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Influence of UV Treatment on β -galactosidase Produced by *Lactobacillus plantarum*

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Lactose hydrolyzing enzyme, α-galactosidases have been used in the dairy industry for the improvement of lactose intolerance. The aim of this study was to detect β-galactosidase enzyme produced by isolated *Lactobacillus Plantarum* from milk and cheese and effect of UV treatment on b-galactosidase. Isolated lactobacilli were cultured on MRS agar. Lactobacilli were identified by Gram stain and standard bacteriological and biochemical methods. Their ability to hydrolyze 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal)andO-nitrophenyl-D galactopyranoside (ONPG) was determined. A protein band of indicated β-galactosidase enzyme was also detected by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) method. The colonies that produced greenish blue color on X-Gal plates were lactobacilli with β-galactosidase enzyme which had ONPG positive results. The highest enzymatic value was observed in 15 minutes UV radiation and also to characterize its probable bactericins of Lactobacillus plantarum. By adding *Lactobacilli* producing β-galactosidase enzyme as probiotic to dairy products, could help lactose intolerant people.

Key words: β-galactosidase, *Lactobacillus Plantarum,* UV treatment.

Lactose is a disaccharide found in milk and other dairy products. Lactose indigestion in the intestinal microflora leads to dizziness, headache and nausea. So milk and other dairy products are manufactured with less lactose content for lactose intolerance people which further adds the cost to about 80% of the normal unhydrolysed milk[1]. One of the glycosidases, is β–galactosidase enzyme that widely used in dairy industry and is produced by most *lactobacilli*.[2, 3] This enzyme hydrolyzes lactose, the main carbohydrate in milk, into glucose and galactose, which can be absorbed across the intestinal epithelium[4]. β–galactosidase has two enzymatic activities: one is responsible for the hydrolysis of lactose and also cleaves cellobiose, cellotriose, cellotetrose and to a certain extent cellulose and the other, splits β–glycosides[5]. Low activity of β–galactosidase causes digestive insufficiency, called lactose intolerance in most cases[6,7]. The symptoms of lactose intolerance such as abdominal pain and diarrhea, nausea, flatulence, and or bloating after the ingestion of lactose or lactose containing food substances which can lead to decrease quality of life, and daily activities. Treatment is relatively simple by eliminating lactose

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from the diet or by using of supplemental β–galactosidase enzyme replacement.[7] The bacterial species currently used by the dairy industry which produced β–galactosidase enzyme belong to genera of Lactobacillus and comprise a limited collection of strains[8, 9].

MATERIALS AND METHODS

Sample Collection

The dairy effluents were collected from the 2010 to 2011 in Varanasi. The samples were brought to the laboratory under aseptic conditions in a sterile container.

Culture media and incubation conditions

For isolation of bacteria, 2 g of cheese and 2 ml of milk was added to 5 ml of MRS broth and incubated anaerobically (24 h at 37°C). Then, 50 ìl of them were spread onto MRS agar. Plates were incubated in anaerobic jars at 37°C for 48 h. To determine β–galactosidase activity, MRS broth without glucose but contained 1% of lactose was used[10].

Identification of isolated bacteria

Bacteria were examined by Gram stain, and identified by standard bacteriological and biochemical methods.[11] Acid production from carbohydrates (glucose, galactose, maltose, mannitol, ribose, sucrose, arabinose, lactose, mannose, raffinose, rhamnose, xylose, sorbitol, salicin) in MRS broth base. The microorganisms were checked whether they belong to lactic acid bacteria or not. Gram staining test, Catalase test. X-gal was used for screening of â-galactosidase activity.

Preparation of diluted inoculums

Pick the isolated colony from plate and grow in 1ml MRS broth and then in 2% modified MRS broth at 37° C for 14hrs. Optical density (OD 600) of inoculum culture was measured before the inoculation into 1 ml modified MRS medium (pH 6.35). By appropriate dilutions turbidity of the inoculum was set up between 0.45 and 0.6 at 600 nm. This solution was labeled as "diluted inoculum". During activation transfers 100 ìl of sample was transferred into 1.5 ml eppendorf tube containing 1 ml fresh MMRS medium.Dilutions of inoculum were carried out by mixing 1 ml cell suspension with 9 ml Peptone water (0.1% g/ml). Blank solution was prepared by mixing 1ml of growth medium with 9 ml peptone water. Absorbance at 600nm was recorded against the blank solution in UV-VIS spectrophotometer.

UV treatment of varying duration on Lactobacillus plantarum

Five plates of MMRS media were prepared in addition to a plate to be used as a control. Each of the plate was sub cultured with Lactobacillus plantarum by spreading 100 µl of diluted inoculum. Plates were incubated for 1hr at 37°C. Except a plate to be used as control, rests of the 5 plates were UV treated for varying durations. All the plates except control were exposed to UV light inside the laminar air flow for 3min, 6min, 9 min, 12min and 15min. Then plates were then kept for incubation at 37°C for 24 hrs.

Basic Tests for Identification of Lactobacilli and β-galactosidase

Production Test

The microorganisms were checked whether they belong to lactic acid bacteria or not. Gram staining test, Catalase test. X-gal was used for screening of β–galactosidase activity.

Gram staining test

The microorganisms that are stained by the Gram's Method are commonly classified as Gram-positive or Gram non-negative and appear purple brown under the microscopic examination. Others are referred to as Gram negative and appeared. It is known that lactic acid bacteria are gram-positive bacteria.

Purification of the Enzyme

After 2 days of incubation period, cell free supernatant was subjected to precipitation by adding chilled acetone 1:1.5%v/v (culture broth to acetone) at 20°C, stirred well and the mixture was kept at 4°C for 12hrs. The precipitate was recovered by centrifugation at 8,850×g for 30 min and dissolved in a minimal volume of 50mM acetate buffer, pH 5.0 and was dialyzed overnight against 5mM acetate buffer of pH 5.0.

Study of β–galactosidase production

X-gal substrate

One colony of isolated bacteria were grown on MRS agar plates containing 60 µl X-gal (5-bromo-4-chloro-3-indolyl-β–D-galacto-pyranoside) and 10 µl of IPTG (iso- propyl-thio-β–D-galactopyranoside) solution as an inducer. Plates were incubated at 37°C for 24 h to 3 days. Colonies producing β–galactosidase were green[10].

Catalase test

One ml 3% H_2O_2 solution was poured over the surface of agar culture, to test whether they form gas bubbles or not. No free oxygen bubble indicates the absence of catalase negative.

Enzyme Assay

The β–galactosidase activity was measured by the method of Gumgumjee and Danial.[14] The enzyme was assayed by using ONPG as a substrate prepared by dissolving 2.5 mg/ml of ONPG in 0.1M sodium acetate buffer (pH-5). 0.2 ml of the culture filtrate was added to 1 ml of the substrate solution and incubated at the temperature of 55°C for 20 min. The reaction was then stopped by adding 1 ml of 10% sodium carbonate. The absorbance was read at 420 nm One unit of enzyme activity was defined as the amount of enzyme that liberates 1 µM of ONP per min at the temperature of 55°C. The amount of protein was estimated by Bradford method using Bovine Serum Albumin (BSA) as a standard according to the instruction manual of Bradford Protein Assay[12].

ONPG substrate

All bacteria were inoculated into tubes containing ONPG (O-nitrophenyl-β–D-galactopyranoside) (0.5 ml) and 0.01 M sodium phosphate buffer (pH 7.0) (5 ml) and peptone water. Production of yellow color was indicated positive ONPG results[13].

Value of β–galactosidase

This test was performed according to the method of Miller (1998)[13, 10]. Briefly, all bacteria were harvested in MRS broth. After centrifugation at 12000 × g for 5 min. at 5°C.

Electrophoretic Analysis

Protein homogeneity of purified β–galactosidase from the isolate was assessed by 10% SDS-PAGE.[15] The proteins were stained with 0.25% (w/v) Commassie Brilliant Blue R-250. Medium molecular weight (Sigma Chemicals Ltd, USA) protein markers were used.

RESULTS AND DISCUSSION

Identification of isolated lactobacillus strains

One *Lactobacillus* strains was isolated from 5 samples. All bacteria grow at the maximum rate at 25°C and weakly growth was detected at 40°C.

Biochemical Test

Most microorganisms that grow aerobically posses the enzyme catalase. The lactic acid bacteria do not normally produce a detectable amount of catalase. The isolated strains were checked for the production of the enzyme catalase. No free oxygen bubble indicates the absence of catalase negative (Fig.1).

β–galactosidase screening with X-gal, ONPG

In this experiment, lactose was used as a main carbohydrate source by the bacteria during the growth. Throughout this study, a unit activity was defined as the amount of the enzyme required to release one µmol of o-nitrophenol in one minute under the assay conditions. In this study ONPG and X-gal were used as substrate for detecting β–galactosidase activity. X-gal was used to test the stains β–galactosidase activity. Greenish blue color was accepted as positive β–galactosidase activity. It was observed that the cultures, which were incubated in modified MRS, could still produce β–galactosidase when they were transferred on the MRS agar. Some of them produced dark green colonies after 24 h incubation (rapid enzymatic activity) and others had delay enzymatic activity after 2 - 4 days of incubation. All bacteria had positive ONPG results (production of yellow color). In ONPG method, values of β galactosidase enzyme were the range of 119.3 to 1,499 (U/ml) in milk. In this study, β–galactosidase production was measured after U.V treatment. Highest value of β–galactosidase enzyme was detected in

Fig. 1. Gram (+ve), Rod shaped bacteria showing Catalase (-ve), no oxygen bubbles evolved

Lactobacillus treated with 15 minutes UV radiation (Fig. 2). Lactobacillus plantarum was selected because they have probiotic activity such as improved digestion of lactose by releasing β–galactosidase into the environment.[10]

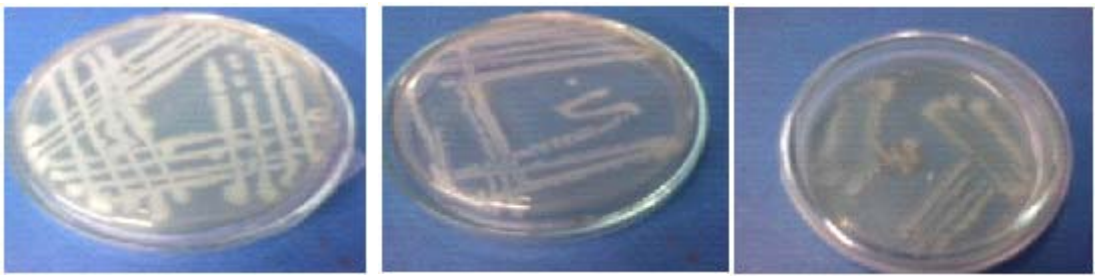
Effect of pH on β–galactosidase activity

pH influences the velocity of an enzyme-catalyzed reaction. Therefore it is important to know effect of pH on enzyme activity. The optimum pH differs when the substrate of the enzyme changes. Therefore if the enzyme is used in milk system for lactose hydrolization, the optimum pH should be found by using lactose as the main substrate. In this study the pH optimum of β–galactosidase was found by using ONPG as substrate. As Wallenfels and Weil (1972) summarized, early studies on the influence of pH on the enzymatic hydrolysis of ONPG had given data which were fitted to a bell-shaped curve with maximal enzymatic activity between pH 7.2 and 7.4.[16] It can be observed that a bell-shaped curve was formed between pH 6.6 and pH 7.6. The optimum pH of β–galactosidase from Lactobacillus plantarum was found as pH 7.2 under the assay conditions. The maximum activity was calculated as 1.58 µmol /min/ml. It was observed that there was decline in activity at above and below the optimum pH. The highest mean value showed at pH 7.0 (.1933±0.01).

Most of the known bacteria can grow on pH 7.6 in this study. However, there may be some that do not and there are probably many others that have not been cultivated at all. Therefore, the numbers given here are likely to underestimate the population size, but comparisons between the pH should be possible and valid. The differences due to pH were significant ($F_{4, 10} = 40.53$; $P < 0.05$) (Fig. 3β–b). Lowest mean value for enzyme activity was estimated for 5.0 pH (0.0633 ± 0.03)

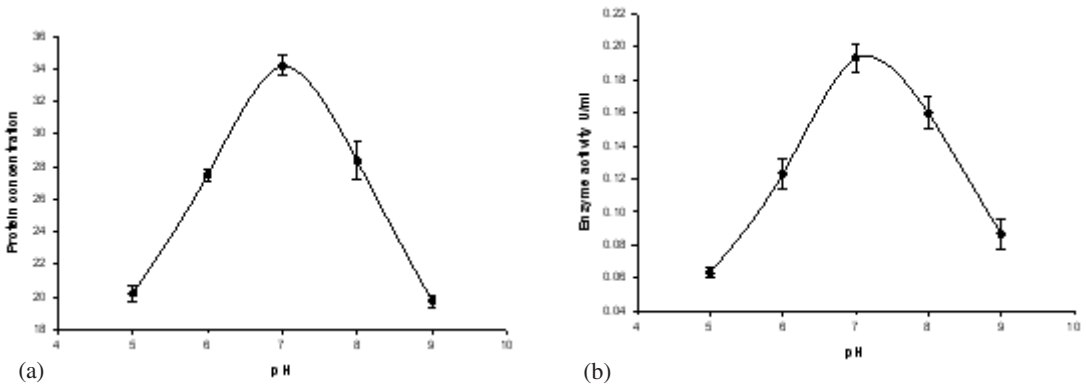
Effect of temperature on β–galactosidase activity

The stability of the purified enzyme was determined between the temperature ranges of 37-42°C where it retained 100% of its activity at the temperature of 45°C and at pH 7 (90%) (Fig. 4). Quyen *et al.*[17] some findings showed that the thermo stability of the enzyme was between the



Control 6 min. UV treatment 15 min. UV treatment

Fig. 2. Colonies of Lactobacillus strains after UV treatment



(a) (b)

Fig. 3(a-b). The effect of pH on β-galactosidase activity

temperature range of 20-37°C when maintained at pH 6.8 -7.0. A slight variation in the thermo stability range was observed at 27-37°C. The maximum activity was calculated as 3.81 ì mol/min/ml at 37°C.

Effect of UV treatment on growth of *Lactobacillus plantarum*

There has been a significant decrease in the growth of *Lactobacillus plantarum* with the increasing durations of UV treatment as compared to the normally grown culture i.e. Control.

Effect of UV treatment on β–galactosidase production

β–galactosidase has catalytic property to hydrolyze lactose into glucose and galactose. In this experiment ONPG was used as substrate, β–galactosidase converts it into ONP. The micromoles of ONP released by the enzyme were determined from the standard curve. Lactobacillus plantarum showed higher production of enzyme on UV treatment by calculating activity of enzyme which was found to be more in 15 minutes UV treated sample as compared to control.

There was a statistically significant difference among treatment as determined by one way analysis of variance of galactosidase production indicates significant difference due to UV treatments at $p<0.05$. A Tukey's post hoc tests of UV treated bacteria at the 15 minutes showed greatest significant with other time intervals. Somewhat 3, 6, 9 minutes UV treatments less significant with control but the difference between 0 minutes UV treatment and control was not significant. The observed production of β–galactosidase in bacteria was consistent with the results of other authors (Fig. 5).

SDS - PAGE of Total Protein Samples

Proteins isolated from all samples are indicated by bands observed in above gel (Fig.6).

SDS PAGE of β–galactosidase

Our results were in accordance with results observed in previous studies (Nichtl et al., 1998). Therefore, the SDS-PAGE method is not suitable for all cases to detect β–galactosidase activity. Since all of the isolated lactobacilli treated with UV that produce high and low or weak β–galac- -tosidase, can be detected by X-gal and ONPG tests, so, biochemical tests, which are rapid, cheap and simple in all laboratory and do not need any expensive molecular materials and apparatus, is recommended. Prominent bands of all samples (control & UV treated) were observed. As the time duration of UV treatment increases, intensity of band increases which shows increase in production of β–galactosidase (Fig. 7).

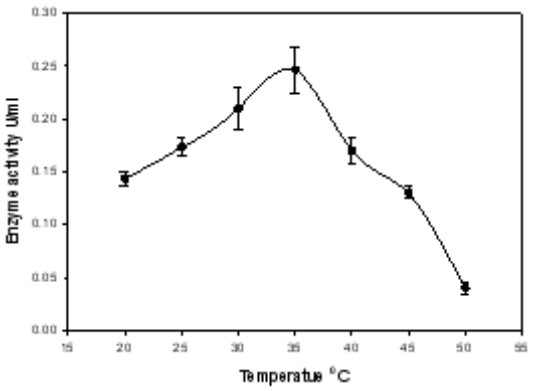
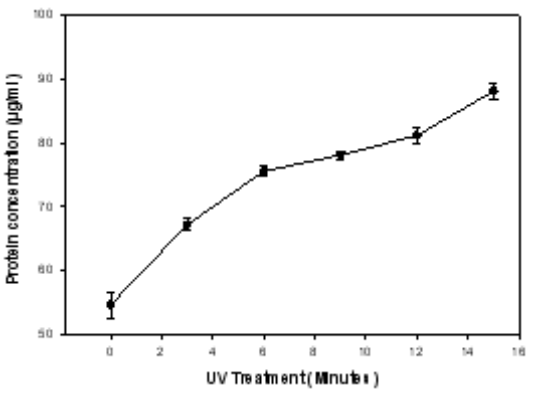
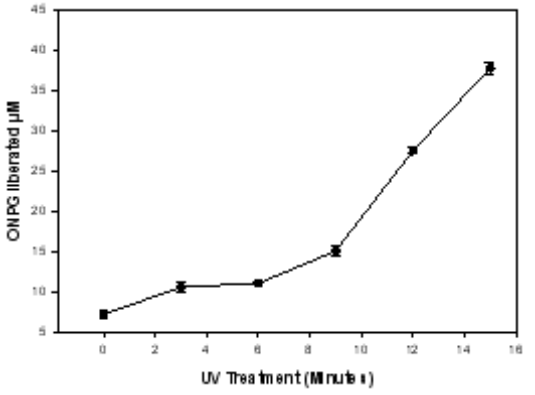


Fig. 4: The effect of temperature on β-galactosidase activity



(a)



(b)

Fig. 5 (a-b). The effect of UV treatment on β-galactosidase production

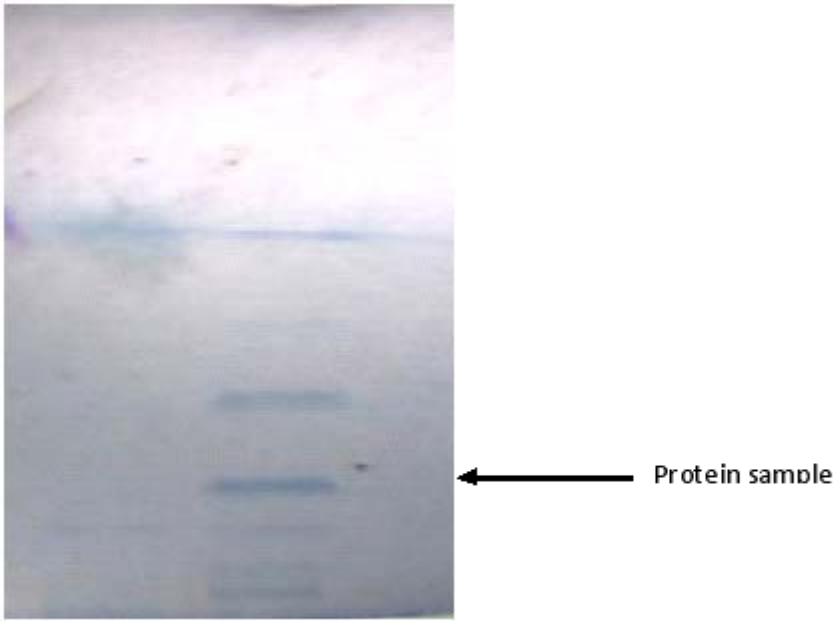


Fig. 6. Protein bands of Lactobacillus strains in SDS-PAGE.

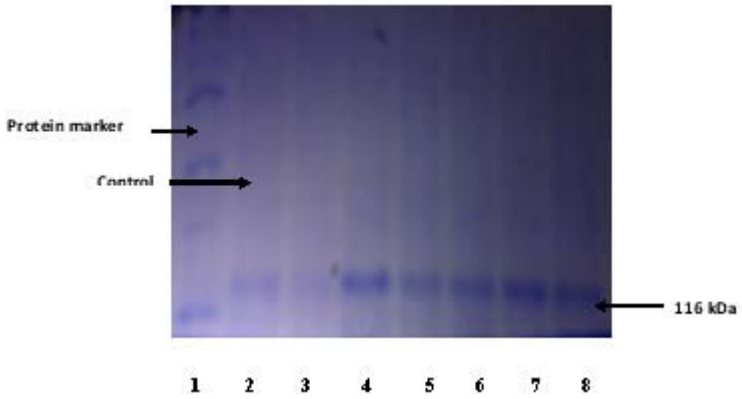


Fig.7: Photographic representation of SDS-PAGE Gel (Lane 1- Marker protein, Lane 2- Control, Lane 3 to 8 showing β-galactosidase single band after UV treatments of 0, 3, 6, 9, 12, 15 minutes respectively)

CONCLUSION

Lactobacillus plantarum is considered as an important organism for dairy industry. In dairy industry, β–galactosidase enzyme is rather used as a crude extract to prevent high cost of purification. Thus it is important to characterize the enzyme in crude extract of β–galactosidase producers.It was reported that β–galactosidase enzyme is tetrameric enzyme which consists of identical subunits with a molecular weight of 116 kDa. In this study, by using of SDS-PAGE method, an intensive 116 kDa protein band was observed in *Lactobacilli* with high values of β–galactosidase enzyme. Our results were in accordance with results observed in previous studies [18]. Therefore, the SDS-PAGE method is not suitable for all cases to detect β–galactosidase activity. Since all of the isolated lactobacilli that produce high and low or weak

β–galactosidase, can be detected by X-gal and ONPG tests, so, biochemical tests, which are rapid, cheap and simple in all laboratory and do not need any expensive molecular materials and apparatus, is recommended.

The study can be concluded that the UV treatment of 15 minutes to *Lactobacillus plantarum* results in higher enzyme i.e. β–galactosidase production, which is more than in normally grown *Lactobacillus plantarum*. On the other hand UV treatment of 3 minutes also exhibits high enzyme activity but is less than UV 15 minutes treated sample. Optimum temperatures, pH of β–galactosidase were found to be 37°C, 7.2 under assay conditions respectively.

Finally, it has been concluded that the UV treatment was able to induce some mutations which resulted in increased β–galactosidase production. The most effective UV treatment was proved to be of 15 minutes as it showed highest activity of enzyme.

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