolysis in buffalo milk

3.5.1 Lactose hydrolysis and reducing sugar release

The hydrolysis of lactose by purified β-galactosidase from *Bacillus aryabhattai* MBC1 was evaluated over 0–6 h using three independent replicates. Reducing sugar release increased with incubation time. The mean concentration was 20.87 ± 1.47 µg/mL at 1 h and increased to 32.73 ± 1.92, 130.81 ± 19.23, 177.60 ± 14.68, 234.65 ± 10.17, and 307.09 ± 4.01 µg/mL at 2, 3, 4, 5, and 6 h, respectively.

A corresponding increase in apparent lactose hydrolysis was observed. Mean hydrolysis increased from 4.17 ± 0.29% at 1 h to 6.55 ± 0.38% at 2 h, 26.16 ± 3.85% at 3 h, 35.52 ± 2.94% at 4 h, and 46.93 ± 2.03% at 5 h. The highest apparent hydrolysis was observed at 6 h (61.42 ± 0.80%). No reducing sugar release or apparent hydrolysis was detected in either control.

Table 2. Mean reducing-sugar release and apparent lactose hydrolysis in buffalo milk. (Source: Authors)

Incubation time (h)	Reducing sugars, Mean ± SD (µg/mL)	Hydrolysis, Mean ± SD (%)	Significance
0	0.00 ± 0.00	0.00 ± 0.00	A
1	20.87 ± 1.47	4.17 ± 0.29	A
2	32.73 ± 1.92	6.55 ± 0.38	B
3	130.81 ± 19.23	26.16 ± 3.85	C
4	177.60 ± 14.68	35.52 ± 2.94	D
5	234.65 ± 10.17	46.93 ± 2.03	E
6	307.09 ± 4.01	61.42 ± 0.80	F
F-value	410.23	—	—
p-value	<0.001	—	Highly significant

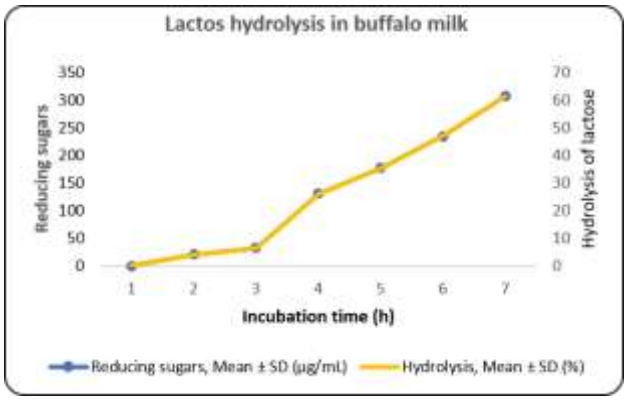


Figure 3. Release of reducing sugars during lactose hydrolysis in buffalo milk by β-galactosidase from *Bacillus aryabhattai* MBC1.

Values are expressed as mean ± SD (n = 3). One-way ANOVA indicated a significant effect of incubation time on reducing-sugar release ($F(6,14) = 410.23$, $p < 0.001$). Different letters indicate statistically significant differences ($p < 0.05$). The ANOVA statistic should be confirmed against the original replicate-level data before submission.

Overall, the milk experiment showed a time-dependent increase in reducing sugar release and apparent lactose hydrolysis. The largest increase occurred between 2 and 3 h, followed by smaller increases up to 6 h. All values are presented as mean ± SD (n = 3).

3.5.2 Lactose hydrolysis in 5% lactose substrate

The hydrolysis of lactose by purified β-galactosidase from *Bacillus aryabhattai* MBC1 increased with incubation time. Based on the glucose calibration curve, reducing-sugar concentrations in the 100-fold-diluted assay samples increased from 21.07 µg/mL at 1 h to 28.64 µg/mL at 2 h, 170.99 µg/mL at 3 h, 240.90 µg/mL at 4 h, 350.21 µg/mL at 5 h, and 407.41 µg/mL at 6 h. The corresponding apparent lactose hydrolysis values were 4.21%, 5.73%, 34.20%, 48.18%, 70.04%, and 81.48%, respectively. No reducing sugars were detected in the zero-time sample or enzyme-free control. Because the DNS assay measures reducing sugars as glucose equivalents rather than residual lactose directly, these percentages are reported as apparent lactose hydrolysis.

Table 3. Effect of incubation time on reducing-sugar release and apparent lactose hydrolysis in a 5% lactose solution.

Incubation time (h)	Reducing sugars (µg/mL)	Apparent lactose hydrolysis (%)

0	0.00	0.00
1	21.07	4.21
2	28.64	5.73
3	170.99	34.20
4	240.90	48.18
5	350.21	70.04
6	407.41	81.48
Control	0.00	0.00

(Source: Authors)

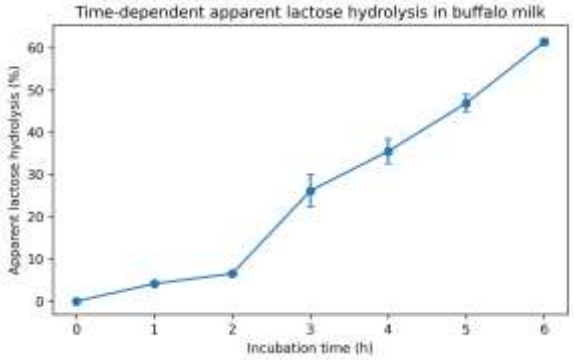


Figure 4. Time-dependent apparent lactose hydrolysis in a 5% lactose solution by purified β-galactosidase from *Bacillus aryabhattai* MBC1.

Table 4. Mean reducing-sugar release and apparent lactose hydrolysis in a 5% lactose solution (mean ± SD).

Incubation time (h)	Reducing sugars (µg/mL), Mean ± SD	Apparent lactose hydrolysis (%), Mean ± SD	Significance
0	0.00 ± 0.00	0.00 ± 0.00	A
1	21.07 ± 2.00	4.21 ± 0.40	B
2	28.64 ± 1.91	5.73 ± 0.38	C
3	170.99 ± 2.91	34.20 ± 0.58	D
4	240.90 ± 7.71	48.18 ± 1.54	E
5	350.21 ± 7.71	70.04 ± 1.54	F
6	407.41 ± 4.80	81.48 ± 0.96	G
Control	0.00 ± 0.00	0.00 ± 0.00	A
F-value	3667.78	—	—
p-value	<0.001	—	Highly significant

(Source: Authors)

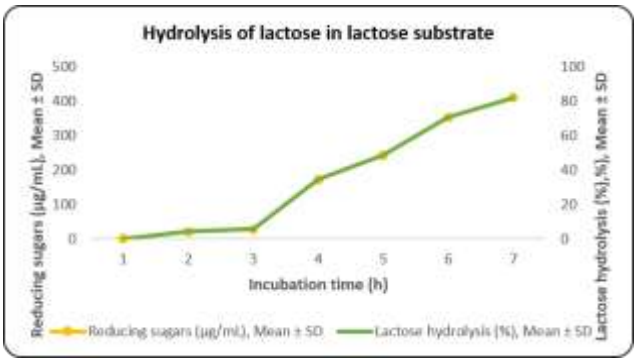


Figure 5. Release of reducing sugars during hydrolysis of a 5% lactose solution by β-galactosidase from *Bacillus aryabhattai* MBC1.

Reported ANOVA result: $F = 3667.776$, $p = 1.51 \times 10^{-21}$ ($p < 0.001$). Confirm the test statistic and degrees of freedom against the original replicate-level data before submission.
Values are expressed as mean ± SD (n = 3). Different letters indicate statistically significant differences ($p < 0.05$). The reported one-way ANOVA indicates an effect of incubation time on reducing-sugar release ($F = 3667.776$, $p < 0.001$); verify this statistic using the original replicate-level data.

Table 5. Glucose standard curve used to estimate reducing sugars by the DNS method.

Glucose concentration (µg/mL)	OD_{540}
0	0.000
20	0.107
40	0.246
60	0.336
80	0.423
100	0.529
120	0.649
150	0.798
200	1.058
250	1.317
300	1.581
350	1.746
400	2.195

(Source: Authors)

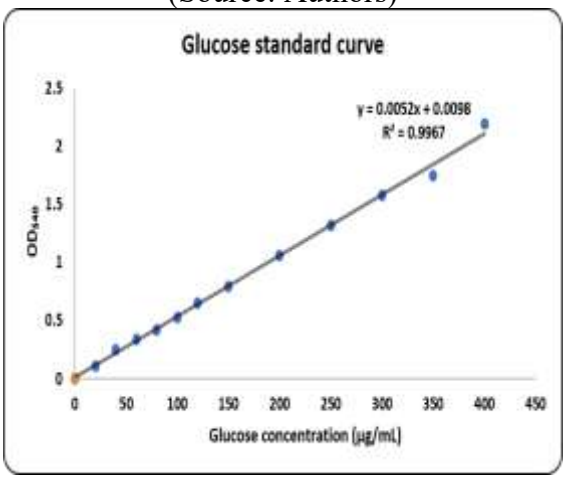


Figure 6. Glucose standard curve for the estimation of reducing sugars by the DNS method.
(Source: Authors)

Table 6. Sensory scores of untreated and β-galactosidase-treated buffalo milk using a nine-point hedonic scale.

Sensory attribute	Untreated milk Mean ± SD	Enzyme-treated milk Mean ± SD	F-value	p-value
Appearance	6.20 ± 0.00	7.37 ± 0.25	64.47	0.0013
Colour	6.40 ± 0.00	7.17 ± 0.15	75.57	0.0010
Aroma	6.00 ± 0.00	7.27 ± 0.12	361.00	<0.001

Taste	5.80 ± 0.00	8.03 ± 0.06	4489.00	<0.001
Sweetness	5.50 ± 0.00	8.43 ± 0.35	209.30	<0.001
Mouthfeel	6.10 ± 0.00	7.50 ± 0.20	147.00	<0.001
Aftertaste	5.90 ± 0.00	7.63 ± 0.15	386.29	<0.001
Overall acceptability	6.00 ± 0.00	8.00 ± 0.00	Not estimable	Not estimable

(Source: Authors)

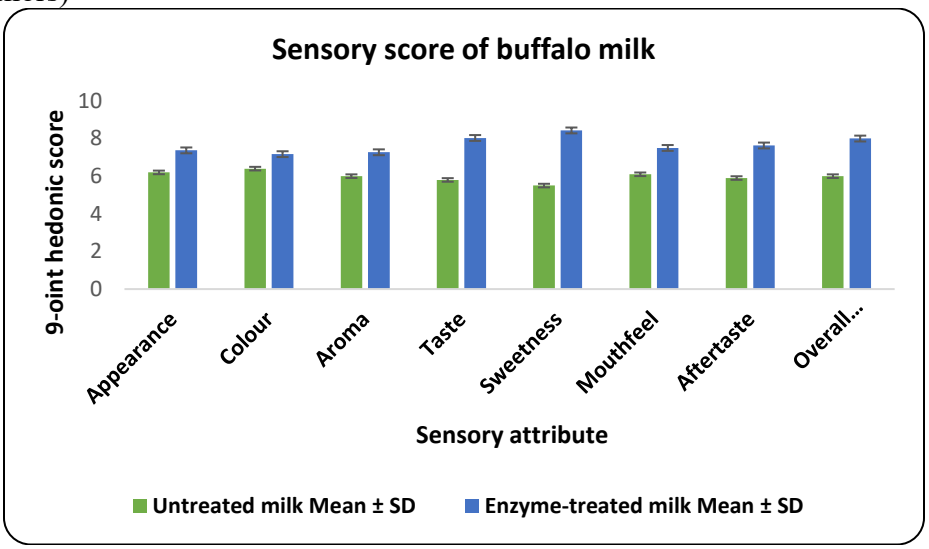


Figure 7. Sensory Evaluation of Beta-Galactosidase-Treated Buffalo Milk

Discussion

This study investigated β-galactosidase production by marine bacteria, followed by purification, characterization, and evaluation of lactose hydrolysis in buffalo milk and a model lactose solution. Of the 24 isolates screened, MBC1 showed the strongest quantitative activity and was identified as *Bacillus aryabhattai*. The purified preparation had an activity of 1326 U/mL, a specific activity of 491.10 U/mg, 6.20-fold purification, and 60% recovery, with an apparent molecular mass of approximately 45 kDa. These results support further investigation of MBC1 as a potential enzyme source but do not by themselves establish suitability for commercial dairy processing. Enzyme source, purity, catalytic properties, and processing conditions all influence performance in food applications (Husain, 2010; Zhou et al., 2024).

Of the 24 marine bacterial isolates screened, MBC1 and MBC3 were positive in the X-Gal/IPTG assay. MBC1 showed the highest activity in the quantitative ONPG assay and was selected for further work. Identification was based on cultural, biochemical, VITEK 2, and 16S rRNA gene analyses. Zhou et al. (2021) characterized β-galactosidase Gal42 from marine *Bacillus* sp. BY02 and evaluated its application in milk. The present study adds another marine *Bacillus* isolate for investigation and includes testing in buffalo milk. Direct comparisons between studies should be made cautiously because enzyme doses, substrates, assay methods, and reaction conditions may differ.

Enzyme production varied with the nutritional and physicochemical conditions tested. Glucose gave the highest reported activity among the carbon sources (398 U/mL), while 1.5% lactose gave 216 U/mL. Yeast extract was the most effective nitrogen source (425 U/mL), and $MgCl_2$ at 1 mM gave 299 U/mL. Among the tested physical conditions, the highest reported activities were associated with 37 °C (487 U/mL), pH 7.5 (399 U/mL), and 36 h of incubation (319 U/mL). The values in Table 1 represent results for individual conditions and should not be interpreted as a

single combined optimum unless that combination was experimentally validated. Nutrient composition and cultivation conditions are known to influence microbial β-galactosidase production (Luan & Duan, 2022; Zhou et al., 2021; Zhou et al., 2024). Luan and Duan (2022), for example, reported different optimum conditions for the GEL-09 enzyme. Differences between strains and experimental designs may explain the variation, and production optima should be distinguished from the catalytic optimum of the purified enzyme.

The metal-ion assay showed different effects on enzyme activity. Mg^{2+} gave the highest reported activity (851 U/mL), followed by Ca^{2+} (759 U/mL) and Mn^{2+} (542 U/mL), whereas lower activities were recorded with Zn^{2+}, Fe^{2+}, and Cu^{2+}. This differs from the findings for Gal42 from marine *Bacillus* sp. BY02, for which Zn^{2+} enhanced enzyme performance and thermostability (Zhou et al., 2021). Such differences can depend on enzyme structure and assay conditions; the effect of Mg^{2+} should therefore be confirmed under the intended application conditions. In buffalo milk, reducing sugars increased from 20.87 ± 1.47 µg/mL at 1 h to 307.09 ± 4.01 µg/mL at 6 h. The corresponding apparent hydrolysis estimates increased from 4.17 ± 0.29% to 61.42 ± 0.80%. The largest increase occurred between 2 and 3 h, followed by continued increases through 6 h. These results indicate time-dependent reducing-sugar release, but DNS does not directly quantify residual lactose. The reported percentages should consequently be interpreted as apparent hydrolysis estimates. The reported ANOVA statistics should be checked against the original replicate-level data before publication.

In the 5% lactose-solution assay, the three replicate OD_{540} readings were converted using the glucose calibration curve. The resulting reducing-sugar concentration at 6 h was 407.39 ± 4.80 µg/mL in the 100-fold-diluted assay sample. Using the equivalent diluted substrate concentration as the denominator, this corresponded to 81.48 ± 0.96% apparent hydrolysis. This estimate was higher than that observed in buffalo milk, although the two systems differ in composition and analytical matrix. Milk contains proteins, fat, minerals, and other components that may affect enzyme performance and DNS measurements, whereas the lactose solution is a simpler substrate. Because DNS measures reducing sugars rather than residual lactose specifically, the percentage should not be presented as a direct measurement of lactose disappearance. A lactose-specific assay is needed to confirm the extent of hydrolysis.

Comparisons with previous studies should account for enzyme dose, substrate concentration, temperature, incubation time, and analytical method. Luan and Duan (2022) reported complete lactose hydrolysis in milk within 4 h using a thermostable recombinant enzyme at 45 °C and an enzyme dose of 8 U/mL. In the present study, the apparent hydrolysis estimate in buffalo milk reached 61.42 ± 0.80% after 6 h under the test conditions. Singh et al. (2022) also showed that enzyme concentration and processing conditions affect lactose hydrolysis and galacto-oligosaccharide formation in concentrated skim milk. These studies emphasize the need to optimize enzyme dose and reaction conditions for each dairy substrate. Because the experimental conditions and analytical approaches differ, the reported percentages should not be used to rank enzyme performance directly.

Sensory evaluation showed higher mean scores for enzyme-treated milk than for untreated milk across the attributes listed in Table 6. For example, the sweetness score was 8.43 for treated milk compared with 5.50 for untreated milk, and overall acceptability was 8.00 compared with 6.00. Increased glucose and galactose formation may contribute to perceived sweetness after lactose hydrolysis. However, several untreated-milk standard deviations are reported as 0.00, and the overall acceptability variability is also zero in the table. These values should be checked against the individual panelists' records before interpreting statistical significance. The panel was

relatively small, so these findings should be treated as preliminary and confirmed with a larger, appropriately designed sensory study.
The glucose calibration curve showed a strong relationship between glucose concentration and absorbance (reported $R^2 = 0.9967$). The reducing-sugar values were estimated from this curve and should be recalculated if the final standard readings or dilution factors change. More importantly, DNS measures reducing sugars and does not directly quantify residual lactose. Therefore, the percentages reported here are apparent hydrolysis estimates. Future work should quantify lactose, glucose, and galactose separately using HPLC or another validated analytical method and should verify the statistical analysis from the original replicate-level observations.

Further work is required before the enzyme can be considered for practical dairy processing. Activity and stability should be evaluated under realistic processing conditions and during refrigerated storage. Enzyme dose, pH, temperature, and reaction time should be optimized directly in buffalo milk rather than inferred from a model lactose solution. Enzyme immobilization and reuse could also be investigated to improve process feasibility (Schulz & Rizvi, 2023; Zhou et al., 2024). Pilot-scale studies should assess residual lactose, glucose and galactose concentrations, microbiological quality, nutritional properties, sensory acceptance with a larger panel, and batch-to-batch variation.

Overall, marine *B. aryabhattai* MBC1 is a potential source of β-galactosidase under the laboratory conditions tested. The study examined enzyme production, purification, biochemical characterization, lactose hydrolysis, and preliminary sensory properties of treated buffalo milk. Limitations include the laboratory scale, the relatively short treatment period, the use of DNS-based reducing-sugar estimates rather than direct lactose measurement, and the small sensory panel. The numerical values and statistical outputs should be confirmed against the original laboratory records before publication. Future work should use a validated lactose-specific method, verify ANOVA and sensory statistics, and evaluate performance under practical dairy-processing conditions. These steps are necessary before drawing conclusions about commercial suitability or consumer acceptance.

Conclusion

Marine isolate MBC1 was identified as *Bacillus aryabhattai* and produced a β-galactosidase preparation with an activity of 1326 U/mL, a specific activity of 491.10 U/mg, a 6.20-fold purification, and 60% recovery. The apparent molecular mass was approximately 45 kDa. Maximum activity was reported at pH 7.0 and 35 °C, and Mg^{2+} produced the highest activity among the metal ions tested. During the treatment of buffalo milk, the reducing-sugar concentration increased over time and reached 307.09 ± 4.01 µg/mL after 6 h, corresponding to an apparent hydrolysis estimate of 61.42 ± 0.80%. In a separate 5% lactose-solution assay, the recalculated apparent hydrolysis estimate was 81.48 ± 0.96% after 6 h, based on the glucose calibration curve and the stated 100-fold dilution; this estimate should still be confirmed against the original laboratory records. Enzyme-treated milk received higher sweetness and overall acceptability scores than untreated milk. These findings suggest potential for further development, but direct lactose quantification and validation under practical processing conditions are needed.

The study was limited by its laboratory scale, a maximum milk treatment period of 6 h, the use of reducing-sugar measurements rather than direct lactose quantification, and a relatively small semi-trained sensory panel. Future research should measure lactose, glucose, and galactose separately; optimize enzyme concentration and reaction conditions in milk; and evaluate enzyme immobilization, reuse, and stability during processing and storage. Pilot-scale trials should also assess microbiological and nutritional quality and include a larger consumer panel. Subject to these

further validations, β-galactosidase from marine *B. aryabhattai* MBC1 may warrant further study for the development of lactose-reduced dairy products.

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References

1. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. Journal of Molecular Biology, 215 (3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2
2. Husain, Q. (2010). β-Galactosidases and their potential applications: A review. Critical Reviews in Biotechnology, 30(1), 41–62. https://doi.org/10.3109/07388550903330497
3. Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680–685. https://doi.org/10.1038/227680a0
4. Lawless, H. T., & Heymann, H. (2010). Sensory evaluation of food: Principles and practices (2nd ed.). Springer. https://doi.org/10.1007/978-1-4419-6488-5
5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry, 193(1), 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6
6. Luan, S., & Duan, X. (2022). A novel thermal-activated β-galactosidase from *Bacillus aryabhattai* GEL-09 for lactose hydrolysis in milk. Foods, 11(3), Article 372. https://doi.org/10.3390/foods11030372
7. Ruiz-Ramírez, S., & Jiménez-Flores, R. (2023). Invited review: Properties of β-galactosidase derived from Lactobacillaceae species and their capacity for galacto-oligosaccharide production. Journal of Dairy Science, 106(12), 8193–8206. https://doi.org/10.3168/jds.2023-23392
8. Schulz, P., & Rizvi, S. S. H. (2023). Hydrolysis of lactose in milk: Current status and future products. Food Reviews International, 39(5), 2875–2894. https://doi.org/10.1080/87559129.2021.1983590
9. Singh, P., Arora, S., Singh Rao, P., Kathuria, D., Sharma, V., & Singh, A. K. (2022). Effect of process parameters on the β-galactosidase hydrolysis of lactose and galactooligosaccharide formation in concentrated skim milk. Food Chemistry, 393, Article 133355. https://doi.org/10.1016/j.foodchem.2022.133355
10. Tamura, K., Stecher, G., & Kumar, S. (2021). MEGA11: Molecular evolutionary genetics analysis version 11. Molecular Biology and Evolution, 38(7), 3022–3027. https://doi.org/10.1093/molbev/msab120
11. Zhou, Z., He, N., Han, Q., Liu, S., Xue, R., Hao, J., & Li, S. (2021). Characterization and application of a new β-galactosidase Gal42 from marine bacterium *Bacillus* sp. BY02. Frontiers in Microbiology, 12, Article 742300. https://doi.org/10.3389/fmicb.2021.742300
12. Zhou, Y., Liu, Y., Gao, F., Xia, Z., Zhang, Z., Addai, F. P., Zhu, Y., Chen, J., Lin, F., & Chen, D. (2024). β-Galactosidase: Insights into source variability, genetic engineering, immobilization, and diverse applications in food, industry, and medicine. International Journal of Dairy Technology, 77(3), 651–679. https://doi.org/10.1111/1471-0307.13098