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Research Article

# Studies on Isolation and Optimization of Lactase from *Kluyveromyces lactis* LPY-08

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## Abstract

β-galactosidase, also referred to as lactase (EC.3.2.1.23), is extensively utilized in the pharmaceutical sector for the management of lactose intolerance, a condition that impacts around 75% of the global population. The present study is centered on the improvement of lactase production derived from the yeast *Kluyveromyces lactis* through the optimization of production and reaction parameters using the one variable at a time (OVAT) approach. The initial screening process for lactase-producing microbes involved cultivating the organisms on an agar plate supplemented with X-gal. The lactase-producing positive isolate exhibits a blue coloration on agar plates. Following the initial selection process, the hyperproducer strain was chosen for subsequent investigations. The cultivation and investigation of *Kluyveromyces lactis* pure cultures were conducted in order to optimize the synthesis of lactase. The study focused on examining specific production characteristics, like the starting pH and incubation temperature, in isolation. The highest level of lactase synthesis (402.74 U/mg dcw) was achieved under the following conditions: an initial pH of 7.0, a temperature of 35°C, the use of galactose and peptone as carbon and nitrogen sources, and the utilization of oNPG (Ortho-nitrophenyl- β-D-galactopyranoside) as the substrate. The application of optimization techniques led to a significant increase in the total activity of the enzyme, with a seven-fold boost seen.

**Keywords:** Lactose Intolerance; *Kluyveromyces lactis*; Cultivation

## Introduction

Lactose intolerance is a clinical disorder characterized by a diminished or missing production of the lactase enzyme in the intestinal mucosa, which is sometimes referred to as the brush border enzyme [1]. The estimated prevalence of lactose intolerance is believed to surpass 75%, with a highly unequal distribution of cases observed globally [2]. This issue can be resolved through the utilization of supplements or the incorporation of lactase enzyme in milk and milk derivatives. The lactase enzyme facilitates the hydrolysis of lactose into glucose and galactose, enabling their absorption across the intestinal epithelia [3]. Lactase exhibits a diverse array of applications among several industries, including dairy, confectionery, baking, and soft drink sectors [4]. The highest level of lactase intake is often through the ingestion of meals and beverages. While it is possible to hydrolyze lactose using acids, this method has been found to result in fouling of the ion exchange resins utilized in the processing, as well as the creation of color. The enzymatic breakdown of lactose by lactase offers a resolution to the challenges related to whey disposal, lactose crystallization in frozen concentrated desserts, and milk consumption among lactose-intolerant individuals [5]. Therefore, enzymes are highly sought after in industrial applications owing to their capacity to catalyze the hydrolysis of lactose without any concurrent reactions.

Various plant species, such as almonds, peaches, apricots, and apples, alongside animal organs like the stomach, brain, and placenta, have been shown to possess lactases. Lactases can be found in bacteria, fungi, and yeasts as well [6]. Microbial sources are prioritized over plant and animal sources in enzymatic research owing to their greater commercial feasibility. Bacterial sources possess favorable characteristics such as their propensity for fermentation, heightened enzyme activity, and enhanced stability, rendering them a viable choice for the extraction of lactases [7]. Nevertheless, the limited applicability of microorganisms such as lactic acid bacteria as probiotics stems from their incapacity to generate spores and endure the acidic conditions prevailing in the gastrointestinal tract, hence confining their therapeutic potential to lactose treatment. Yeasts such as *Kluyveromyces marxians*, *Candida pseudotropicalis*, and *Kluyveromyces lactis*, as well as fungi including *Neurospora crassa*, *Aspergillus foetidus*, *Aspergillus phoenicis*, *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus oryzae*, *Mucor pusillus*, and *Mucor meihei*, have the capacity to produce lactase.

Yeasts predominantly utilize lactases for the processing of milk, sweet whey, and dairy products with a neutral pH. The optimization of media components and cultural parameters is of paramount importance in the biological process, since the composition of the medium significantly affects the culture conditions for enzyme production. The primary objective of this work was to optimize the production parameters in order to increase lactase production derived from *Kluyveromyces lactis*.

## Materials and Methods

Analytical-grade chemicals and reagents were obtained from SRL,India and E. Merck. The substrate o-nitrophenyl-β-D-galactopyranoside (oNPG) and bacteriological media were purchased from Himedia (Mumbai). The chemicals used for protein purification were molecular biology and electrophoresis grade. All other chemicals were of analytical grade.

### Enzyme assay

Lactase assay was performed using a colorimetric method by quantifying released reducing sugar in a spectrophotometric analysis used with a few modifications [7]. The assay of both intra- and extra-cellular lactase activity was performed with 1 mM oNPG as the substrate in 0.1 M phosphate buffer (pH 7.0). The activity of lactase was measured by incubating 10 µL of the suitably diluted enzyme with 20 µL oNPG at 35oC for 15 min. The reaction was stopped by adding 1 mL of 0.4 M Na2CO3 and the concentration of o-nitrophenol (oNP) released was determined by spectrophotometry at 420 nm. One unit (U) of lactase activity was defined as the amount of enzyme which catalyzed the release of one micromole of glucose per min under assay conditions.

### Isolation and screening of lactase producing organism

Selection of lactase producing microorganism was done by X-gal plating method. Colonies which produced lactase imparted blue coloration on the agar plates. Screened isolates were further studied for the lactase production. The isolate which showed maximum lactase activity was selected for further studies.

### Optimization of carbon sources

To investigate the effect of carbon sources on lactase production various carbon sources were used in a concentration of 2% (w/v). To optimize the concentration, varying concentrations (1-10% w/v) of selected carbon sources (galactose) were used in the production medium (pH 7.0). Lactase activity was obtained using harvested cells after 48 h and optimized galactose concentration was considered for further studies.

### Optimization of nitrogen source

Various organic and inorganic nitrogen sources i.e., soya peptone, tryptone, urea, $KNO_3$, gelatin, ammonium chloride, ammonium sulfate, and ammonium nitrate were evaluated for their effect on growth and lactase production. Further, to find out the optimum concentration of the optimized nitrogen source, it was added at a different concentration ranging from (0-1%) in the production medium. Harvested cells were utilized to measure the enzyme activity after 48 hours, and the medium was used for future studies with an optimized nitrogen source concentration.

### Effect of pH and temperature

The enzyme production was carried out at various temperatures (25, 30, 35, 40, 45, and 50°C) in shaking conditions to see the effect of temperature on lactase production. The selected isolate was cultivated in the pre-optimized medium and the pH of the medium was varied from (3.0-10.0). Lactase activity was calculated using cells harvested after 48 h.

### Effect of inoculum size

To check the effect of inoculum size on enzyme production medium was supplemented with different concentrations of inoculum and it was varied from 2-10%. Enzyme activity was determined using harvested cells after 48 h and optimized inoculum size was considered for further studies.

### Growth and enzyme production profile

To find out the optimal time of incubation for the maximum lactase production, the medium (pH 6.0) containing yeast extract (0.30%), peptone (0.7%), malt extract (0.2%), and galactose (3.5%) was inoculated with 4% (v/v) inoculum and incubated in an orbital shaker for 4 days at 35°C. The pH profile, lactase activity, and O.D. were checked after an interval of 6 h.

### Shelf life of enzyme

Storage stability of lactase was investigated at 4°C and room temperature for 60 days. The enzyme was stored at 4°C and room temperature and enzyme activity was checked after an interval of 10 days.

## Results

### Isolation and screening of Lactase enzyme

The isolate which showed maximum lactase activity was named as LPY-08 was identifed as *Kluyveromyces lactis* at MTCC Chandigarh and production and characterization of lactase was further carried out with *Kluyveromyces lactis* LPY-08.

### Effect of carbon source

Yeast culture was grown in a medium containing different carbon sources (2%). The carbon sources used for the production of lactase were fructose, glucose, lactose, maltose, galactose, starch, arabinose, cellobiose, glycerol, sorbitol, xylose, and sucrose. Although yeast utilized all the carbon sources for its growth, the

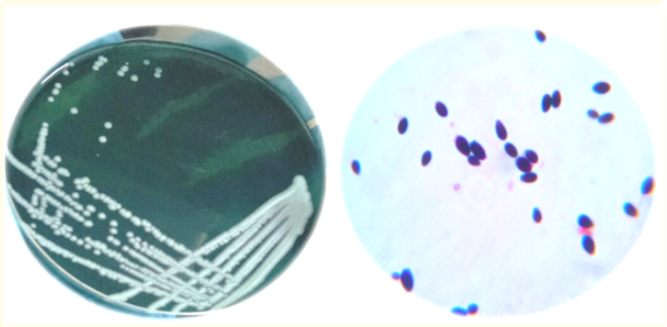

**Figure 1:** Morphological characteristics of *Kluyveromyces lactis*: (a) growth pattern on agar plate (b) Gram staining of *Kluyveromyces lactis* LPY-08 (100X magnification).

maximum production of lactase (56.133 ± 2.8066 U/mg dcw) was found in the medium containing galactose as a carbon source as shown in Figure 2.

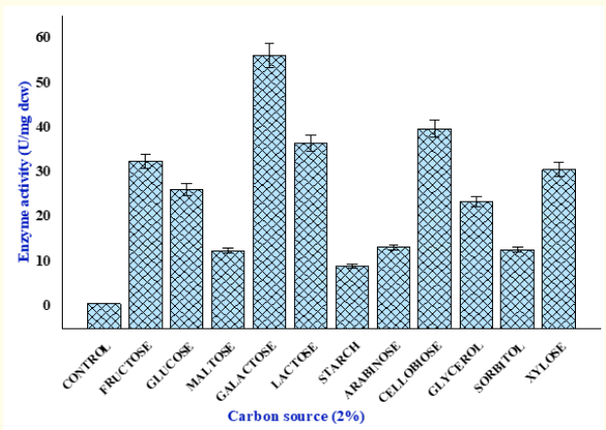


**Figure 2:** Effect of different carbon sources on production of lactase from *Kluyveromyces lactis* LPY-08.

## Effect of nitrogen sources

Various organic and inorganic nitrogen sources such as soya peptone, tryptone, urea, KNO3, gelatin, ammonium chloride, ammonium sulfate, ammonium nitrate, and peptone were used at 0.7% (w/v) concentration in the production medium. The highest enzyme activity (240.80 ± 2.715 U/mg dcw) was observed with peptone as shown in Figure 3.

## Effect of production temperature

The influence of temperature on the production of lactase was studied by growing the *Kluyveromyces lactis* at different temperatures ranging from 25°C to 50°C. The enzyme produced an appreciably higher amount at 35°C (252.92 ± 5.0584 U/mg dcw) as

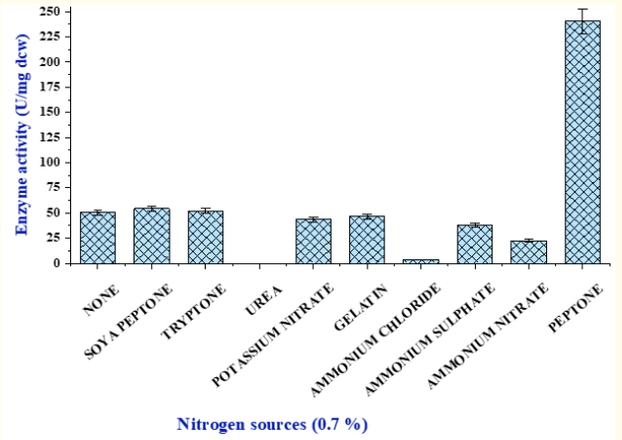


**Figure 3:** Effect of nitrogen source on the production of lactase from *Kluyveromyces lactis* LPY-08.

compared to other temperatures (Figure 4). However, a decrease in enzyme activity was observed with an increase in temperature beyond 35°C.

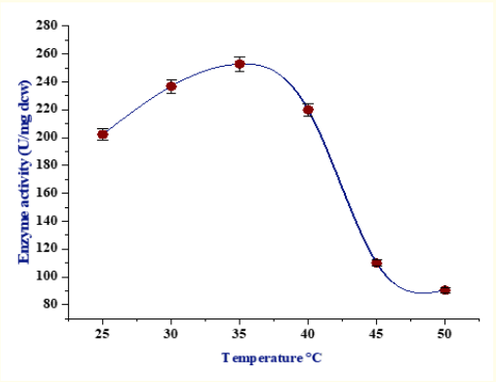


**Figure 4:** Effect of incubation temperature on the production of lactase from *Kluyveromyces lactis* LPY-08.

## Effect of medium pH

The organism was cultured in a selected medium at a pH of 3.0-10. At pH-6.0, the greatest enzyme activity (294.30 ± 5.886 U/mg dcw) was observed as shown in (Figure 5).

## Effect of inoculum size on the Production of Lactase

The inoculum level for optimum production of lactase by *Kluyveromyces lactis* was worked out. 4% v/v inoculum gave the highest titer (273.59 ± 5.4718 U/mg dcw) in 48 h (Figure 6). At low concentrations, the number of cells was not well enough to utilize essential nutrients to produce the enzyme.

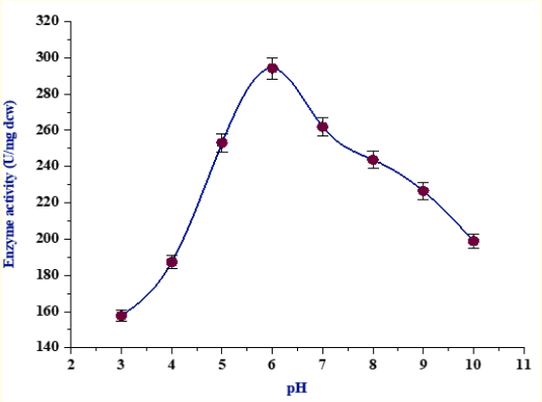


**Figure 5:** Effect of medium pH on the production of lactase from *Kluyveromyces lactis* LPY-08.

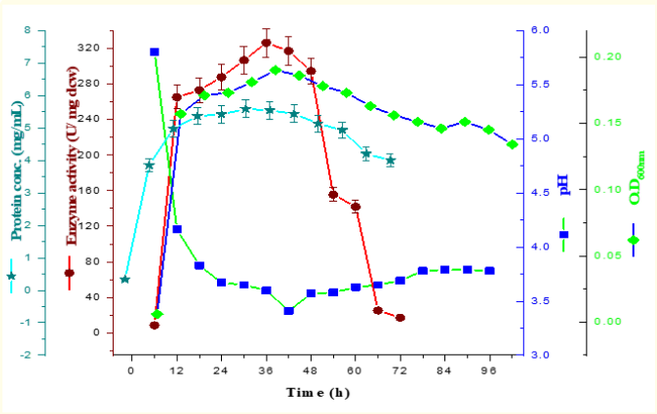


**Figure 7:** Time course of lactase production from *Kluyveromyces lactis* LPY-08.

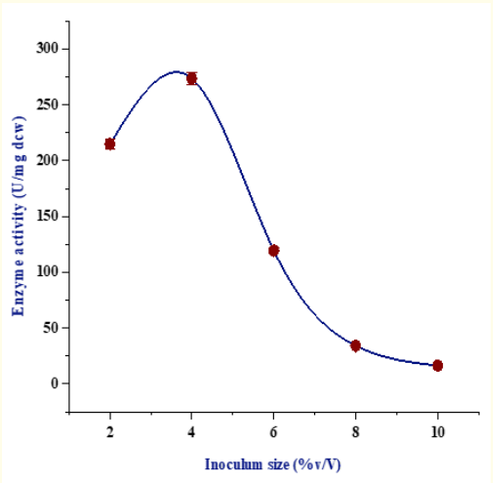


**Figure 6:** Effect of inoculum size on the production of lactase from *Kluyveromyces* lactis LPY-08.

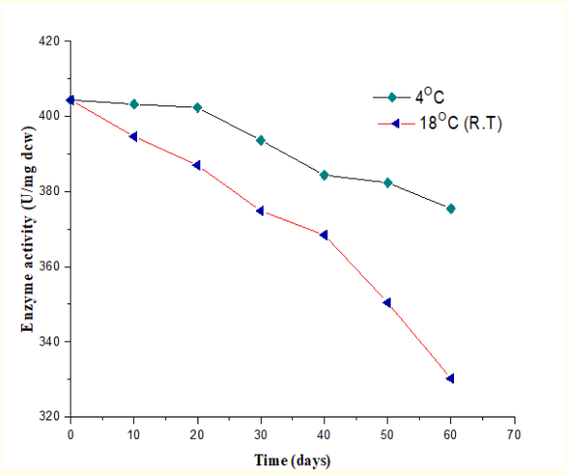


**Figure 8:** Shelf life of lactase enzyme at 4°C and room temperature.

## Time course of lactase production from *Kluyveromyces lactis*

Lactase production was studied in the optimized medium containing yeast extract (0.3%), malt extract (0.3%), peptone (0.7%), galactose (3.5 %), inoculum size of 4%, and pH 6.0. The active cell growth continued up to 48 h and then started decreasing after the increase in time. The optimum incubation time for maximum production of lactase was obtained at 36 h. The maximum lactase activity was observed to be 326.06 U/mg dcw with 5.58 mg/mL of protein and pH declined from 6.0 to 3.65 (Figure 7).

## Shelf life of lactase

Lactase enzyme was kept at both room temperature (18°C) and 4°C to determine its shelf life. After every 10 days, the enzyme activity was evaluated. After 60 days of storage at 4°C, around 93% of activity was still retained (Figure 8). However, after 60 days at room temperature, roughly 82% of the activity was still present. This result leads to the conclusion that storing an enzyme at a lower temperature and in a dry environment can extend its shelf life.

The optimization of physicochemical parameters was conducted in order to maximize the synthesis of lactase by *Kluyveromyces lactis*. The composition and concentration of carbon sources in culture media play a crucial role in the growth and synthesis of lactase enzyme. The biosynthesis of lactase in different bacteria is regulated by the carbon source. The variability and dependence of carbon sources in the production of lactase may be observed in different microorganisms [11]. Yeast demonstrated the ability to utilize various carbon sources for its growth. However, the medium containing galactose as a carbon source exhibited the highest lactase synthesis, reaching a maximum of 56.133 U/mg dcw. The researchers Hsu., *et al*. (2005) conducted a study on the highest level of lactase activity observed when lactose was present, followed by galactose, and the lowest activity observed when glucose was used as the carbon source. The majority of organisms employ both organic and inorganic nitrogen sources. The nitrogen source that resulted in the highest enzyme activity was peptone, with a recorded value of 240.80 ± 2.215 U/mg dcw. The present study investigated the im-

pact of temperature on lactase production, revealing a significantly higher enzyme yield at 35°C (252.92 ± 5.0584 U/mg dcw) compared to alternative temperature conditions. Nevertheless, an inverse correlation was detected between temperature and enzyme activity, potentially indicating the enzyme's partial deactivation. The study determined that the highest level of enzyme activity was observed within the temperature range of 27-30°C, as reported by *K. marxianus* [13]. The equilibrium of acid-base reactions is influenced by variations in pH, leading to notable consequences for the absorption of nutrients within the medium. Consequently, it is imperative to monitor the pH levels prior to inoculation, throughout the growth process, and throughout the harvest stage. The pH level at which the maximum enzyme synthesis was seen was 6.0, with a reported value of 294.30 ± 5.886 U/mg dcw. The pH optimum of commercially available intracellular *K. fragilis* β-galactosidase was determined to be approximately 6.5 by scientific investigation. The study conducted by [13] revealed that the highest level of extracellular lactase activity was observed at a pH of 5.5, originating from the microorganism *Kluyveromyces marxianus*. The optimal inoculum level for the synthesis of lactase by *Kluyveromyces lactis* was determined. The greatest titer was achieved within 48 hours using an inoculum concentration of 4% v/v. At lower concentrations, the cellular population exhibited insufficient capacity to effectively utilize vital nutrients for enzyme production. The viscosity of the fermentation media increased significantly at a high concentration of inoculum, mostly due to the rapid proliferation of yeast. This excessive growth of yeast led to a nutritional imbalance within the medium. The utilization of more potent inoculums has the potential to diminish the duration of the lag phase and yield a substantial reduction in fermentation time. Nevertheless, it is imperative to allocate an adequate amount of time for the secretion of β-galactosidase in the yeast culture [14]. The highest level of lactase synthesis was seen in *Aspergillus sp.* when the inoculum age was 6% v/v, and this was achieved during a period of 7 days [15]. The investigation focused on determining the highest level of enzyme activity by utilizing a 6% (v/v) inoculum of the yeast culture [4]. Cell proliferation remained active for a duration of 48 hours, following which it exhibited a decline subsequent to a period of expansion. The optimal duration of incubation for achieving the highest lactase synthesis was determined to be 36 hours. The highest measured level of lactase activity was 326.06 U/mg dcw when the protein concentration was 5.58 mg/mL, and the pH decreased from 6.0 to 3.65. In addition to this temporal threshold, an extended duration of incubation did not result in an increase in the production of enzymes. The occurrence described might be attributed to the denaturation of the enzyme, which was likely induced by interactions with various elements present in the medium. Additionally, it is probable that the reduction in available nutrients for bacteria had a role in this phenomenon [16,17]. The highest level of lactase activity was seen after a 5-day incubation period. Altering the duration of the incubation period, either by increasing or decreasing it, resulted in a decrease in lactase production [15]. In conclusion, it can be inferred that the aforementioned evidence supports the notion that the stated argument The absence of intestinal lactase secretion during the transition from breastfeeding to solid food consumption has presented challenges for researchers in their efforts to mitigate lactose intolerance, a condition that predominantly affects adults and is prevalent in around 75% of the global population. The utilization of lactase-producing strains on a broad scale is now employed in the dairy industry to cater to individuals with lactose intolerance. Consequently, there is a significant growth in the market for lactase. Given the universally recognized safety of *Kluyveromyces lactis*, our objective was to augment the synthesis of lactase from this microorganism. The strain was chosen for the purpose of conducting optimization tests on the culture medium to enhance enzyme production. The strain was cultivated in a medium consisting of yeast extract (0.30%), peptone (0.7%), malt extract (0.2%), and galactose (2%) at a pH of 6.5 and a temperature of 35°C. The incubation period lasted for 36 hours. The highest level of lactase activity observed was 402.74 U/mg dcw. This was achieved by using a substrate concentration of 4mM oNPG and an enzyme concentration of 4 µg/mL. The reaction time for this optimal activity was 5 minutes. The optimization tests yielded a significant increase in the overall enzyme activity, almost seven times higher than the initial value. Therefore, the significance of this study lies in the investigation of microbial origins as potential catalysts for enhanced lactase production. Hence, the importance of the study is to explore microbial sources for higher production of lactase.

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