



Optimization and Characterization of β -Galactosidase from *Bacillus aryabhatai* – A Marine Bacterium

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Abstract

β -Galactosidase (EC 3.2.1.23) is an industrially important enzyme used in the dairy and food industries, particularly for lactose hydrolysis. The aim of the present study was to isolate and identify a marine bacterial β -galactosidase producer, optimize enzyme production, and purify and characterize the enzyme. Marine seawater and sediment samples were subjected to microbiological isolation; β -galactosidase-producing isolates were screened by an X-Gal chromogenic assay and quantitatively evaluated using the ONPG assay. Among 24 morphologically distinct isolates, MBC1 and MBC3 were β -galactosidase-positive, and MBC1 showed the highest intracellular activity (29 U/mL). MBC1 was identified as *Bacillus aryabhatai* by VITEK 2 biochemical profiling, 16S rRNA gene analysis, and phylogenetic analysis (GenBank accession MG831954). One-factor-at-a-time optimization identified glucose as the preferred carbon source (398 U/mL), 1.5% lactose as the optimum lactose concentration (216 U/mL), yeast extract as the preferred nitrogen source (425 U/mL), MgCl_2 at 1 mM (299 U/mL), pH 7.5 (399 U/mL), 37 °C (487 U/mL), 36 h incubation (319 U/mL), 5% inoculum (269 U/mL), 180 rpm agitation (219 U/mL), and 3% NaCl (148 U/mL). Corn steep liquor produced 248 U/mL among the alternative substrates tested. The crude extract showed 573 IU/mL activity and 220.38 IU/mg specific activity; after ammonium sulphate precipitation and dialysis, specific activity increased to 778.75 IU/mg with a 3.53-fold purification. Following DEAE-cellulose and Sephadex G-200 chromatography, the final preparation showed 1326 IU/mL activity, 491.10 IU/mg specific activity, 2.23-fold purification, and 60% overall yield. SDS-PAGE indicated an apparent molecular mass of approximately 45 kDa. These findings support the potential of marine-derived *B. aryabhatai* MBC1 as a source of β -galactosidase for further food and dairy biotechnology studies.

Keywords: β -galactosidase, marine bacteria, *Bacillus aryabhatai*, enzyme production, optimization, purification, lactose hydrolysis

1. Introduction

Lactase, also known as β -galactosidase (EC 3.2.1.23), is a crucial glycoside hydrolase that catalyzes the breakdown of lactose into glucose and galactose. Due to its uses in lactose hydrolysis, the creation of lactose-reduced dairy products, and whey utilization, the enzyme is highly significant in the food and dairy sectors. Lactose hydrolysis by enzymes can improve the digestibility of dairy products and provide a practical approach for producing lactose-reduced products for consumers with lactose intolerance (Kalathinathan et al., 2023; Ruiz-Ramírez & Jiménez-Flores, 2023).

Because they can be grown under controlled conditions and produce enzymes with diverse biochemical characteristics, microorganisms are important sources of β -galactosidase. The characteristics of β -galactosidases derived from bacteria, yeasts, and fungi vary according to the microbial source and growth conditions, and these enzymes have been studied for food and industrial applications (Ruiz-Ramírez & Jiménez-Flores, 2023; Bechtel et al., 2024).

Recent studies have demonstrated the potential of microbial β -galactosidases for lactose hydrolysis and other biotechnological applications (Bechtel et al., 2024; Liu et al., 2025).

β -Galactosidase production is influenced by several nutritional and physicochemical parameters, including lactose concentration, carbon and nitrogen sources, pH, temperature, incubation period, inoculum size, agitation, and mineral supplementation. Optimization of these parameters is important for enhancing enzyme production and improving microbial fermentation efficiency (Neti & Peshwe, 2025; Duan et al., 2022). In the present study, β -galactosidase production by the selected marine isolate MBC1 was evaluated under these nutritional and physicochemical conditions.

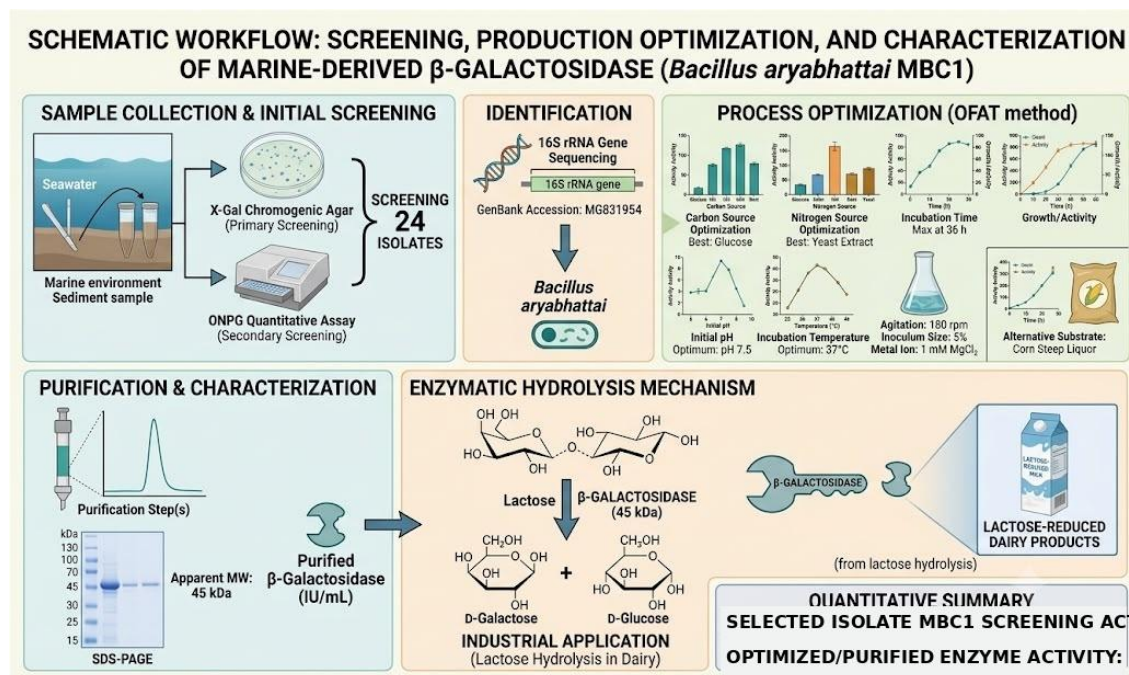


Figure 1. Schematic representation of the production of marine-derived β -galactosidase.

2. Materials and Methods

2.1 Collection and Isolation of Marine Bacteria

Marine bacteria were isolated from seawater and sediment samples collected from Manginapudi Beach, Machilipatnam, Andhra Pradesh, India. One millilitre of seawater or 1 g of sediment was transferred aseptically into 9 mL of sterile marine water and homogenized. Serial dilutions were prepared up to 10^{-6} , and 0.1 mL aliquots were plated onto Marine Agar 2216 supplemented with 1% (w/v) lactose. Plates were incubated aerobically at 30 °C for 48 h.

2.2 Primary Screening of β -Galactosidase-Producing Marine Bacteria

β -Galactosidase-producing isolates were screened using an X-Gal chromogenic plate assay. Isolates were spot-inoculated onto Marine Agar containing 1% (w/v) lactose, 40 $\mu\text{g mL}^{-1}$ X-Gal, and 0.1 mM IPTG, followed by incubation at 35 °C for 48 h. Development of blue coloration indicated β -galactosidase activity, and isolates showing distinct blue colonies were selected for secondary screening and quantitative enzyme assays (Li et al., 2012; Kalathinathan et al., 2023; Liu et al., 2025).

2.3 Secondary Screening of β -Galactosidase-Producing Marine Bacteria

Selected isolates were inoculated into Marine Broth containing 1% (w/v) lactose and incubated at 37 °C for 36 h at 180 rpm. Cultures were centrifuged at 10,000 rpm for 15 min at 4 °C. The cell-free supernatant was collected as the extracellular enzyme fraction, while the cell pellet was used for intracellular enzyme extraction. β -Galactosidase activity was measured using ONPG as the substrate (Li et al., 2012; Neti & Peshwe, 2025).

For intracellular enzyme extraction, the cell pellet was washed twice with chilled 0.1 M phosphate buffer (pH 7.0) and resuspended in the same buffer. Cells were disrupted by probe sonication at 20 kHz using 30 s ON/30 s OFF cycles for 6–8 cycles while maintaining the suspension in an ice bath. The lysate was centrifuged at 10,000 rpm for 15 min at 4 °C, and the clear supernatant was collected as the crude intracellular enzyme extract and kept on ice until analysis (Neti & Peshwe, 2025).

2.4 β -Galactosidase Activity

Intracellular β -galactosidase activity was measured using ONPG as the chromogenic substrate. A 0.5 mL aliquot of crude intracellular enzyme extract was mixed with 0.5 mL ONPG prepared in 0.1 M phosphate buffer (pH 7.0) and incubated at 37 °C for the specified time. The reaction was stopped with 1 mL of 1 M Na₂CO₃, and the yellow color developed due to o-nitrophenol formation was measured at 420 nm. Enzyme activity was expressed in Miller Units (MU) (Li et al., 2012).

2.5 Identification of Marine Bacterial Strains

Selected potent β -galactosidase-producing isolates were characterized based on cultural, morphological, and biochemical characteristics using standard microbiological methods prior to molecular identification (Ligozzi et al., 2002; Neti & Peshwe, 2025).

2.5.1 Cultural and Morphological Characterization

Purified isolates were grown on Marine Agar 2216 at 30 °C for 24–48 h. Colony characteristics, including size, shape, color, pigmentation, surface, texture, opacity, elevation, and margin, were observed and recorded (Neti & Peshwe, 2025).

Fresh cultures were examined by Gram staining and light microscopy using a 100 $\times$ oil-immersion objective. Gram reaction, cell shape, cellular arrangement, and other features such as spore formation and motility were recorded (Neti & Peshwe, 2025).

2.5.2 Biochemical and Molecular Identification of Marine Bacterial Strains

2.5.3 Automated Biochemical Identification by VITEK 2

Selected marine bacterial isolates were identified using the VITEK® 2 automated microbial identification system (bioMérieux, France). Fresh pure cultures were prepared on marine agar, and bacterial suspensions were adjusted to the required turbidity. Appropriate VITEK 2 identification cards were inoculated according to the manufacturer's instructions. The cards were incubated and monitored automatically for biochemical and enzymatic reactions. The resulting biochemical profiles were compared with the VITEK 2 database to identify the isolates (Ligozzi et al., 2002).

2.5.4 DNA Extraction

The selected potent isolate was cultivated in Marine Broth at 35 °C for 24 h. Cells were centrifuged at 10,000 rpm for 10 min and washed twice with sterile PBS. Genomic DNA was extracted using a DNA extraction kit according to the manufacturer's instructions. DNA concentration and purity were measured spectrophotometrically at 260 and 280 nm, and DNA integrity was assessed by 1% agarose gel electrophoresis (Altschul et al., 1990).

2.5.5 DNA Sequencing and Sequence Analysis

The purified 16S rRNA gene amplicons were subjected to DNA sequencing for molecular identification of the bacterial isolate. The acquired nucleotide sequences were assessed against closely related bacterial strains in the NCBI database using the BLASTN algorithm (Altschul et al., 1990). Sequence alignment and similarity analysis were performed by comparing the obtained sequences with reference sequences available in the database (Altschul et al., 1990; Sayers et al., 2024). The top 10 closely related sequences identified through BLAST analysis were recorded and used for subsequent molecular identification and phylogenetic analysis (Altschul et al., 1990; Sayers et al., 2024).

2.5.6 Phylogenetic Analysis

The obtained 16S rRNA gene sequence of the potent marine bacterial isolate MBC1 was compared with closely related bacterial sequences retrieved from the NCBI database using BLASTN. Reference sequences showing high sequence similarity were selected for phylogenetic analysis. Multiple sequence alignment was performed, and a Neighbor-Joining (NJ) phylogenetic tree was constructed using MEGA 11 software based on the Kimura 2-parameter (K2P) distance model. Phylogenetic relationships were evaluated using 1,000 bootstrap replicates. The resulting sequence was submitted to GenBank for accession assignment (Sayers et al., 2024).

2.5.7 GenBank Submission of 16S rRNA Gene Sequence

The obtained 16S rRNA gene sequence was compared with NCBI sequences using BLAST. The validated sequence was submitted to the NCBI GenBank database through BankIt, and a GenBank accession number was assigned (Sayers et al., 2024).

2.6 Optimization of Culture Medium for β -Galactosidase Production

Culture conditions were optimized to enhance β -galactosidase production by the selected marine bacterial isolate. Individual nutritional and physicochemical parameters were varied, and the condition producing maximum enzyme activity was selected for subsequent studies (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.1 Optimization of Carbon Source

Galactose, lactose, sucrose, fructose, starch, and glucose were individually added to the production medium at 1% (w/v). A 5% (v/v) seed culture was inoculated into the medium and cultivated at 37 °C for 36 h at 180 rpm. β -Galactosidase activity was measured by the ONPG assay, and the carbon source giving maximum activity was selected (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.2 Optimization of Lactose Concentration

Lactose concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5% (w/v) were evaluated in the optimized production medium. Cultures were incubated under standard conditions for 36 h, and β -galactosidase activity was determined. The concentration producing maximum activity was selected as optimal (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.3 Optimization of Nitrogen Source

The effects of tryptophan, yeast extract, beef extract, peptone, casein, ammonium sulphate, and ammonium chloride were evaluated at 1% (w/v). Cultures were incubated under optimized conditions, and enzyme activity was measured by the ONPG assay. The nitrogen source producing maximum activity was selected (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.4 Optimization of Metal Ions

The effects of MgSO_4 , CaCl_2 , ZnSO_4 , FeSO_4 , MnSO_4 , and MgCl_2 were evaluated at 1 mM. Following incubation under the standard conditions, β -galactosidase activity was measured, and the metal ion producing the maximum activity was selected.

2.6.5 Optimization of Incubation Time

Cultures were incubated for 12, 24, 36, 48, 60, and 72 h under standard conditions. Samples collected at each interval were analyzed for β -galactosidase activity. The incubation period corresponding to maximum enzyme production was selected (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.6 Optimization of Inoculum Size

Inoculum levels of 1, 2, 3, 4, 5, 6, and 7% (v/v) were evaluated. Cultures were incubated under optimized conditions, and β -galactosidase activity was determined. The inoculum level yielding maximum enzyme production was selected (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.7 Optimization of pH

The initial pH of the production medium was adjusted to 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, and 9.0 using 1 N HCl or 1 N NaOH. After incubation under standard conditions, enzyme activity was measured, and the pH yielding maximum production was selected (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.8 Optimization of Temperature

The effect of incubation temperature was evaluated at 25, 30, 37, 40, 45, and 50 °C. Following incubation, β -galactosidase activity was measured by the ONPG assay. The temperature corresponding to maximum enzyme production was considered optimal (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.9 Optimization of Agitation

Cultures were incubated under static conditions and at 100, 120, 150, 180, and 200 rpm. Enzyme activity was determined after incubation, and the agitation condition producing maximum β -galactosidase activity was selected (Kalathinathan et al., 2023; Neti & Peshwe, 2025; Duan et al., 2022).

2.6.10 Evaluation of Alternative Substrates

Low-cost agro-industrial by-products, including whey powder, whey, molasses, rice bran, and corn steep liquor, were evaluated as alternative substrates for β -galactosidase production. Each substrate was incorporated at 2% (w/v) into the production medium and incubated under optimized conditions. Enzyme activity was measured by the ONPG assay and compared with lactose as the control substrate.

2.7 Purification of β -Galactosidase

Crude β -galactosidase from the optimized fermentation broth was purified using ammonium sulphate precipitation, dialysis, ion-exchange chromatography, and gel-filtration chromatography. All purification steps were performed at 4 °C. At every stage, β -galactosidase activity, protein concentration, purification fold, and enzyme recovery were determined (Bechtel et al., 2024; Neti & Peshwe, 2025; Duan et al., 2022).

2.7.1 Ammonium Sulphate Precipitation

The supernatant was kept at 4 °C overnight after being saturated with 80% ammonium sulphate and continuously stirred. Precipitated proteins were recovered by centrifugation at 12,000 rpm for 20 min at 4 °C. For additional purification, the pellet was dissolved in 0.1 M phosphate buffer (pH 7.0) (Bechtel et al., 2024; Neti & Peshwe, 2025).

2.7.2 Dialysis

A 10–12 kDa molecular-weight-cutoff membrane was used to dialyze the ammonium sulphate precipitate against 0.1 M phosphate buffer (pH 7.0) at 4 °C for 24 h, with regular replacement of the buffer. The dialyzed preparation was collected for chromatographic purification (Neti & Peshwe, 2025; Duan et al., 2022).

2.7.3 Ion-Exchange Chromatography

A DEAE-cellulose column equilibrated with 0.1 M phosphate buffer (pH 7.0) was loaded with the dialyzed enzyme preparation. A linear gradient of 0–1.0 M NaCl was used to elute bound proteins. Fractions were assayed for β -galactosidase activity and monitored at 280 nm. The purified enzyme preparation was obtained by pooling fractions with the highest enzyme activity (Bechtel et al., 2024; Neti & Peshwe, 2025; Duan et al., 2022).

2.7.4 Gel-Filtration Chromatography

A Sephadex G-200 column (2.5 × 50 cm) was loaded with the dialyzed enzyme and equilibrated with 0.1 M phosphate buffer (pH 7.0). Proteins were eluted at approximately 0.5 mL min⁻¹, and 2–3 mL fractions were collected. Protein content was measured at 280 nm, and β -galactosidase activity was determined by the ONPG assay. Active fractions were collected for subsequent analysis (Bechtel et al., 2024; Neti & Peshwe, 2025; Duan et al., 2022).

2.7.5 Determination of Protein Concentration and Purification Yield

Protein concentration at each purification stage was measured by the Lowry method using bovine serum albumin (BSA) as the standard. β -Galactosidase activity was measured using the ONPG assay, and specific activity was expressed as U mg⁻¹ protein. Purification fold and percentage recovery were calculated using standard equations (Lowry et al., 1951).

2.8 Characterization of Purified β -Galactosidase

2.8.1 Determination of Molecular Weight by SDS-PAGE

Purified β -galactosidase molecular mass was determined by SDS-PAGE according to Laemmli, 1970. The enzyme sample was mixed with sample loading buffer, heated at 95 °C for 5 min, and electrophoresed on a 10–12% polyacrylamide gel alongside molecular-weight markers. The gel was stained with Coomassie Brilliant Blue R-250 after electrophoresis. The apparent molecular mass of the purified enzyme was estimated by comparing its migration distance with those of the standard protein markers (Laemmli, 1970).

3. Results

3.1 Isolation of Marine Bacteria and Primary Screening

Marine bacterial isolation from seawater and sediment samples yielded 24 morphologically distinct bacterial isolates. Most isolates exhibited circular, smooth, convex, opaque, cream to pale-yellow colonies and showed good growth on Marine Agar 2216 supplemented with lactose

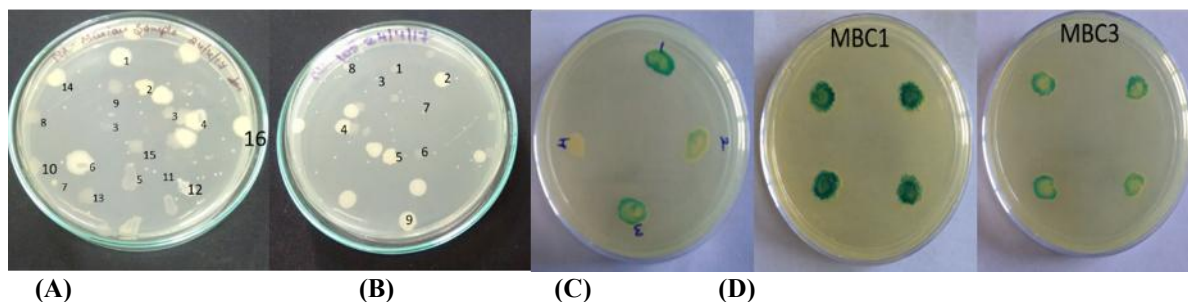


Figure 2. Isolation and primary screening of marine bacteria for β -galactosidase production.

(A) Isolation of marine bacteria; (B) screening of marine bacteria;

(C) Positive green-coloured MBC1; (D) Positive light-green-coloured MBC3.

Primary screening of the 24 purified marine bacterial isolates using the X-Gal chromogenic assay identified two β -galactosidase-positive isolates, MBC1 and MBC3.

3.2 Secondary Screening and β -Galactosidase Activity

MBC1 showed 8.0 U/mL extracellular and 29.0 U/mL intracellular β -galactosidase activity, whereas MBC3 showed 3.0 U/mL extracellular and 7.0 U/mL intracellular activity.

Table 1. Secondary screening of β -galactosidase-producing marine bacterial isolates.

Isolate	X-Gal screening	Colony colour	Extracellular activity (U/mL)	Intracellular activity (U/mL)
MBC1	Positive (+)	Green/Blue	8.0	29.0
MBC3	Positive (+)	Green	3.0	7.0

Source: Authors' experimental data.

3.3 Morphological and Biochemical Characterization

The selected isolates showed circular, smooth, convex and opaque colonies with cream-to-white pigmentation. Gram staining revealed Gram-positive, rod-shaped bacterial cells, confirming their distinct morphological characteristics.



Figure 3. Morphological characteristics of the potent marine bacterial isolate MBC1.

(A) Colony morphology (B) Gram staining.

3.4 Biochemical and Molecular Identification

VITEK 2 showed a distinct biochemical profile for MBC1. 16S rRNA sequencing and BLAST analysis showed close similarity to *Bacillus* sequences in the NCBI database.

Phylogenetic analysis using the Neighbor-Joining method in MEGA 11, the Kimura 2-parameter model, and 1,000 bootstrap replicates showed that MBC1 (MG831954) clustered most closely with *Bacillus aryabhatai* DSM 21047 (NR_115953.1). MBC1 was positioned within the *Bacillus* clade containing *B. megaterium*, *B. simplex*, *B. flexus*, and *B. lentus*.

Table 2. Biochemical identification profile of the selected marine bacterial isolate MBC1 using the VITEK 2 system.

S.NO	Test	Results
1	Beta-Xylosidase	+
3	L-Lysine Arylamidase	-
4	L-Aspartate Arylamidase	+
5	Leucine Arylamidase	+
7	Phenylalanine Arylamidase	+
8	L-Proline Arylamidase	-
9	Beta – Galactosidase	+
10	L-Pyrrolydonyl- Arylamidase	+
11	Alpha-Galactosidase	+
12	Alanine Arylamidase	+
13	Tyrosine Arylamidase	+

14	Beta-N-Acetyl-Glucosaminidase	+
15	Ala-Phe-Pro Arylamidase	+
18	Cyclodextrin	-
19	D-Galactose	+
21	Glycogen	+
22	Myo-Inositol	-
24	Methyl-A-D-Glucopyranoside Acidification	+
25	Ellman	+
26	Methyl-D-Xyloside	-
27	Alpha-Mannosidase	-
29	Maltotriose	+
30	Glycine Arylamidase	-
31	D-Mannitol	+
32	D-Mannose	-
34	D-Melezitose	-
36	N-Acetyl-D-Glucosamine	-
37	Palatinose	+
39	L-Rhamnose	-
41	Beta-Glucosidase	+
43	Beta-Mannosidase	-
44	Phosphoryl Choline	-
45	Pyruvate	+
46	Alpha Glucosidase	+
47	D-Tagatose	-
48	D-Trehalose	+
50	Inulin	-
53	D-Glucose	+
54	D-Ribose	-
56	Putrescine Assimilation	+
58	Growth in 6.5%NaCl	+
59	Kanamycin Resistance	-
60	Oleandomycin Resistance	+
61	Esculin Hydrolysis	+
62	Tetrazolium Red	+
63	Polymixin B Resistance	-

Source: Authors' experimental data.

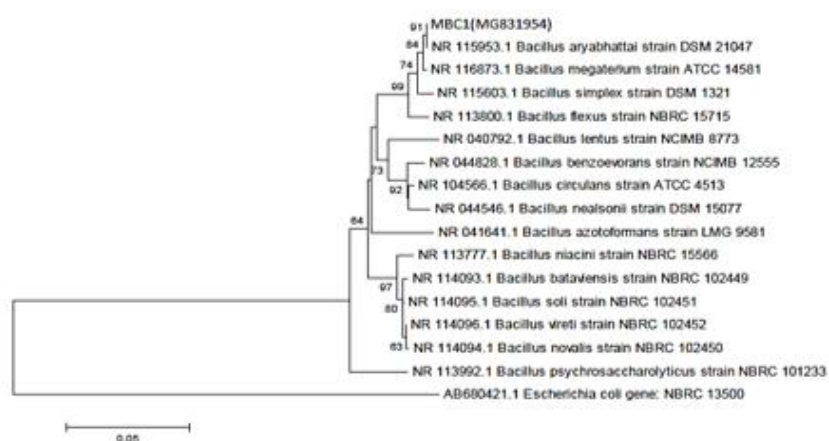
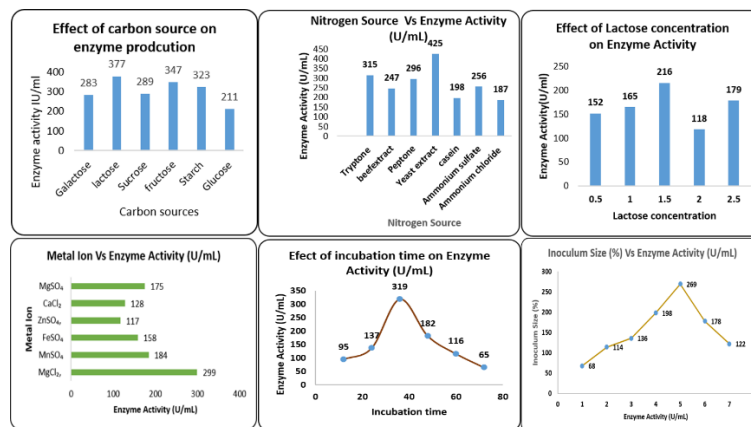


Figure 4. Phylogenetic tree of the β -galactosidase-producing marine bacterial isolate MBC1 based on 16S rRNA gene sequences using the Neighbor-Joining method.

3.5 Optimization of Culture Conditions for β -Galactosidase Production

Among the carbon sources tested, glucose supported the highest β -galactosidase production (398 U/mL). Maximum production was observed at 1.5% (w/v) lactose (216 U/mL), with yeast extract as the nitrogen source (425 U/mL) and $MgCl_2$ (1 mM) as the metal ion (299 U/mL). Maximum activities were observed at 36 h (319 U/mL) and 5% (v/v) inoculum (269 U/mL).

Figure 5. Effect of nutritional and culture conditions on β -galactosidase production by *Bacillus aryabhattai* MBC1.



β -Galactosidase production was maximum at pH 7.5 (399 U/mL) and 37 °C (487 U/mL). The maximum activity under agitation conditions was noted at 180 rpm (219 U/mL), while corn steep liquor supported 248 U/mL among the alternative substrates.

Figure 6. Effect of pH, temperature, alternative substrates, and agitation on β -galactosidase production by *Bacillus aryabhattai* MBC1.

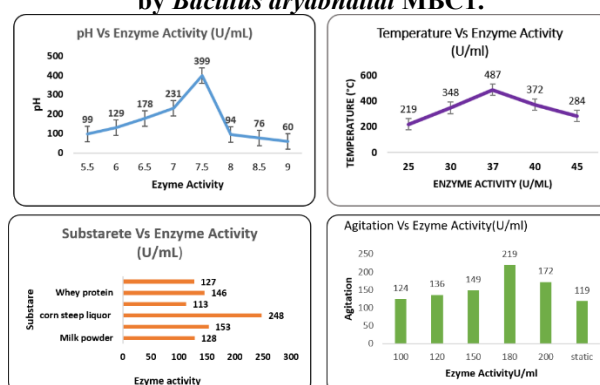


Table 3. Optimized culture conditions and β -galactosidase production by *Bacillus aryabhattai* MBC1.

Parameter	Optimum condition	β -Galactosidase activity
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
Shaking speed	180 rpm	219 U/mL
NaCl concentration	3% (w/v)	148 U/mL
Alternative substrate	Corn steep liquor (2%)	248U/mL

Source: Authors' experimental data.

3.6 Purification of β -Galactosidase

The crude extract showed 573 IU/mL enzyme activity with 2.6 mg/mL protein and a specific activity of 220.38 IU/mg. After ammonium sulphate precipitation and dialysis, enzyme activity was 1246 IU/mL and protein concentration was reduced to 1.6 mg/mL, giving a specific activity of 778.75 IU/mg, 3.53-fold purification, and 89% yield. DEAE-cellulose chromatography yielded an active preparation with 921 IU/mL activity and 2.1 mg/mL protein, corresponding to 438.57 IU/mg specific activity, 1.99-fold purification, and 72% yield. Following Sephadex G-200 gel filtration, the final active preparation showed 1326 IU/mL activity, 2.7 mg/mL protein, and 491.10 IU/mg specific activity, corresponding to 2.23-fold purification and 60% overall yield. The final Sephadex G-200 preparation was therefore characterized by the highest measured volumetric enzyme activity, whereas the highest specific activity and purification fold were observed after ammonium sulphate precipitation and dialysis.

Table 4. Purification profile of β -galactosidase from *Bacillus aryabhattai* MBC1.

Purification step	Enzyme activity (IU/mL)	Protein (mg/mL)	Specific activity (IU/mg)	Purification fold	Yield (%)
Crude extract	573	2.6	220.38	1.00	100
Ammonium sulfate precipitation + dialysis	1246	1.6	778.75	3.53	89

DEAE-cellulose chromatography	921	2.1	438.57	1.99	72
Sephadex G-200 chromatography	1326	2.7	491.10	2.23	60

Source: Authors' experimental data.

3.7 Molecular Weight Determination by SDS-PAGE

SDS-PAGE showed a specific protein band corresponding to an apparent molecular mass of approximately 45 kDa.

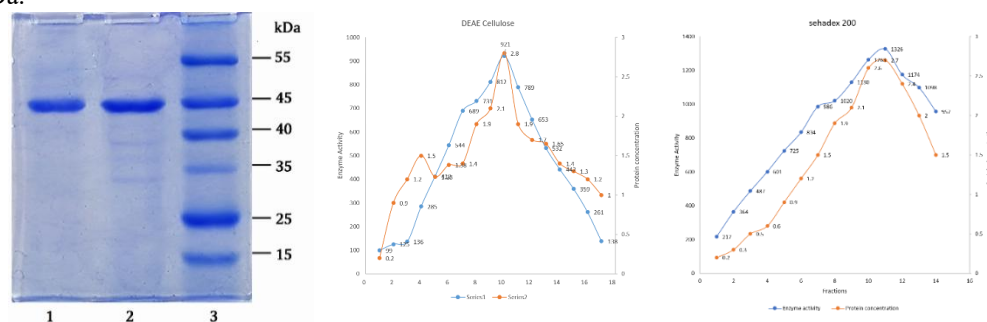


Figure 7. SDS-PAGE profile of purified β -galactosidase from *Bacillus aryabhattai*

(A) Lane 1: Dialyzed protein; Lane 2: Crude protein; Lane 3: Protein marker.

(B) DEAE-cellulose ion-exchange chromatography.

(C) Sephadex G-200 gel-filtration chromatography.

4. Discussion

Marine environments represent an important source of metabolically diverse microorganisms with potential for the production of industrially relevant enzymes. In the present study, isolation of marine bacteria resulted in 24 morphologically distinct bacterial isolates. Primary screening using the X-Gal assay identified two isolates, MBC1 and MBC3, as β -galactosidase-positive. During secondary screening, MBC1 showed 8.0 U/mL extracellular and 29.0 U/mL intracellular activity, whereas MBC3 showed 3.0 U/mL extracellular and 7.0 U/mL intracellular activity. The higher intracellular activity of MBC1 provided the basis for selecting this isolate for further optimization, purification, and characterization. The finding supports the value of marine microbial screening for identifying strains with enzyme-producing potential.

The potent isolate MBC1 was identified as *Bacillus aryabhattai* using complementary phenotypic and molecular approaches. The VITEK 2 biochemical profile provided phenotypic evidence, while 16S rRNA gene sequencing, BLAST analysis, and phylogenetic placement supported the molecular identification. MBC1 (MG831954) clustered most closely with *B. aryabhattai* DSM 21047 in the phylogenetic analysis. The combined approach provides stronger taxonomic evidence than reliance on a single identification method. The identification is also relevant because *Bacillus* species are established sources of industrial enzymes, although the biochemical properties of β -galactosidases can differ substantially among species and strains.

The one-factor-at-a-time experiments showed that β -galactosidase production was affected by nutritional and physicochemical variables. Glucose supported the highest activity among the tested carbon sources (398 U/mL), while 1.5% lactose gave the highest activity among the lactose concentrations tested (216 U/mL). Lactose is particularly relevant because it can serve as a substrate and, depending on the microorganism, contribute to induction of β -galactosidase synthesis. Reviews of microbial β -galactosidases emphasize that enzyme production and properties vary with microbial source and cultivation conditions (Kalathinathan et al., 2023; Neti & Peshwe, 2025). The present response to glucose and lactose therefore indicates that the nutritional requirements of *B. aryabhattai* MBC1 are strain dependent and should be established experimentally rather than inferred from other microorganisms.

Yeast extract was the most effective nitrogen source, producing 425 U/mL. Yeast extract supplies readily assimilable nitrogen, amino acids, peptides, vitamins, and other growth-promoting components, which can support biomass formation and enzyme biosynthesis. The strong response to yeast extract is consistent with the general importance of nitrogen availability in microbial enzyme production. The observed result also demonstrates why optimization of individual medium components is important before downstream processing. The use of a complex nitrogen source may provide multiple nutrients simultaneously, although the present study did not determine which individual components of yeast extract were responsible for the observed response.

Among the tested metal ions, MgCl_2 at 1 mM gave the highest activity (299 U/mL). Magnesium participates in numerous cellular processes, including nucleic acid metabolism, ribosome function, and enzyme-associated reactions. The response observed in this study suggests that Mg^{2+} at the tested concentration provided a favorable environment for enzyme production. Because metal-ion effects are concentration dependent, the result should be interpreted as an optimum under the experimental conditions rather than as a universal requirement for β -galactosidase production.

The physicochemical variables also produced distinct activity profiles. Maximum production was observed at pH 7.5 (399 U/mL) and 37 °C (487 U/mL). The pH optimum is within the range reported for several microbial β -galactosidases, although substantial strain-to-strain variation exists. For example, a β -galactosidase from the marine-related bacterial genus *Arthrobacter* showed an optimum pH of 7.5 for its GH2 enzyme, while a *Bacillus licheniformis* β -galactosidase reported previously had an optimum pH of 5.7, illustrating the influence of enzyme source on biochemical properties (Wanarska et al., 2024; Tran et al., 1998). Thus, the pH 7.5 optimum observed for MBC1 is compatible with some bacterial β -galactosidases but differs from other *Bacillus*-derived enzymes.

The temperature response showed maximum production at 37 °C (487 U/mL). This moderate temperature is suitable for active growth of many mesophilic bacteria and may provide a balance between cellular metabolism and enzyme biosynthesis. In contrast, previously characterized bacterial β -galactosidases have shown markedly different temperature optima; for example, the *B. licheniformis* enzyme reported by Tran et al. (1998) showed an optimum at 45 °C, whereas the GH2 β -galactosidase from *Arthrobacter* sp. S3* showed an optimum at 22 °C (Wanarska et al., 2024). These differences reinforce that the optimal temperature cannot be generalized across microbial β -galactosidases and must be determined for each strain.

The optimum incubation period was 36 h (319 U/mL), while a 5% inoculum produced the highest activity (269 U/mL). The increase in production up to the optimum incubation period may reflect active biomass formation and enzyme biosynthesis, whereas prolonged cultivation can lead to nutrient depletion, accumulation of metabolic products, changes in medium conditions, or enzyme degradation. Similarly, an appropriate inoculum size is necessary to establish active growth without causing excessive competition for nutrients at the beginning of cultivation. Agitation at 180 rpm gave the highest activity (219 U/mL), consistent with the importance of mixing and oxygen transfer in aerobic microbial cultivation.

Because the organism was isolated from a marine environment, salinity was also evaluated. The optimized NaCl concentration was 3% (w/v), at which β -galactosidase activity reached 148 U/mL. This response indicates that MBC1 retained enzyme-producing capability under a saline condition compatible with its marine origin. The salinity result is particularly relevant when considering cultivation of marine-derived microorganisms because ionic strength can influence cellular growth, transport processes, protein folding, and enzyme stability. The observed optimum should, however, be interpreted specifically for the tested strain and medium.

Among the alternative low-cost substrates evaluated, corn steep liquor at 2% supported 248 U/mL activity. This result indicates that CSL can support β -galactosidase production under the tested conditions. However, because CSL was evaluated as an alternative substrate rather than through a dedicated factorial comparison with the optimized medium, its independent contribution to the final optimized production level cannot be quantified from the present data. It is therefore more appropriate to regard CSL as a potential low-cost fermentation component requiring further economic and process validation rather than to claim a specific enhancement attributable to CSL alone.

Collectively, the OFAT experiments established a reproducible set of nutritional and physicochemical conditions for *B. aryabhattai* MBC1: glucose as the preferred carbon source, 1.5% lactose, yeast extract as the nitrogen source, MgCl_2 at 1 mM, pH 7.5, 37 °C, 36 h incubation, 5% inoculum, 180 rpm agitation, and 3% NaCl. The individual activities reported for these parameters should be interpreted as responses obtained in separate one-factor-at-a-time experiments; they should not be treated as a single combined activity value unless the complete combination was experimentally validated. This distinction is important when describing the optimization process. The purification results demonstrated enrichment of β -galactosidase through sequential downstream processing. The crude extract showed 573 IU/mL activity, 2.6 mg/mL protein, and 220.38 IU/mg specific activity. After ammonium sulphate precipitation and dialysis, activity increased to 1246 IU/mL and protein decreased to 1.6 mg/mL, resulting in a substantially higher specific activity of 778.75 IU/mg, a 3.53-fold purification, and 89% yield. The increase in specific activity indicates enrichment of β -galactosidase relative to total protein. Ammonium sulphate precipitation is commonly used as an initial protein concentration and fractionation step, followed by dialysis to remove excess salt and low-molecular-weight components.

DEAE-cellulose chromatography produced an active preparation with 921 IU/mL activity, 2.1 mg/mL protein, and 438.57 IU/mg specific activity, corresponding to 1.99-fold purification and 72% yield. The decrease in specific activity relative to the preceding step should not be concealed; it may reflect differential recovery of enzyme and protein during column separation, fraction selection, or losses during handling. DEAE-cellulose separates proteins according to charge, providing a purification principle different from precipitation. Further purification by Sephadex G-200 produced the final preparation with 1326 IU/mL activity, 2.7 mg/mL protein, and 491.10 IU/mg specific activity, corresponding to 2.23-fold purification and 60% overall yield. Gel filtration separates proteins primarily according to molecular size.

The purification data demonstrate why volumetric enzyme activity and specific activity should be interpreted separately. Sephadex G-200 gave the highest measured activity (1326 IU/mL), whereas ammonium sulphate precipitation plus dialysis gave the highest specific activity (778.75 IU/mg) and purification fold (3.53). Therefore, the Sephadex G-200 step should not be described as producing the highest degree of purification. Instead, it yielded the final active preparation with the highest measured volumetric activity. The final 60% overall yield indicates that a substantial proportion of the starting enzyme activity was retained through the complete purification sequence. The industrial significance of β -galactosidase is well established, and current research increasingly focuses on stability, immobilization, recovery, and reuse to improve process feasibility (Duan et al., 2022).

SDS-PAGE revealed an apparent protein band of approximately 45 kDa in the purified preparation. The molecular mass of microbial β -galactosidases varies widely among organisms and enzyme families. For example, a *B.*

licheniformis β -galactosidase was reported at approximately 80 kDa, while recent work on *Arthrobacter* sp. S3* identified β -galactosidases with much larger native oligomeric molecular masses (Tran et al., 1998; Wanarska et al., 2024). Thus, the approximately 45-kDa band observed for MBC1 represents an apparent SDS-PAGE molecular mass and highlights the diversity of β -galactosidase structures. SDS-PAGE alone does not establish the native oligomeric state or prove that the band is the catalytic subunit; further biochemical or proteomic characterization would be required for definitive structural assignment.

The combined use of precipitation, dialysis, ion-exchange chromatography, gel filtration, and SDS-PAGE provided complementary approaches for enzyme enrichment and preliminary molecular characterization. Recent studies also emphasize the importance of developing microbial β -galactosidases with properties suitable for lactose hydrolysis and subsequent food-processing applications (Bechtel et al., 2024; Senger et al., 2024). The present enzyme therefore warrants further evaluation of kinetic parameters, thermal and pH stability, storage stability, substrate specificity, and actual lactose hydrolysis in milk and whey before industrial application can be established.

Overall, the study demonstrates that marine-derived *B. aryabhatai* MBC1 is a β -galactosidase-producing bacterium with measurable responses to nutritional, saline, and physicochemical optimization. The final purified preparation showed 1326 IU/mL activity, 491.10 IU/mg specific activity, 2.23-fold purification, and 60% overall yield, with an apparent molecular mass of approximately 45 kDa. These findings provide a basis for further characterization and application studies, while the laboratory-scale nature of the work means that industrial performance should not yet be inferred without additional validation.

5. Conclusion

The present study identified *Bacillus aryabhatai* MBC1 as a marine bacterial producer of β -galactosidase through biochemical screening, VITEK 2 profiling, 16S rRNA gene sequencing, and phylogenetic analysis. Among the 24 marine bacterial isolates screened, MBC1 showed the highest intracellular activity (29 U/mL) during secondary screening and was selected for further investigation. One-factor-at-a-time optimization identified glucose, 1.5% lactose, yeast extract, MgCl_2 at 1 mM, pH 7.5, 37 °C, 36 h incubation, 5% inoculum, 180 rpm agitation, and 3% NaCl as the respective optimum conditions under the tested experimental ranges. Among the alternative substrates, 2% corn steep liquor supported 248 U/mL activity. The purification sequence yielded a final Sephadex G-200 preparation with 1326 IU/mL activity, 491.10 IU/mg specific activity, 2.23-fold purification, and 60% overall yield; the highest specific activity and purification fold were obtained after ammonium sulphate precipitation and dialysis (778.75 IU/mg and 3.53-fold, respectively). SDS-PAGE indicated an apparent molecular mass of approximately 45 kDa. A major limitation of the study is that enzyme production and purification were evaluated at laboratory scale and the individual effects and interactions of optimized factors were not evaluated using a multivariable statistical design. In addition, enzyme stability, kinetic parameters, storage stability, and performance during actual dairy processing were not comprehensively established. Future studies should therefore investigate kinetic parameters, thermal and pH stability, storage stability, enzyme immobilization or reuse, fermentation scale-up, and lactose hydrolysis efficiency in milk and whey. Application under simulated and pilot-scale dairy-processing conditions will be important for determining practical suitability. Overall, the findings provide a basis for further development of marine-derived *B. aryabhatai* β -galactosidase as a potential biocatalyst for lactose hydrolysis and food and dairy biotechnology.

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