

Production, Purification, Immobilization, and Characterization of a Thermostable β -Galactosidase from *Aspergillus alliaceus*

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Abstract A fungal strain isolated from rotten banana and identified as *Aspergillus alliaceus* was found capable of producing thermostable extracellular β -galactosidase enzyme. Optimum cultural conditions for β -galactosidase production by *A. alliaceus* were as follows: pH 4.5; temperature, 30 °C; inoculum age, 25 h; and fermentation time, 144 h. Optimum temperature, time, and pH for enzyme substrate reaction were found to be 45 °C, 20 min, and 7.2, respectively, for crude and partially purified enzyme. For immobilized enzyme–substrate reaction, these three variable, temperature, time, and pH were optimized at 50 °C, 40 min, and 7.2, respectively. Glucose was found to inhibit the enzyme activity. The K_m values of partially purified and immobilized enzymes were 170 and 210 mM, respectively. Immobilized enzyme retained 43 % of the β -galactosidase activity of partially purified enzyme. There was no significant loss of activity on storage of immobilized beads at 4 °C for 28 days. Immobilized enzyme retained 90 % of the initial activity after being used four times.

Keywords β -Galactosidase · Fungal · Immobilization · Thermostable · Reusability

Introduction

β -D-galactosidase (E.C. 3.2.1.23), commonly known as lactase, has wide application in biotechnology processes. It is used for production of lactose hydrolyzed milk and whey hydrolysate to obtain the glucose and galactose. Concerning the health issue, lactose becomes a problem when there is insufficient intestinal β -galactosidase enzyme, and lactose is passed into the blood, finally appearing in the urine and in the large intestine resulting in several disorders [1]. Lactose-intolerant people are restricted to the daily intake of lactose amount in food. Because of these problems, the reduction of the lactose content in milk and dairy products is of prime importance, and the enzyme β -D-galactosidase is commercially important in food and dairy

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industries [2, 3]. The enzyme is also used to prevent the lactose crystallization in dairy industry using lactose hydrolyzed milk. This hydrolyzed milk can be consumed directly or produces many products such as cheeses, yoghurts, and buttermilk especially suitable for the lactose intolerant. Therefore, the use of β -galactosidase is one of the most promising applications of enzymes in food industries [4, 5]. From environmental point of view, hydrolysis of lactose in whey can solve the pollution problem caused by disposal of whey, and whey permeates in local water streams. High concentration of lactose in dairy waste is a major environmental problem, as it increases the biological oxygen demand in water stream [6]. β -Galactosidase can be employed directly to the cheese whey/permeate to produce high sweetness syrups, which is used as additive in ice creams, desserts, etc. [7]. Applications for β -galactosidase enzyme such as in the recovery of biologically active oligosaccharides from milk have also been described in literature [8]. Because of commercial importance of this enzyme, it has attracted keen interest of the researchers. β -Galactosidase occurs widely in nature and is produced by a number of microorganisms. However, their properties are very different, and it varies with the source microorganism. The properties like pH and thermal stability, activity, and optimum assay condition are very important for the enzyme. For most of the studies, metal ions are known to be important to the catalytic activity and stability of many β -galactosidases [9, 10]. Biocatalyst process economics is directly related to the enzyme reusability and improvement of enzyme stability afforded by immobilization. Immobilization improves the stability of enzyme. Use of immobilized enzyme is better to control the operation and flexibility in reactor design and to facilitate product recovery without catalyst contamination [11–13]. Immobilization can also alter the enzyme performance; therefore, the study with immobilized enzyme is required [14]. Lactose hydrolysis is usually carried out using immobilized β -galactosidase. Continuous product formation using a column reactor and the reusability are the two major advantages in using immobilized enzyme. The application of immobilized β -galactosidases has commercial importance and has been widely discussed in the literature [15, 16]. It is used in food industry for conversion of lactose, the major component of milk, and whey to other products to provide a lactose-free diet to the lactose-intolerant people. Immobilization helps to reduce the cost of lactose hydrolysis [17, 18].

In view of the above, the main objective of this study was the isolation of a β -galactosidase-producing strain and characterization of this enzyme. For that, a screening process from natural sources was carried out to isolate β -galactosidase producing strains. Among different isolates, one potential fungal species was selected, which produces β -galactosidase, and the species was identified as *Aspergillus alliaceus*. In present study, different process variables were optimized for β -galactosidase production. Besides, this paper describes studies on partial purification, characterization, and immobilization of the enzyme β -galactosidase using agar gel matrix. The properties of the immobilized enzyme was investigated and compared with those of partially purified enzyme. Different properties of the enzyme such as thermal, pH stability, metal ion effect, etc. were explored to characterize the enzyme for suitable commercial application.

Material and Methods

Microorganism

A thermostable β -galactosidase-producing fungal strain was isolated from rotten banana and identified as *A. alliaceus* was used for the present study. It was maintained by monthly subculturing at 30 °C and stored at 4 °C. The medium for isolation and maintenance of the culture was composed of the following (in g/L): lactose, 1; KH_2PO_4 , 0.1; KCL, 0.05; NaNO_3 , 0.2; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001; malt extract, 0.1; yeast extract, 0.1;

agar, 30; and pH of medium, 4.5. The composition of inoculum and fermentation medium was the same except agar.

Isolation of β -Galactosidase Producing Strain

Twenty-five strains were isolated from soil, spoiled orange, and banana by plate and dilution technique [19]. Each isolate was tested for β -galactosidase activity.

Taxonomical Study

The selected strain was identified following Bergey's manual of determinative bacteriology [20] followed by determination of DNA base composition done by Bangalore Genei, India.

Production of Crude Enzyme

Inoculum was prepared by transferring one loopful of organism from fresh 24 h slant culture to 50 ml inoculum medium in 250-ml Erlenmeyer flask and incubating the flask at 30 °C at 120 rpm for 24 h. Fermentation medium (50 ml) in 250-ml Erlenmeyer flask was inoculated with 5 % (v/v) inoculum and incubated at different temperature for optimizing these variables. Inoculum age and fermentation time were also taken as variable for optimization of crude enzyme production. Experiments were carried out varying one variable at a time while keeping the other three variables constant. Medium pH was varied from 4 to 6, temperature was varied from 28 to 37 °C, inoculum age was varied from 20 to 48 h, and fermentation time was varied from 48 to 168 h for optimization. The cell-free supernatant obtained by centrifugation at 5,000 rpm for 15 min was used to determine the extracellular β -galactosidase activity.

Characterization of Crude Enzyme

The culture broth was filtered to get the crude enzyme solution, which was used for characterization. Crude enzyme–substrate reactions were carried out separately, varying one factor at a time while keeping others constant for optimizing the variables. Three variables, namely pH, temperature, and reaction time of assay, were varied from 6 to 8, 30–60 °C, and 5–40 min, respectively.

Partial Purification of Crude β -D-Galactosidase

Crude enzyme solution was treated with appropriate amount of ammonium sulfate under chilled condition and kept for 2 h. After that, it was centrifuged, and the precipitate was dissolved in 0.2 M phosphate buffer (pH 7.25) [21]. β -Galactosidase activity and protein concentration were estimated as usual.

Protein Estimation

Protein content of the partially purified enzyme was estimated by Folin–Lowry method [22].

Characterization of Partially Purified Enzyme

Partially purified enzyme–substrate reactions were carried out separately varying pH, temperature, and reaction time from 6 to 8, 30–60 °C, and 5–60 min, respectively, for optimizing the

assay condition. To study the pH stability, partially purified enzyme was stored at different pH (using 0.2 M phosphate buffer), and for thermal stability, enzyme was incubated at different temperatures (4–70 °C) for 1 h. For studying the effect of metal ions, Ca^{2+} and Mg^{2+} on enzyme activity, reactions were carried out using different concentrations of metal ions in enzyme substrate reaction mixture during assay. The effect of glucose and galactose on enzyme activity was studied by carrying out enzyme substrate reaction in presence of glucose and galactose varying from 5 to 100 mM separately. Various protective agents, namely ascorbic acid, EDTA, glutathione, and sodium azide, were added to partially purified enzyme–substrate reaction mixture during assay procedure to study their effect on enzyme activity.

Immobilization of Partially Purified Enzyme

The partially purified enzyme solution was immobilized by entrapping into agar gel. Two milliliters of partially purified enzyme solution was mixed thoroughly with 5 ml molten agar (final agar concentration, 2.5 %). The mixture was cast into bead shape by injecting drop by drop using hypodermic syringe into ice-cold toluene–chloroform (3:1) mixture and allowed to stand for 2 h at 4 °C [23]. The beads were then repeatedly washed with phosphate buffer (0.2 M, pH 7.2), air dried and used immediately. Beads may be dipped into phosphate buffer (0.2 M, pH 7.2) and stored in refrigerator for future use. The average diameter of the resultant beads was 0.38 cm. Immobilized beads formed were used as immobilized enzyme equivalent to 2 ml partially purified enzyme. The activity of immobilized enzyme was determined as usual.

Characterization of Immobilized Enzyme

To get the optimal condition of reaction time, pH, and temperature, immobilized enzyme–substrate reactions were carried out varying reaction time from 5 to 60 min, pH from 6 to 8 and temperature from 30 to 60 °C. Immobilized enzyme–substrate reactions were carried out varying one parameter and keeping others constant.

Enzyme Assay

Assay was performed using lactose as substrate. For enzyme assay, 2 ml of β -galactosidase enzyme solution was mixed with 4 ml of lactose solution (0.25 M) and 4 ml of sodium phosphate (0.35 N, pH 7.25) and incubated at 30 °C for 20 min. The released glucose was measured by enzymatic method using glucose oxidase (glucose kit, BioLab diagnostic, Mumbai). One unit of enzyme activity (IU) was defined as the micromole of glucose released per minute.

Bead Size Measurement

The size of the bead was measured using an optical microscope equipped with ocular and stage micrometer [24]. The diameter of each bead was measured at three different angles. Ten beads were used to give average bead size.

Reusability of Immobilized Enzyme

In order to test the reusability of agar entrapped β -galactosidase, the beads were washed with 0.2 M phosphate buffer (pH 7.25). For each run, the activities of the immobilized enzyme were measured and then reintroduced into a fresh enzyme–substrate mixture. The activity of freshly prepared beads in the first run was used as control.

Storage Stability

Immobilized beads were dipped in 0.2 M phosphate buffer (pH 7.25) and kept in a refrigerator. Definite amount of beads were taken out at definite time interval and tested for residual enzyme activity.

Results and Discussion

Isolation, Screening, and Identification of β -Galactosidase-Producing Strain

Of the 25 isolates from different natural sources, B₅ was selected as a potential strain for β -galactosidase production and considered for further study. Based on nucleotide homology and phylogenetic analysis, the isolate B₅ was identified as *A. alliaceus* (GenBank accession number EF661567), and its nearest homolog species was found to be *Aspergillus lanosus* (GenBank accession number EF661553) (Fig. 1).

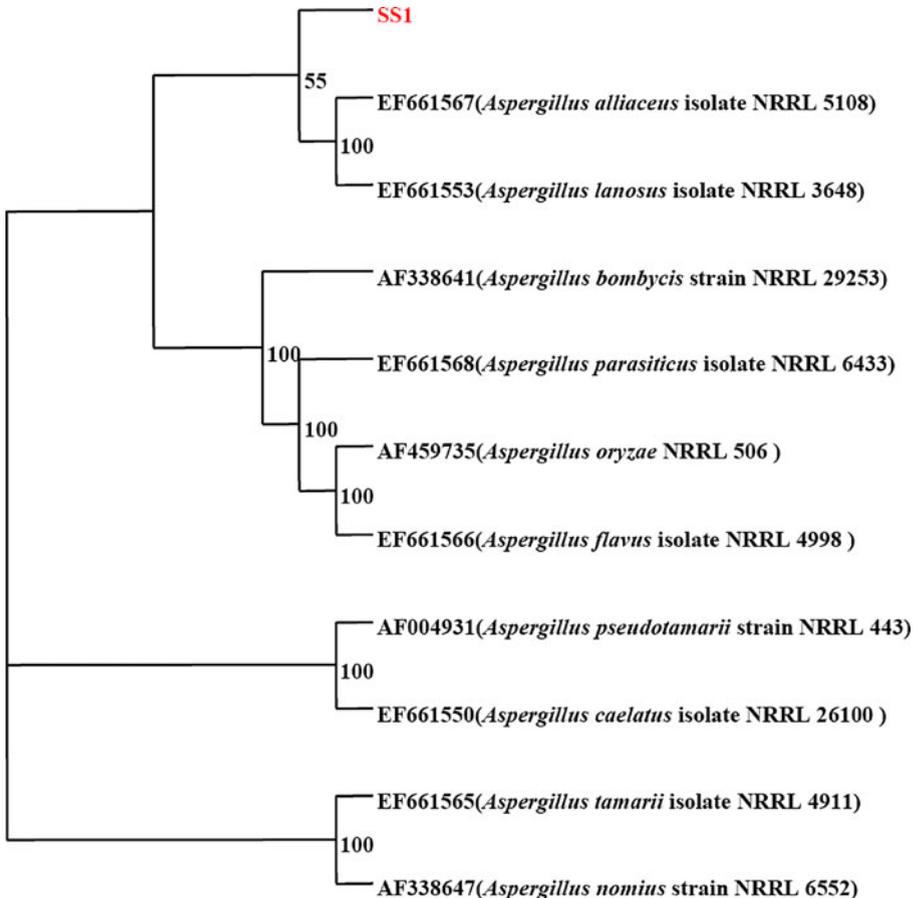


Fig. 1 Phylogenetic tree (using neighbor joining method)

Optimization of Crude β -Galactosidase Production

Effect of initial pH of the culture medium on β -galactosidase production was studied varying pH from 4 to 6, and maximum production was found at pH 4.5 (Fig. 2a). Thereafter, pH of the fermentation medium was adjusted to 4.5 in subsequent experiments. β -Galactosidase production was carried out at different temperature from 28 to 37 °C. Maximum enzyme activity was achieved by growing the selected strain at 30 °C (Fig. 2b). Effect of inoculum age and fermentation time were also studied, and most suitable conditions were found to be 25 and 144 h (Fig. 2c, d), respectively. From the optimization of fermentation condition, the maximum β -galactosidase activity of the isolated strain was found to be 0.0486 IU/ml at pH 4.5, temperature 30 °C, fermentation time of 144 h, and 25 h old inoculum culture. Various process variables, such as temperature, inoculum age, pH, and fermentation time, were optimized by varying respective variable in fermentation process, as these variables are important factors for microbial growth as well as enzyme production. The pH 5.0 was optimum for many yeast strains fermentation for β -galactosidase production [25]. However pH 5.5 was used for *Kluyveromyces marxianus* fermentation for enzyme production [26], whereas pH 4.68 was found optimum for production was optimum for *Kluyveromyces lactis* enzyme [27]. Temperature in the range of 28–30 °C was used for many organisms for β -galactosidase production [26, 28, 29]. Matheus and Rivas found 30.3 °C as optimal temperature for β -galactosidase production by *K. lactis* [27]. Inoculum of 24 h old of *K. marxianus* strains for β -galactosidase production has been used [29]. However, 20-h-old

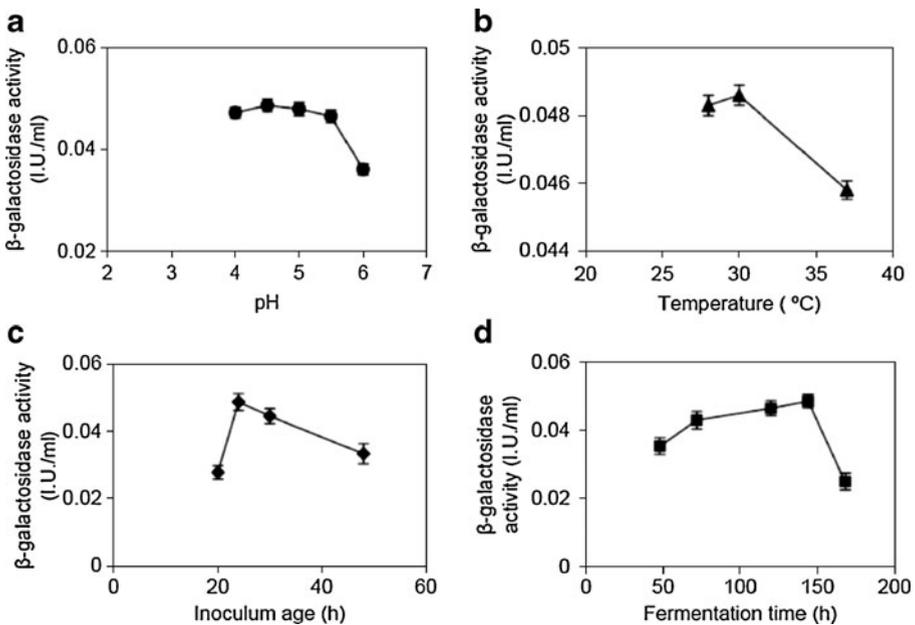


Fig. 2 Production of β -galactosidase enzyme by *Aspergillus alliaceus*. Reaction conditions for **a** pH optimization (temperature, 30 °C; inoculum age, 24 h; and fermentation time, 144 h), **b** temperature optimization (pH 4.5; inoculum age, 24 h; and fermentation time, 144 h), **c** inoculum age optimization (pH 4.5; temperature, 30 °C; and fermentation time, 144 h), and **d** fermentation time optimization (temperature 45 °C and pH 7.2)

culture of *Kluyveromyces fragilis* strain has been found for maximum enzyme activity also [30]. Different optimal conditions found by various researchers for enzyme production could be explained by the difference of source strains, their metabolic behavior, and different media composition used for fermentation.

Characterization of Crude Enzyme

Effect of Reaction Temperature

Enzyme substrate reaction was carried out at different temperatures keeping at pH 7.2 for 20-min incubation time. The maximum β -galactosidase activity was found at 45 °C, but further increase in reaction temperature resulted in reduced enzyme activity (Fig. 3a).

Effect of pH

The enzyme substrate reaction was carried out at 45 °C for 20 min varying the pH from 6.0 to 8.0. Maximum enzyme activity was obtained at pH 7.2 (Fig. 3b).

Effect of Reaction Time

Enzyme substrate reaction was carried out at 45 °C and pH 7.2 for different time periods, and maximum enzyme activity was found to be 0.0652 IU/ml at 20-min incubation time (Fig. 3c).

Partial Purification of the Enzyme

Partial purification of the enzyme was carried out by salting out method using $(\text{NH}_4)_2\text{SO}_4$ in 30 % saturation. Purity of the partially purified enzyme was increased by 2.74-fold.

Characterization of Partially Purified Enzyme

Effect of Incubation Time, pH, and Temperature

From different partially purified enzyme–substrate reactions, the optimum enzyme activity was found to be 0.25 IU/mg for 20-min incubation time at pH 7.2 and temperature of 45 °C (Fig. 4a–c).

pH Stability and Thermal Stability

Partially purified enzyme was stored at different pH from 5.5 to 8.0 using 0.2 M phosphate buffer for 1 h prior to assay, and residual enzyme activity was determined using standard assay procedure. The optimal pH for enzyme storage was found at pH 7.2 (Fig. 5a). For thermal stability, enzyme was incubated at different temperatures from 4 to 70 °C for 1 h. The enzyme activity was maximally maintained at 4 °C, but there was no further significant decrease in enzyme activity with increase in storage temperature from 35 to 70 °C (Fig. 5b), which indicated thermostable nature of the enzyme.

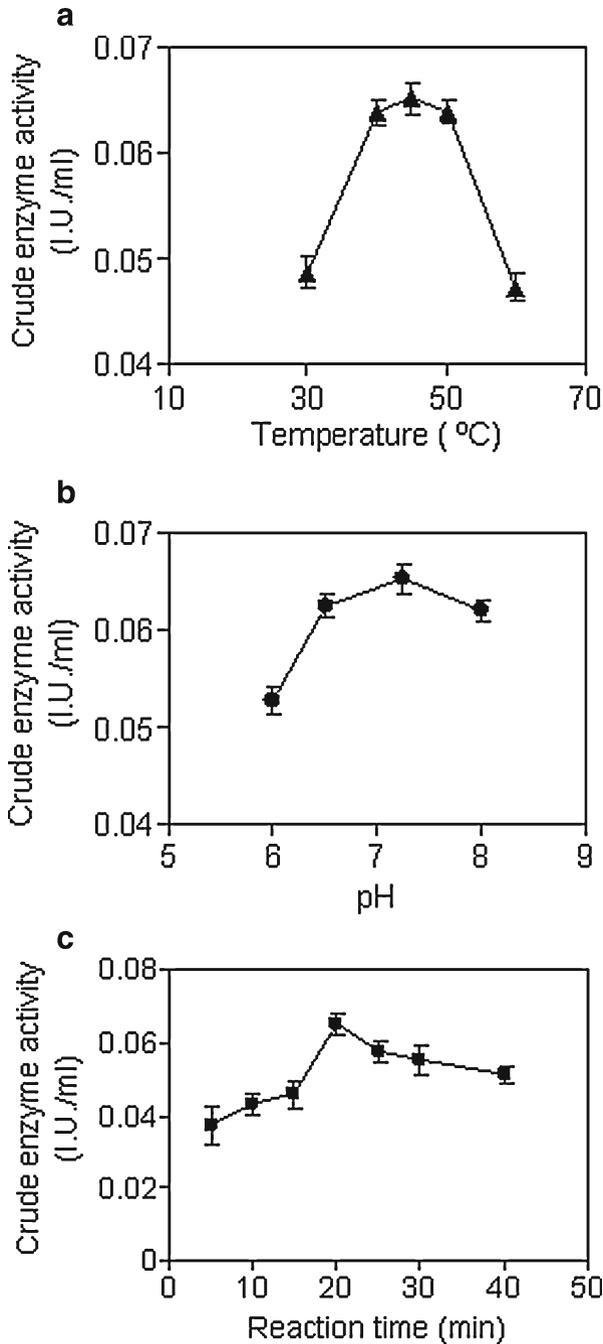


Fig. 3 Optimization of crude enzyme activity. Reaction conditions for **a** temperature optimization (pH 7.2, incubation time 20 min), **b** pH optimization (temperature, 45 °C; incubation time, 20 min), and **c** incubation time optimization (temperature 45 °C and pH 7.2)

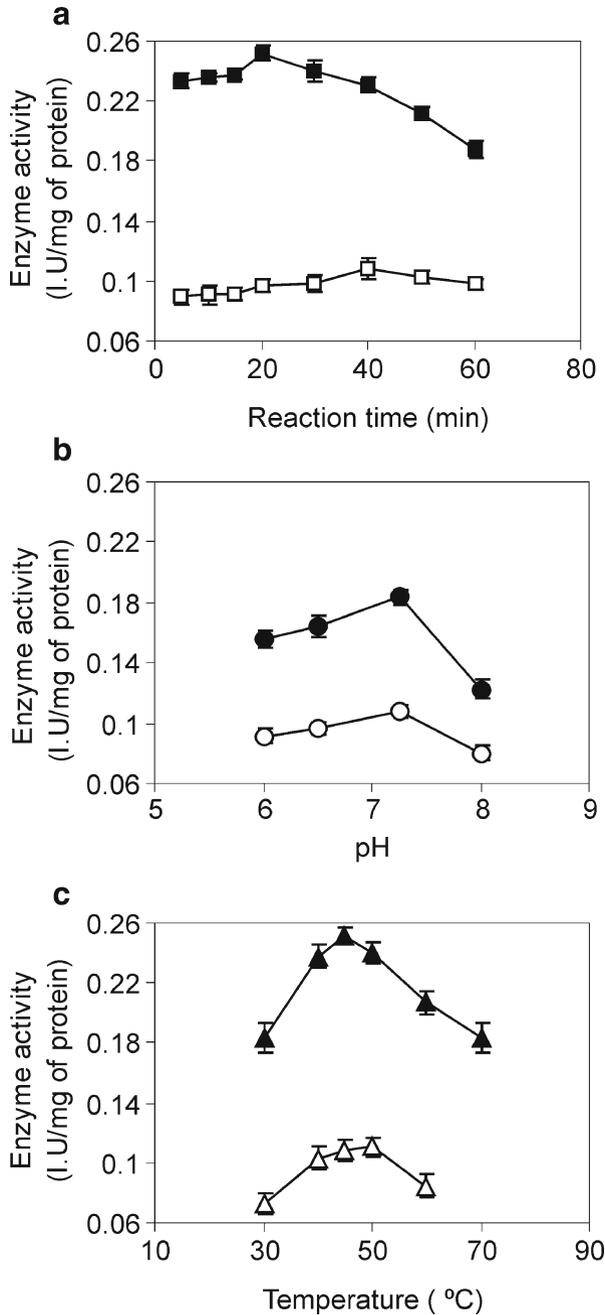


Fig. 4 Optimization of partially purified and immobilized enzyme activity. Reaction condition for **a** incubation time optimization (temperature, 45 °C, and pH 7.2), **b** pH optimization (temperature, 45 °C; incubation time, 40 min), and **c** temperature optimization (pH 7.2; incubation time, 40 min). *Filled symbol* indicates for partially purified enzyme, and *open symbol* indicates for immobilized enzyme

Effect of Metal Ion

From Fig. 6, it was observed that Ca^{2+} ion showed slight stimulatory effect, whereas Mg^{2+} had inhibitory effect on β -galactosidase activity.

Effect of Sugars on β -Galactosidase Activity

The effect of end products, i.e., glucose and galactose, on enzyme activity was studied. In the presence of glucose, the enzyme activity decreased gradually with increase in glucose concentration, and activity decreased by 35 % in presence of 50 mM glucose (Fig. 7). However, in the presence of galactose, enzyme activity was not affected.

Effect of Protective Agents

Effect of various protective agents, viz., ascorbic acid, EDTA, glutathione, and sodium azide, on enzyme substrate reaction was studied, and in the presence of ascorbic acid, the

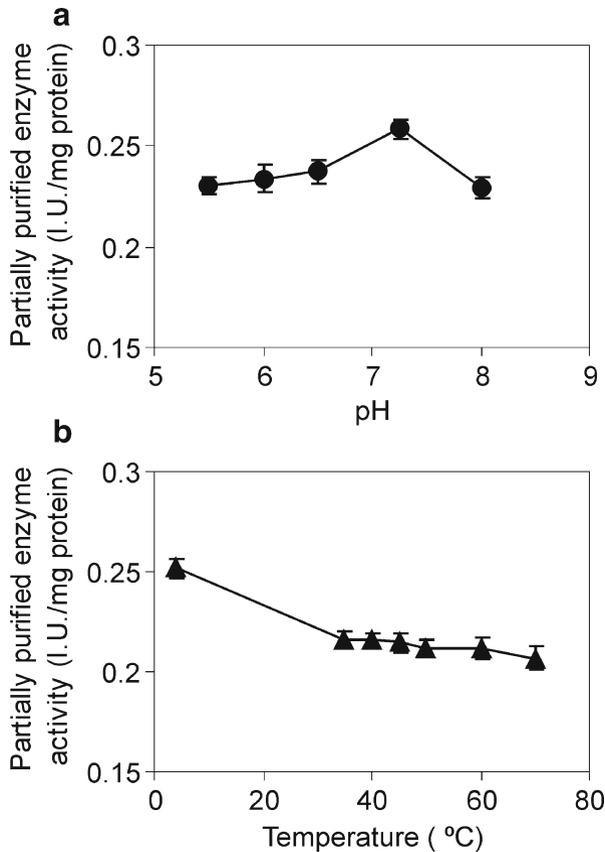


Fig. 5 pH and thermal stability of partially purified enzyme. **a** pH stability: stored at different pH at 4 °C. **b** Thermal stability: incubated at different temperatures at pH 7.2. Reaction condition for measuring activity was pH 7.2, temperature at 45 °C, and incubation time of 20 min

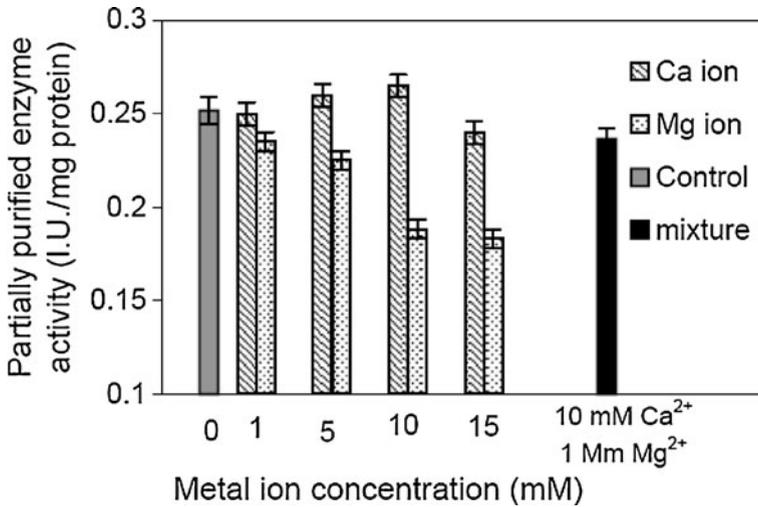


Fig. 6 Effect of metal ion on partially purified enzyme activity. Reaction condition was pH 7.2, temperature at 45 °C, and incubation time of 20 min

enzyme activity decreased by 37 % compared to control, whereas other protective agents have no significant effect on enzyme activity (Fig. 8).

Characterization of Immobilized Enzyme

Effect of Incubation Time, pH, and Temperature

Immobilized enzyme–substrate reactions were carried out to standardize assay conditions. Maximum enzyme activity was found to be 0.108 IU/mg for 40-min incubation time

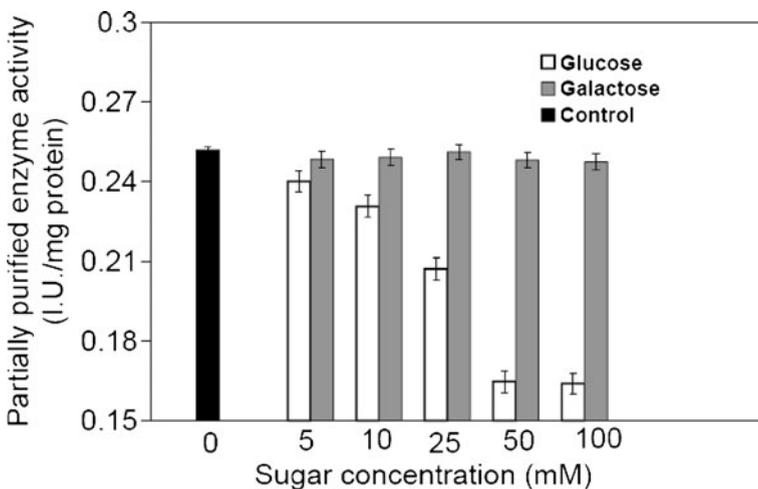


Fig. 7 Effect of glucose and galactose on enzyme activity. Reaction condition was pH 7.2, temperature at 45 °C, and incubation time of 20 min

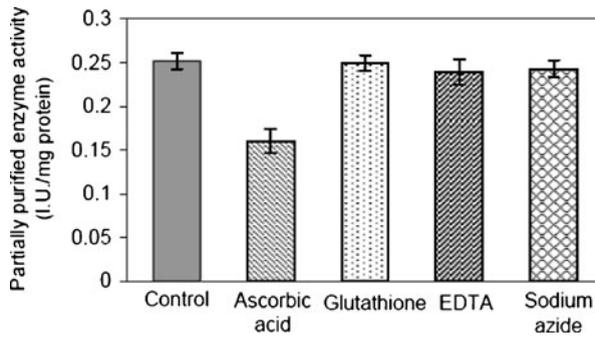


Fig. 8 Effect of protective agents on partially purified enzyme activity. Reaction condition was pH 7.2, temperature at 45 °C, and incubation time of 20 min

(Fig. 4a), keeping other conditions constant, whereas the optimal incubation time for crude enzyme was 20 min (Fig. 3c). This may be due to entrapment of enzyme in agar, and it takes longer incubation time for substrate to reach enzyme active center due to extra permeability barrier. Immobilized enzyme–substrate reactions were carried out at different pH, and the optimal pH was found to be 7.2 (Fig. 4b). Crude and partially purified enzyme also showed optimum pH at 7.2 (Figs. 3a and 4b). The optimal temperature for immobilized enzyme was 50 °C (Fig. 4c), while that for crude enzyme and partially purified enzyme were 45 °C (Figs. 3b and 4c). The increase in optimum temperature for immobilized enzyme may be due to diffusional effects as the immobilized enzyme can be more easily contacted by the substrates with the increase in the bulk temperature.

Activity Yield of Immobilized Enzyme

For determining extent of decrease in enzyme activity due to immobilization, activity yield was calculated.

$$\text{Activity yield} = \frac{\text{activity of immobilized enzyme}}{\text{activity of partially purified enzyme}}$$

It was found that 2.5 % agar-entrapped enzyme retain 43 % of the β -galactosidase activity (Fig. 9). This lowering of activity was due to the diffusional effect in the immobilized bead.

Reusability of Immobilized Enzyme

Reusability of immobilized enzyme is one of the important parameters to improve process economics. Repeated use brought about activity loss in all cases. This might be mainly due to damage of the active sites of the immobilized enzyme during repeated use or leakage of the enzyme from the gel beads. The agar entrapped β -galactosidase retained 90 % of the initial activity after four cycles (Fig. 10).

Storage Stability

It was observed from Fig. 11 that the immobilized enzyme did not loss activity significantly after 4-week storage at 4 °C (Fig. 11).

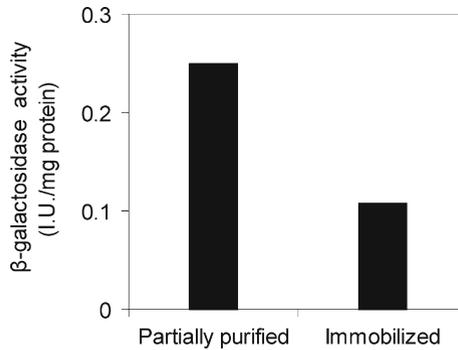


Fig. 9 Activity reduction due to immobilization of enzyme. Reaction condition was pH 7.2, temperature at 50 °C, and incubation time of 40 min

Determination of Kinetic Parameters of Partially Purified and Immobilized Enzymes

The kinetics of β -galactosidase activity of partially purified and immobilized enzyme was investigated by conducting enzyme–substrate reaction with varying lactose concentrations (100–333 mM) under optimized assay condition. The Lineweaver–Burk plots are shown in Fig. 12. Michaelis constant (K_m) and maximum velocity (V_{max}) of both the partially purified and immobilized enzymes were determined. The kinetic parameters calculated from the Lineweaver–Burk plots are listed in Table 1. Both the K_m and the V_{max} values seemed to be affected by the immobilization process. The K_m values for the partially purified and immobilized enzyme were 170 and 210 mM, respectively. The $1/K_m$ value is known as the criterion for the affinity of enzymes to substrates [31], and the lower value of K_m indicated the higher affinity between partially purified enzyme and substrate than the immobilized enzyme. The decrease in the affinity of the immobilized enzyme to the substrate was probably caused by lower accessibility of the substrate to the active site of the immobilized enzyme due to steric hindrance by the agar support and diffusional resistance of lactose transport to the active site of enzyme in matrix. The lower affinity of immobilized enzyme may also be caused by conformational changes of active site due to immobilization [32]. In this study, V_{max} value of immobilized enzyme was 2.2 times lower than that of the partially purified enzyme. The decrease in V_{max} values proved that activity

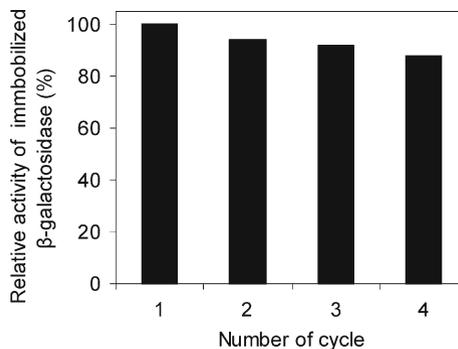


Fig. 10 Relative activities of immobilized enzyme for the hydrolysis of lactose with multiple reuses. Reaction condition was pH 7.2, temperature at 50 °C, and incubation time of 40 min

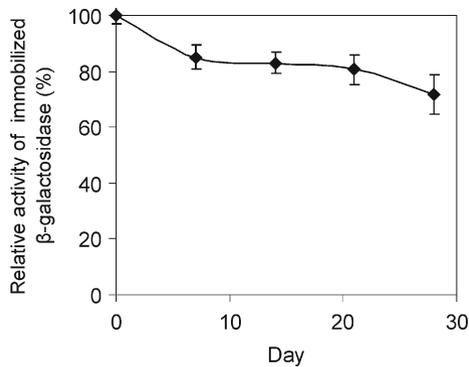


Fig. 11 Storage stability of immobilized enzyme. Reaction condition for measuring activity was pH 7.2, temperature at 50 °C, and incubation time of 40 min

was reduced in immobilization. This loss in activity may be attributed to either a loss in native conformation of enzyme or shielding effect of the matrix.

The optimal pH for β -galactosidase activity is very important for dairy industry as the milk pH is 6.8 [33]. The optimal pH of this enzyme activity is different for enzyme produced from different source microorganism. Palumbo et al. [34] observed that the optimum pH for the *Aspergillus oryzae* produced β -galactosidase for hydrolysis in liquid milk systems was 5.0. β -Galactosidase activity from *Saccharomyces lactis* and *Bifidobacterium adolescentis* DSM 20083 shows maximum activity at pH value close to 6 [35, 36]. The optimum pH zones for lactose was acidic (pH between 2.6 and 3.0) for β -galactosidase from *Aspergillus fonsecaeus* [37] where as the optimum pH for *Seudoalteromonas haloplanktis* TAE 79 β -galactosidase activity (pH 8.5) and *Escherichia coli* enzyme activity (pH 8.0) were basic [38]. However, in the present study, optimal activity of newly isolated *A. alliaceus* β -galactosidase is at pH 7.2, and activity was found to be decreased only 10 % at pH 6.5 (Fig. 4b). In addition, this enzyme is stable in the pH range (Fig. 5a). Therefore, the optimal pH range of enzyme activity for *A. alliaceus* β -galactosidase is very close to milk pH.

Other important property of this newly β -galactosidase is thermal stability. The activity of the *Kluyveromyces lactis* β -galactosidase decreased by half of its maximal value in 17.5 min at 30 °C and in <1 min at 45 and 60 °C [39]. In another study, *K. lactis* β -galactosidase retained only 27 % catalytic activity at 60 °C [40]. In the current study *A. alliaceus* β -galactosidase has

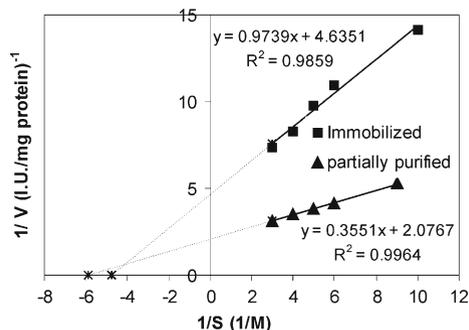


Fig. 12 Lineweaver–Burk plots for partially purified enzyme and immobilized enzyme

Table 1 Kinetic parameters of partially purified and immobilized enzymes

Enzyme	K_m (mM)	V_{max} (IU/mg of protein)
Partially purified	170	0.48
Immobilized	210	0.22

a higher thermal stability. It retains 82 % activity, after maintaining the temperature at 70 °C for 1 h compared to maximum activity at 4 °C (Fig. 5b), whereas it has been reported that a commercially available preparation from *A. oryzae* lost 50 % activity in 10 min at 60 °C [37]. *A. alliaceus* β -galactosidase activity is retained without appreciable loss over periods of several weeks of storage in the refrigerator (Fig. 11), and also it is unaffected by several protective agents except ascorbic acid. Moreover, this enzyme can be reused for several times (Fig. 10).

Conclusion

A new fungal strain *A. alliaceus* capable of producing β -galactosidase enzyme was isolated. The values of optimum β -galactosidase activity were 0.0486 IU/mL, 0.25 IU/mg, and 0.108 IU/mg for crude, partially purified enzyme, and immobilized enzyme, respectively. The enzyme was quite thermostable retaining 82 % enzyme activity at 70 °C compared to activity at 4 °C. The partially purified enzyme was entrapped in 2.5 % agar bead to make it stable and reused successfully. Furthermore, immobilized beads were stored for 28 days without any significant loss of activity. The pH of milk is around 6.8, and the enzyme has maximum activity in the pH range of 6.5–7.2. Therefore, this enzyme can be applied in dairy sector to prepare low lactose milk and lactose-hydrolyzed milk-based products for patients suffering from lactose intolerance.

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