International Journal of Microbial Science, ISSN (online), 2582-967X, Volume 7, Issue 1, February 2026, pp. 11-18
Available at https://internationaljournalofmicrobialscience.com/

Original Article

Screening, Production, and Optimization of β-galactosidase enzyme from *Alcaligenes* Species Isolated from Dairy Industry Effluents in Baramati, Pune District, Maharashtra

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Article Info

Article history:
Received: December 11, 2025
Accepted: January 23, 2026
Published: February 5, 2026

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Keywords: β-galactosidase, DPE, ONPG, *Alcaligenes faecalis*, Optimization studies

Abstract

β-galactosidase (E.C. 3.2.1.23) is an enzyme of great industrial importance, mainly used to hydrolyze lactose into glucose and galactose for the preparation of lactose-free dairy products for lactose intolerant individuals, to prevent lactose crystallization in frozen milk products and to reduce the environmental water pollution resulting from dairy whey disposal. Beyond its role in dairy processing, β-galactosidase is also widely exploited to synthesize prebiotic galacto-oligosaccharides (GOS) that support gut health, underlining its broader value across the food, pharmaceutical, and biotechnology sectors. In this study, efficient β- galactosidase producing bacterial strains were isolated from dairy industry effluents and optimized for maximum production. Biochemical characterization and 16S rRNA gene sequencing identified the producing strains as *Alcaligenes* spp. Five isolates DPE-1, DPE-2, DPE-3, DPE-4 and DPE-5 were screened by colorimetric assay at 430 nm, using o-nitrophenyl-β-D-galactopyranoside (ONPG) as a chromogenic substrate. DPE-3 and DPE-5 were found to be the most effective producers and hence, used for optimization and kinetic studies (Km, Vmax). The obtained results indicate the potential of these isolates from wastewater, especially, the acid-tolerant DPE-5, for customized uses in food biotechnology and sustainable dairy waste management. Further work with statistically designed optimization trials would be useful for confirming and refining these findings for practical application

1. Introduction:

β-galactosidase (E.C. 3.2.1.23), also known as lactase, is an enzyme of considerable industrial and biotechnological interest. It is found in plants, animals and in a number of microorganisms, such as fungi, yeasts and bacteria [1]. Its primary role is to catalyze the hydrolysis of lactose, the main sugar in milk, to glucose and galactose. This reaction is the basis for producing lactose-free and lactose-reduced dairy products, it is of great significance because a large population of adults worldwide are lactose intolerant [2,3]. Although intestinal lactase activity naturally declines after weaning in most humans, a genetically determined trait known as lactase non-persistence, this does not eliminate the physiological value of dairy in adulthood; milk and dairy products remain major

dietary sources of calcium, protein, and vitamin D, and public health bodies recommend that even lactose-intolerant adults continue consuming dairy to maintain adequate nutrient intake and bone health, which is why lactose-hydrolyzed, easily digestible dairy alternatives produced using β-galactosidase remain nutritionally important across all adult age groups [8]. β-galactosidase enzyme is also used in food industry. It prevents lactose crystallization in condensed and frozen milk, which may leave a granular texture. It also improves solubility and sweetness without the addition of extra sugar [4]. There is an environmental side to this as well. Cheese whey, a common byproduct of the dairy processing industry, carries very high BOD and COD levels and poses a real pollution risk if discharged untreated [5]. Treating it with β-galactosidase turns this waste into a usable substrate for making value-added, bio-based products, solving two problems at once [5,6]. In addition to its role in dairy processing and waste recycling, β-galactosidase's transgalactosylation activity is harnessed industrially to produce galacto-oligosaccharides (GOS), functional prebiotic ingredients used in functional foods and to promote beneficial gut microbiota, further extending the enzyme's relevance to human health well beyond simple milk digestion [9].

Commercial enzyme production from microbial sources is preferred over plant or animal sources mainly because microorganisms grow rapidly, have high yield and are relatively easy to manipulate under laboratory or industrial conditions. Specifically, isolates from dairy processing environments are more likely to show adaptations appropriate to those environments [4]. However, to turn a microbial strain into a useful commercial entity, it requires systematic optimization of pH, temperature, carbon and nitrogen sources, and inducers, as well as determining the enzyme's kinetic parameters (K_m, V_{max}) to understand its actual behavior [4,7]. Lactase production has been studied for decades, but there is still a need for bacterial strains resistant to real processing conditions, such as acid environments in fermented dairy waste, which has been given comparatively less attention. We tried to fill this gap by using local dairy effluent as a source of bacteria that might be already adapted to such conditions. The approach was to isolate bacterial strains from dairy effluent and then screen for β-galactosidase activity, quantify the activity, and then optimize the nutritional and environmental conditions required for each strain to perform well. Of the isolates, DPE-5 was found to be most effective as it was highly tolerant to acid conditions with optimum pH 4.0, and its kinetics were significantly different (V_{max} 2.8 U/mL, K_m 3.05 mM).

2. Materials and Methods:

2.1 Collection of sample

Dairy effluent samples were collected in sterile containers from industrial waste site from Baramati, District Pune, Maharashtra, India and immediately transported to the laboratory. The samples were stored at 4°C to maintain sample integrity prior to bacterial enrichment and isolation procedures.

2.2 Isolation and culture conditions

Bacterial strains were isolated from the collected effluent samples using standard serial dilution and spread plate techniques on sterile nutrient agar. Five distinct isolates (DPE-1 to DPE-5) were selected based on colony morphology and preserved for further screening. For enzyme production, isolates were inoculated into 250 mL Erlenmeyer flasks containing nutrient broth with IPTG (3 µM/mL), added as a stable, non-metabolizable lactose analog to induce enzyme production and incubated at 30°C for 24 hours.

2.3 Screening and enzyme assay

Isolates were screened for β-galactosidase activity using the ONPG method, with o-nitrophenyl-β-D-galactopyranoside (ONPG) as the chromogenic substrate. Following incubation, cultures were centrifuged, and the cell-free supernatant was used as the crude enzyme source. The assay mixture contained 200 µL crude enzyme, 0.1 M phosphate buffer, and 500 µL of 6 mM ONPG, incubated at 37°C for 30 minutes. The reaction was terminated with 0.5 mL of 1 M Na_2CO_3, and the amount of o-nitrophenol (ONP) released was measured spectrophotometrically at 430 nm. One unit (U) of β-galactosidase activity was defined as the amount of enzyme releasing 1 µmol of ONP per minute under assay conditions, based on a standard curve prepared using ONP concentrations of 0.1–1.0 mM [7]

2.4 Optimization and enzyme kinetics

The optimum conditions for maximum enzyme yield were determined by changing one parameter at a time while keeping the other parameters constant to study the effects of pH, temperature, carbon and nitrogen supplies and inducers on β-galactosidase activity. The enzyme activity was determined at different concentrations of ONPG substrate and the kinetic

parameters (K_m and V_{max}) were calculated by Lineweaver-Burk plot.

3. Results:

3.1 Qualitative and quantitative assay of β-galactosidase enzyme

The five bacterial isolates were screened qualitatively, and some isolates showed a visible yellow color in the reaction tubes. This confirmed the β-galactosidase activity by the release of o-nitrophenol (ONP) from ONPG hydrolysis (figure 1). The quantitative estimation of enzyme activity was carried out spectrophotometrically at 430 nm, showing variation in β-galactosidase production among the isolates (DPE 1 to DPE 5) (table 1). Based on these results, isolates DPE-3 was **13.9 U/mL** and DPE-5 was found to be **24.8 U/mL**, Hence, they were the most efficient producers of β-galactosidase and were selected for further optimization studies, in which, the effects of pH, temperature, carbon sources, nitrogen sources, and inducers on enzyme production were evaluated.

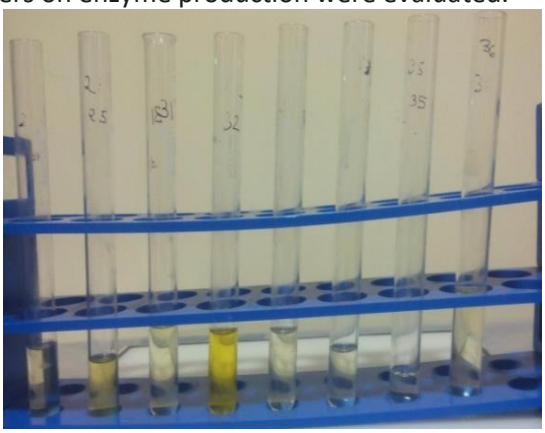

Figure 1. Qualitative assay of β-galactosidase enzyme

Table 1: Quantitative assay of β-galactosidase enzyme

Culture No	β-galactosidase enzyme Units/mL of crude enzyme
DPE -1	0.0
DPE -2	5.8
DPE -3	**13.9**
DPE -4	7.5
DPE -5	**24.8**

3.2 Effect of pH on β-galactosidase enzyme activity

The optimization study of effect of pH on β-galactosidase activity, indicated that the bacterial isolate DPE-3 (*Alcaligenes* spp. AMT-03 gene for 16S rRNA, partial sequence), produced β-galactosidase enzyme **2.9 U/mL** at pH – 7.0 (i.e. optimum pH – 7.0) whereas the bacterial isolate DPE-5 (*Alcaligenes* spp. SR24-2 16S ribosomal RNA gene, partial sequence), produced β-galactosidase enzyme **156.2 U/mL** in acidic conditions at pH – 4.0 (i.e. optimum pH – 4.0).

Table 2: Effect of pH on β-galactosidase enzyme activity

pH	β –galactosidase enzyme Units / mL	
	DPE -3	**DPE -5**
pH – 4	1.0	**156.2**
pH – 7	**2.9**	67.3
pH – 9	0.2	0.00

3.3 Effect of temperature on β –galactosidase enzyme activity

Thermal optimization studies suggest that both isolates have similar thermal optima for enzymatic catalysis. Isolate DPE-3 produced **19.3 U/mL** of β-galactosidase activity, whereas isolate DPE-5 produced **120.8 U/mL** at the same ideal temperature of 37^{o}C.

Table 3: Effect of temperature on β –galactosidase enzyme activity

Temperature	β –galactosidase enzyme activity	
	DPE -3	**DPE -5**
30 ^{0}C	0.00	71.6
37 ^{0}C	**19.3**	**120.8**
42 ^{0}C	0.00	9.6

3.4 Effect of carbon source on β -galactosidase enzyme activity

Optimization studies were carried out to study effect of different carbon sources on β-galactosidase production by bacterial isolates. Starch significantly improved the synthesis of β-galactosidase compared to glucose and sucrose for both bacterial isolates. Isolate DPE-3 produced a maximum enzymatic activity of **6.4 U/mL** in presence of starch. Isolate DPE-5 showed more activity of **12.7 U/mL** within same conditions, and **9.5 U/mL** in presence of glucose and sucrose respectively. DPE-3 isolate, on the other hand, completely inhibited the enzyme activity in the presence of glucose and sucrose.

Table 4: Effect of carbon source on β-galactosidase enzyme activity

Carbon source	β -galactosidase enzyme Units / mL	
	DPE -3	DPE -5
Glucose	0.00	9.5
Sucrose	0.00	9.5
Starch	**6.4**	**12.7**

3.5 Effect of nitrogen source on β -galactosidase enzyme activity

The bacterial isolate DPE-3 showed **0.22 U/mL** in the presence of Ammonium sulfate (an inorganic nitrogen source) compared to Peptone and Beef extract (an organic nitrogen source), while the isolate DPE-5 showed **12.99 U/mL** in the presence of Ammonium sulfate compared to Peptone and Beef extract.

Table 5: Effect of nitrogen source on β -galactosidase enzyme activity

Nitrogen source	β -galactosidase enzyme Units / mL	
	DPE -3	DPE -5
Peptone	0.16	6.33
Beef extract	0.127	9.49
Ammonium sulfate	**0.22**	**12.99**

3.6 Effect of inducers on β -galactosidase enzyme activity

The bacterial isolate DPE-3 demonstrated **8.4 U/mL** of β-galactosidase activity in the presence of skim milk when compared to IPTG, while isolate DPE-5 demonstrated **27.0 U/mL.**

Table 6: Effect of inducers on β -galactosidase enzyme activity

Inducer	β -galactosidase enzyme Units / mL	
	DPE -3	DPE -5
Skim milk	**8.4**	**27.0**
IPTG	7.2	3.5

3.7 Km and Vmax study (Michaelis Menton constant & maximum velocity)

The kinetic properties and the substrate affinity of the improved enzyme were determined by measuring the initial reaction rates at various concentrations of ONPG. The kinetic parameters, K_m and V_{max} were then determined from the Lineweaver–Burk reciprocal double plot. The results revealed that the reaction velocity increased with substrate concentration with a maximum velocity (V_{max}) of **2.8 U/ml** at the optimum substrate concentration of 24 mM and the calculated Michaelis-Menten constant (K_m) was **3.05 mM** of ONPG.

4. Discussion:

The most efficient producers identified in this study, DPE-3 and DPE-5, showed a pattern of inter-isolate variability, consistent with recent screening studies. Screening of 13 bacterial isolates from dairy products resulted in a maximum crude yield of **2245 U/ml**, which increased to more than **14,000 U/ml** after statistical medium optimization [10]. A *Lactobacillus* fermentum kefir isolate produced **4254 U/ml** before and **6232.13 U/ml** after optimization [11], both considerably higher than the crude yields obtained here. In contrast, a yeast isolate (*Kluyveromyces marxianus*) from milk and cheese samples yielded a maximum crude activity of only **277 U/ml** [4], and *Lactobacillus leichmannii* 313 showed an unoptimized activity of **4.5 U/mg** protein, improving to **23.13 U/mg** after optimization [12], values corresponding to the dimensions of the present isolates. The differences are probably due to differences in bacterial species/strain, extraction method, substrate concentration and optimization status of the reported activity since the present values are crude and pre-optimization yields. The selection of DPE-3 and DPE-5 for further optimization of pH, temperature, carbon source, nitrogen source and inducers follows the same strategy used in these studies to significantly improve enzyme yield beyond baseline crude activity.

The distinctly acidic pH optimum observed for DPE-5, in contrast to the neutral optimum for DPE-3, reflects the strain-dependent pH preferences reported for bacterial β-galactosidases elsewhere, with a *Lactobacillus* fermentum kefir isolate showing optimum activity at neutral pH 7.0 [11], versus the characterization of two β-galactosidase isozymes from the halophilic bacterium *Gracilibacillus dipsosauri* which revealed one isozyme was more active under acidic conditions whilst the other favored neutral to alkaline pH, demonstrating that co-occurring β-galactosidases within related bacterial genera can differ considerably in their pH-activity profiles [13]. Optimization of a *Bacillus licheniformis* isolate from dairy effluent showed optimal β-galactosidase activity at nearly neutral pH [10], supporting isolates such as DPE-3 which prefer neutral pH. The strong tolerance of DPE-5 to strong acids and the high activity at pH 4.0 demonstrate its

special and potentially useful characteristics for applications involving whey substrates or acidic dairy wastewater.

The optimum temperature of 37°C for growth observed for both the isolates is in agreement with recent reports on β-galactosidase producing bacteria from similar dairy/soil environments wherein the maximum lactase activity was also recorded at 37°C with ONPG as substrate [14]. Further supporting the mesophilic optimum, studies on *Lactobacillus leichmannii* 313 showed that β-galactosidase production and specific activity were optimal in the 37°C mesophilic growth range typical of dairy-processing bacteria and with a sharp decline in activity above 45°C, consistent with the decline in activity in the present isolates past their optimum [12]. However, some recently characterized β-galactosidases have higher thermal optima, demonstrated by a novel thermostable β-galactosidase cloned from *Bacillus aryabhattai* GEL-09 that showed maximum activity at 45°C, underlining the fact that thermal optima can vary significantly even among *Bacillus*-related producers and emphasizing the comparatively moderate, mesophilic character of the DPE-3 and DPE-5 isolates in this study [15].

This better performance of starch over glucose and sucrose and the complete repression of DPE-3 activity by these simple sugars is in agreement with the recent report on carbon-source dependent regulation [18]. Simple and easily metabolized sugars such as glucose result in repression of β-galactosidase synthesis through carbon catabolite repression and complex or alternative carbon sources result in higher levels. In *Lactobacillus casei* MB2 isolated from a traditional dairy product, glucose failed to induce β-galactosidase production significantly due to catabolite repression and lactose as a carbon source resulted in significantly higher enzyme yield [16]. Similarly, in a screening of lactic acid bacterial isolates from a homemade curd, only particular carbon sources among the tested isolates significantly increased β-galactosidase production, simple sugars being generally less effective than the more favorable carbon source which is consistent with the strain-dependent, selective carbon source preference found for DPE-3 and DPE-5 in the present study [17].

The higher activity achieved with ammonium sulfate than the organic nitrogen sources used agrees with a screening of *Lactiplantibacillus* isolates from home-made curd where ammonium sulfate and beef extract were found to be the two most effective nitrogen sources for maximum β-galactosidase production, similar to the pattern observed here [17]. Conversely, a study of optimization of *Lactobacillus acidophilus* isolated from dairy industrial effluent showed that peptone extract, an organic nitrogen source was the most effective for production of β-galactosidase [18]. Thus, the preferred nitrogen source for maximal enzyme yield is strain-dependent and not universal across β-galactosidase producing bacteria.

The higher enzyme activity obtained with skim milk than with IPTG for both isolates is in agreement with recent findings that although IPTG is a common and highly effective inducer of β-galactosidase expression, it is expensive and has inhibitory effects on bacterial cells at higher concentrations whereas natural inducers such as lactose avoid such disadvantages while still inducing high enzyme synthesis [19]. This is also supported by the results of *Lactiplantibacillus* isolates from homemade curd, where lactose, a natural component of skim milk, significantly increased the production of β-galactosidase compared with other substrates studied, consistent with the higher response of skim milk compared to IPTG for both DPE-3 and DPE-5 in the present study [17].

5. Conclusion:

This study showed that effluent of dairy industry from Baramati, Pune district is a potential source of β-galactosidase producing bacteria which was identified as *Alcaligenes* spp. by biochemical and 16S rRNA characterization. Among the five screened isolates, DPE-3 and DPE-5 were the most efficient producers and DPE-5 exhibited a unique acid-tolerant profile making it amenable to application with acidic whey substrates and dairy wastewater. Results of this study suggest that isolates of bacteria from wastewater could be used specifically in food biotechnology and sustainable management of dairy waste. Further work with statistically designed optimization trials would help confirm and enhance these results for practical large scale application.

Author contributions: SS: Developed the idea, wrote and verified the manuscript.

Competing interest: The author declare that no conflict of interest exists.

Ethical Statement: This work does not violate ethical guidelines.

Grant Support Details: The authors did not get funding from any agency to complete this work.

Acknowledgement: The authors are thankful to Dr. Bharat Shinde and Dr. Lalasaheb Kashid, Vice Principal, Vidya Pratishthan's Arts, Science and Commerce College, Baramati, Maharashtra, India. They are thankful to Nilima Pendharkar (HOD), Mrs. A. Neelima Devi, Dr. Amarja Bhosale, Ms Komal Kokare, Ms.Pallavi Rajguru, and Ms. Ruchita Honarao for their support in publishing this work.

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Cite this article as:
Suryawanshi SN. Screening, Production, and Optimization of β-galactosidase enzyme from *Alcaligenes* Species Isolated from Dairy Industry Effluents in Baramati, Pune District, Maharashtra. *Int. J. Micro. Sci.* 2026;7(1):11-18.

Abbreviations:

DPE: Dairy Processing Effluent (DPE-1 to DPE-5)
AMT: Ammonium transporter
BOD : Biochemical Oxygen Demand
COD : Chemical Oxygen Demand
E.C. : Enzyme Commission (number)
GOS : Galacto-Oligosaccharides
IPTG : Isopropyl β-D-1-thiogalactopyranoside
Km : Michaelis-Menten Constant
ONP : o-Nitrophenol
ONPG : o-Nitrophenyl-β-D-Galactopyranoside
rRNA : Ribosomal Ribonucleic Acid
U : Unit (of enzyme activity)
U/mL : Units per Milliliter
Vmax : Maximum Reaction Velocity