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IDENTIFICATION OF *BETA-GALACTOSIDASE* PRODUCING BACTERIA FROM SOIL SAMPLES AND ENZYME OPTIMIZATION

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Abstract
β Galactosidase are hydrolases that can be found in microorganisms like fungi, bacteria, and yeasts, as well as plants, animal cells, and recombinant sources. The enzyme is used for two purposes: eliminating lactose from milk products for lactose intolerant persons and creating galactosylated goods. This study aimed to isolate and optimize beta-galactosidase-producing microbe from a soil sample collected near a dairy farm. For screening X-gal (5-bromo-4-chloro-3-indoyl-β-d-galactopyranoside), a chromogenic substrate essential as an indicator of glycosidase activity by giving it a blue colour, is used. Maximum production of enzyme was obtained with pHat7 and temperature at37ºC.Other factors of maximum production were observed in sucrose, ammonium sulphate, magnesiumsulphate and wheat flour. In enzyme assay ONPG (Ortho-Nitrophenyl-β-galactoside) was used as substrate. These results reveal a *Lactobacillus* spp. producing β-galactosidase obtained from soil samples with favorable characteristics has a vital role in foodindustries.

Introduction
Beta-galactosidase is a glycoside hydrolase enzyme, commonly known as lactase. This enzyme is responsible for engendering the hydrolysis of β-galactosidase by breaking the glycosidic bond in the presence of water, breaking it into simple monosaccharides; galactose and alcohol. Being an active group of enzymes, beta-galactosidases can separate the residues of β linked galactose from various compounds, so that lactose is sundered into galactose and glucose. The lactose-hydrolyzing enzyme, β-galactosidase is an enzyme that hydrolyzes lactose and thus has been recognized as a fundamental enzyme for the dairy industry. One of the first hydrolyses to be discovered was β-galactosidase (Husain, 2010). β-galactosidase is an extremely essential enzyme which completely digests milk by breaking lactose, a milk-sweetening sugar. This type of enzyme majorly emerges in microorganisms (Burn, 2012), animal organs and plants such as almonds, apples, peaches, and apricots. Apart from its hydrolyzing role, it is used to produce food products containing a lesser amount of lactose for people who are intolerant to lactose. It's also crucial for the utilization of an environmental pollutant cheese whey (Gandhi *et al*., 2018),
Consumption of dairy products by lactose intolerant people is made possible by reducing the

content of lactose in such foods through its enzymatic hydrolysis by galactosidase which is one of the most prominent technologies for the production of lactose-reduced milk. β-Galactosidase is entrenched in food processing operations as it's used to determine lactose in biological fluids. β-Galactosidase has the capability to improve sweetness, solubility, flavor and digestibility of dairy products and thus it is extensively used in food industries (Citron *et al*., 2015). The enzyme also plays a crucial role as a reporter enzyme for monitoring gene activation and transcription. β-Galactosidase also has good characteristics when conjugated to antibody molecules or streptavidin for use in ELISA systems. The various sources of β-galactosidase include microorganisms such as fungi, bacteria and yeasts; animal and plants cells (Bhowal*et al*., 2018).

Materials and Methods

Collection of sample

The organism was isolated from soil sample collected near dairy farm in a sterile polythene bag and precautions were taken to minimize the contamination. Sample used in dilution was 1g in 100ml distilled water (Usman *et al*., 2023).

Morphological identification of bacteria

The structural and functional attributes of bacteria were examined under microscope. (Hajra*et al*., 2023).

Assay of beta-galactosidase

For enzyme assay Ortho-Nitrophenyl-β-galactoside (ONPG) is used as substrate to convert enzyme into product.

Extracellular enzymeactivity

The extracellular beta galactosidase activity was measured by adding 0.5 mM ONPG as substrate prepared by dissolving 7.5 mg/5ml injection water

Intracellular enzymeactivity

The intracellular beta-galactosidase activity was measured by taking pellet and adding chloroform 500µl. Vortex mixer is used for 15 min to suspend the cells. Afterwards it is centrifuged for 10 min at 6000rpm.

OPTIMIZATION OF CULTURE CONDITIONS FORBETA-GALACTOSIDASE

Effect of time course on cell growth and lactaseproduction

The effects of time course on the production of the enzyme was studied. The cell growth at each time interval was monitored by using absorbance at 420 nm on spectrometer (Kumari*et al.*, 2011).

Effect ofpH

Effect of initial pH was studied on lactase production by varying the pH of culture medium the pH of the medium was adjusted to 2 - 4 by HCl and 7 – 13 by NaOH.

Effect oftemperature

Effect of temperature on lactase production and cell growth was studied by incubating 5ml of culture medium at various temperatures. A temperature range of 30°C to 60°C was used (Raazia*et al*., 2023).

Effect of Various CarbonSources

The isolate was grown in medium containing various carbon sources (1% m/v) including glucose, lactose, maltose, sucrose, dextrose and ribose on the production of lactase at every specific interval to study their effect on enzyme production. The carbon sources were taken and added 0.5% in 5ml Lb broth. The media was autoclaved and inoculum of pure culture was placed (Akcan, 2011).

Effect of Various NitrogenSources

The production medium was supplemented with different nitrogen sources (1% m/v) including inorganic nitrogen sources such as urea, sodium nitrate, ammonium nitrate and ammonium sulphate to investigate their effect on enzyme production (Rosenberg, 2006).

Effect of Various MetalIons

The effect of metal ions such as $MgSO_4$, K_2SO_4 and $FeSO_4$ added to the culture medium on enzyme production was determined (El-Shebawy*et al.*, 2007).

Effect of NaturalSubstrates

Natural substrates such as corn flour and wheat flour were studied at 1% m/v and the medium was optimized for the maximum production of the enzyme (Irkin*et al.*, 2008).

Results

Isolation of bacteria was done through lactose agar media that produce gram positive bacteria. And different tests like gram staining, endospore test and catalase test were performed. Lactase producing bacteria were identified with blue colonies on X-gal. Soil sample near dairy farm was spread on lactose agar plates supplemented with X-gal. 137 colonies showed blue coloration (beta-galactosidase activity) having round, opaque, entire margin colonies (table 1). A single colony was picked and re-streaked on lactose agar plates for thrice. Pure colony was further used for identification and characterization of beta-galactosidase.

Beta-galactosidase producing colony were found to be gram positive rods under microscope (Table 2). The bacteria were found to be non-motile, non-endospore former and showed no catalase activity.

Characterization of beta-gal activity

The optimization of beta gal activity was checked by blue colony.

Enzyme activity units

1 unit (U) is the amount of enzyme that catalyzes the reaction of 1 nmol of substrate per minute. So enzyme activity is quoted in units per ml (U/ml), in other words nmol per min per ml. Activity of enzyme is calculated by using following formula.

$$\text{Enzyme activity U/ml} = \frac{\text{OD at 420nm}}{\text{Incubation time} \times \text{Volume per ml}}$$

Figure 1: Streaking of beta-galactosidase producing colony (blue coloration) on lactose agar medium supplemented with x-gal.

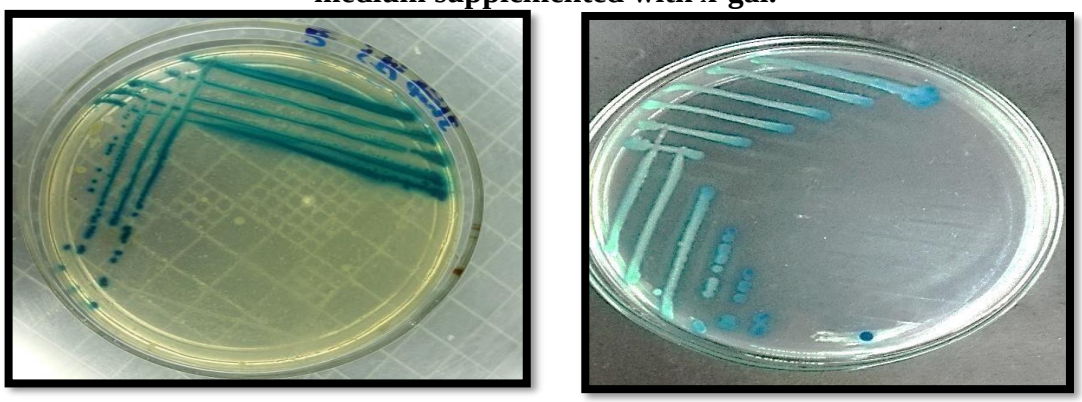


Table 1 Colony morphology characteristics of bacterial isolates from soil on lactose agar medium.

Isolate (soil samples) No.	Color	No. of Colonies of lactose agar with X-gal	Shape of colonies	Size of colonizes	Opacity	Margins of colonies	Eleva-Tion of colonies
1	Blue	137	Round	Small	Opaque	entire	raised
2	White	200	Round	Small	Opaque	entire	raised
3	Creamy	68	Filamentous	Small	transparent	undulated	flat
4	Off white	25	Irregular	Small	translucent	undulated	flat
5	DarkBlue	30	Round	Large	Opaque	entire	convex

These colonies were observed on three different dilution of soil sample as 10^4, 10^5 and 10^7. The number of colony were counted on colony counter. The colony morphology chart is compared with colonies on lactose agar medium after spreading

Figure 2: Gram staining of *beta-galactosidase*

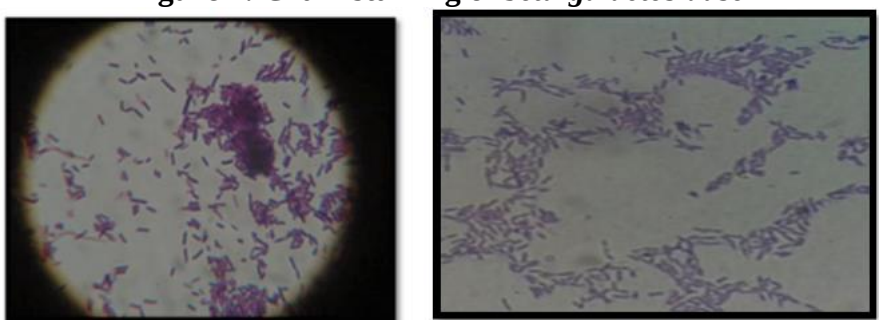


Table 2: Morphological identification of pure colony of microbe producing beta-galactosidase

Identification	*Lactobacillus* spp
Color	Blue
Shape	Round
Size	Medium
Opacity	Opaque
Margins	Entire
Elevation	Raised
Gram staining	Gram +ve, rod shape
Motility test	Non-motile
Endospore test	Non-endospore forming
Catalase test	Negative

Colony morphology and morphological test identified *Lactobacillus* spp. these are gram positive, rod shape bacteria. These lactic acid bacteria are found in dairy products, animal guts andsoil

Table 3: Effect of different pH on extracellular and intracellular cell growth at 420nm and beta-galactosidase activity U/ml

pH	OD at 420nm (extracellular)	Extracellular enzyme activity (U/ml)	OD at 420nm (intracellular)	Intracellular enzyme activity (U/ml)
13	0.106	0.054	0.119	0.079
11	0.181	0.363	0.154	0.102
9	0.296	0.197	0.177	0.078
7	0.893	0.595	0.987	0.658
4	0.064	0.042	0.085	0.056
2	0.090	0.060	0.109	0.072

The effect of different pH in the β-galactosidase production was analyzed. B galactosidase production increased concentration up to a range of 7.0 and a decrease in enzyme production with respect to increase in pH was also observed.

Figure 3: Graphical representation of the effect of pH on cell growth at 420nm of extracellular and intracellular and *lactase* enzyme growth after 24 hours at 37°C in shaking incubator and enzyme assay with ONPG as a substrate.

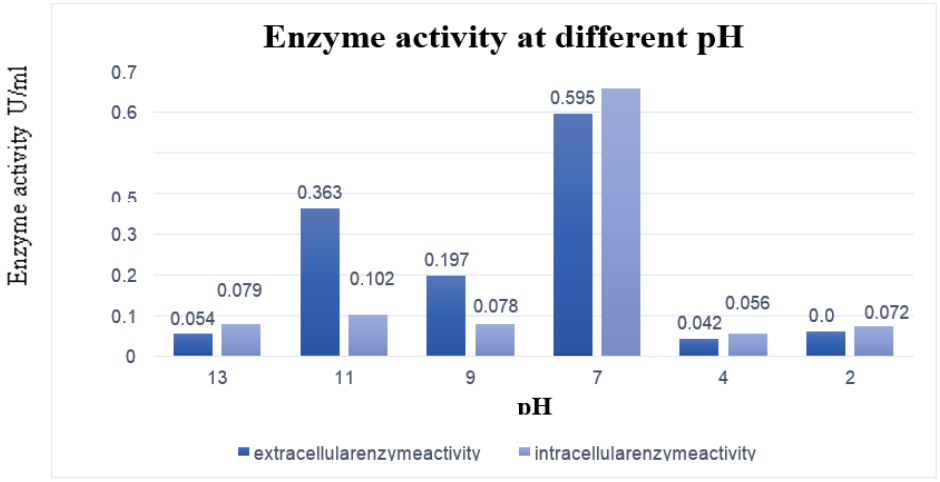


Table 4: Effect of different ranges of temperature on extracellular and intracellular beta-galactosidase activity U/ml

Temperature oC	OD at 420nm (extracellular)	Extracellular enzyme activity (U/ml)	OD at 420nm (intracellular)	Intracellular enzyme activity (U/ml)
30	0.291	0.194	0.378	0.252
37	0.590	0.393	0.890	0.593
40	0.370	0.246	0.390	0.260
45	0.201	0.134	0.281	0.187
50	0.113	0.075	0.267	0.178
55	0.102	0.068	0.234	0.156

This table is showing the effects of different ranges temperature on lactase enzyme activity has been measured in which U/ml and at 37ºC the lactase concentration is maximum.

Figure 4: Graphical representation of effect of different ranges of temperature on cell growth at 420nm of extracellular and intracellular and lactase enzyme growth after 24 hours at 37°C in shaking incubator and enzyme assay with ONPG as a substrate.

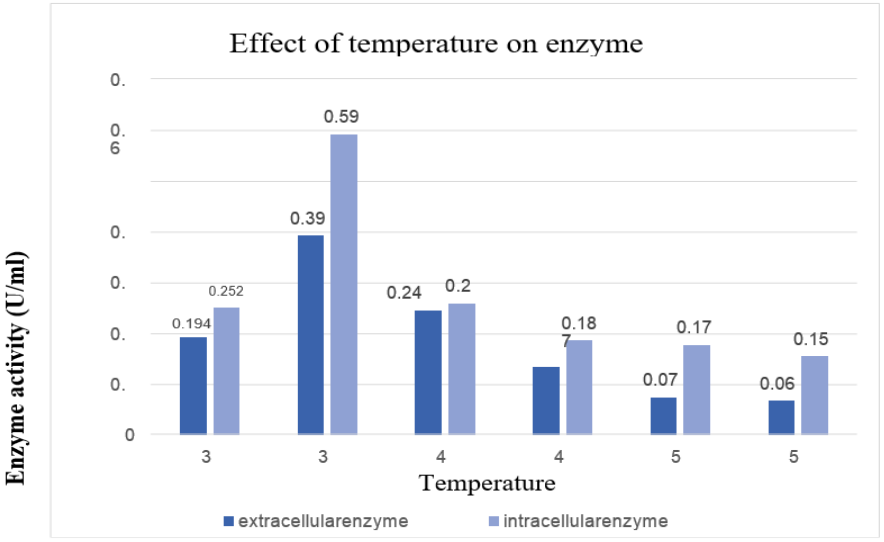


Table 5: Effect of incubation period on extracellular and intracellular on extracellular and intracellular cell growth at 420nm and beta-galactosidase activity U/ml

Incubation period (hours)	OD at 420nm (extracellular)	Extracellular enzyme activity (U/ml)	OD at 420nm (intracellular)	Intracellular enzyme activity (U/ml)
20	0.234	0.158	0.337	0.224
24	0.590	0.393	0.482	0.321
48	0.907	0.604	0.984	0.656
72	0.228	0.151	0.299	0.199

The maximum enzyme activity was obtained at 48th hour of incubation. Beyond this, the enzyme productivity was remains constant and no further increase in production was observed.

Figure 5: Graphical representation of effect of incubation period on cell growth at 420nm of extracellular and intracellular and *lactase* enzyme growth after 24 hours at 37°C in shaking incubator and enzyme assay with ONPG as a substrate.

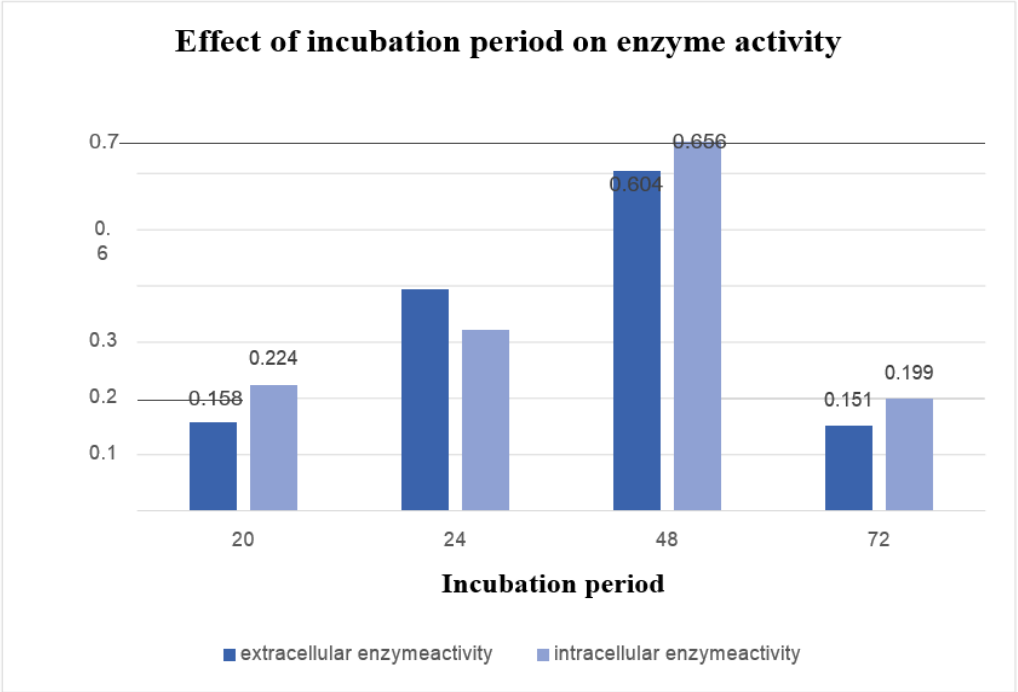


Table 6: Effect of carbon sources on extracellular and intracellular on extracellular and intracellular cell growth at 420nm and beta-galactosidase activity U/ml

Carbon sources	OD at 420nm (extracellular)	Extracellular enzyme activity (U/ml)	OD at 420nm (intracellular)	Intracellular enzyme activity (U/ml)
Sucrose	0.183	0.122	0.538	0.358
Glucose	0.156	0.104	0.347	0.231
Ribose	0.144	0.096	0.290	0.193
Lactose	0.104	0.069	0.159	0.106
Maltose	0.173	0.115	0.321	0.214
Dextrose	0.134	0.089	0.250	0.166

Figure 6: Graphical representation of effect of carbon sources on cell growth at 420nm of extracellular and intracellular and lactase enzyme growth after 24 hours at 37°C in shaking incubator and enzyme assay with ONPG as a substrate.

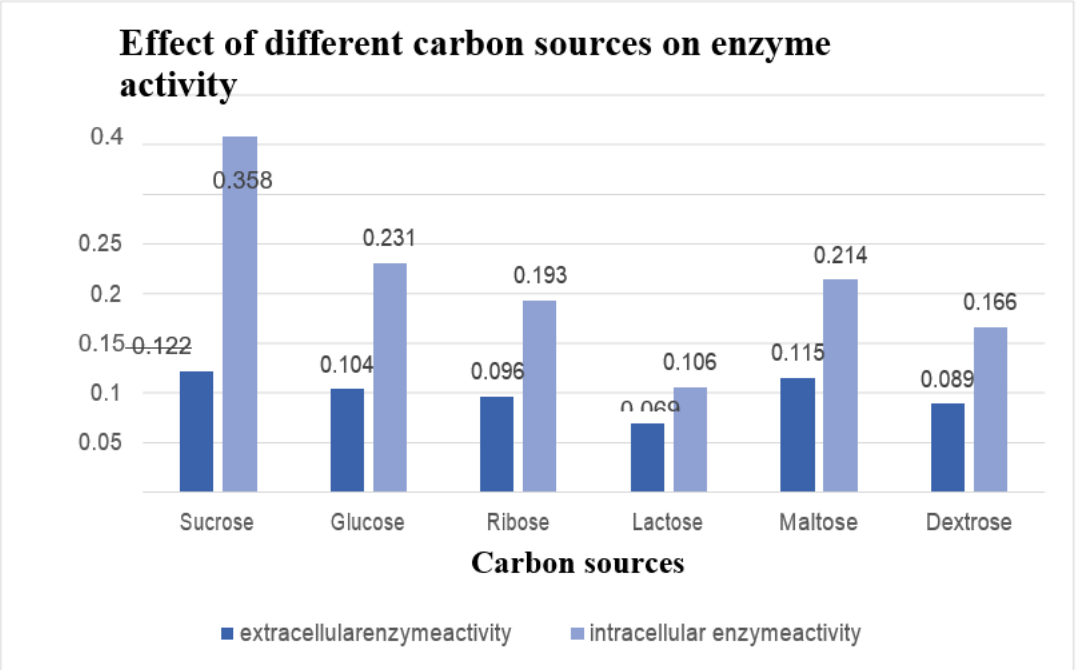


Table 7: Effect of nitrogen sources on extracellular and intracellular on extracellular and intracellular cell growth at 420nm and beta-galactosidase activity U/ml

Nitrogen sources	OD at 420nm (extracellular)	Extracellular enzyme activity (U/ml)	OD at 420nm (intracellular)	Intracellular enzyme activity (U/ml)
NH4NO3	0.850	0.566	0.902	0.601
(NH4)2SO4	0.761	0.507	0.899	0.599
Urea	0.245	0.163	0.450	0.300
NaNO3	0.440	0.293	0.560	0.370

Figure 7: Graphical representation of effect of nitrogen sources on cell growth at 420nm of extracellular and intracellular and lactase enzyme growth after 24 hours at 37°C in shaking incubator and enzyme assay with ONPG as a substrate.

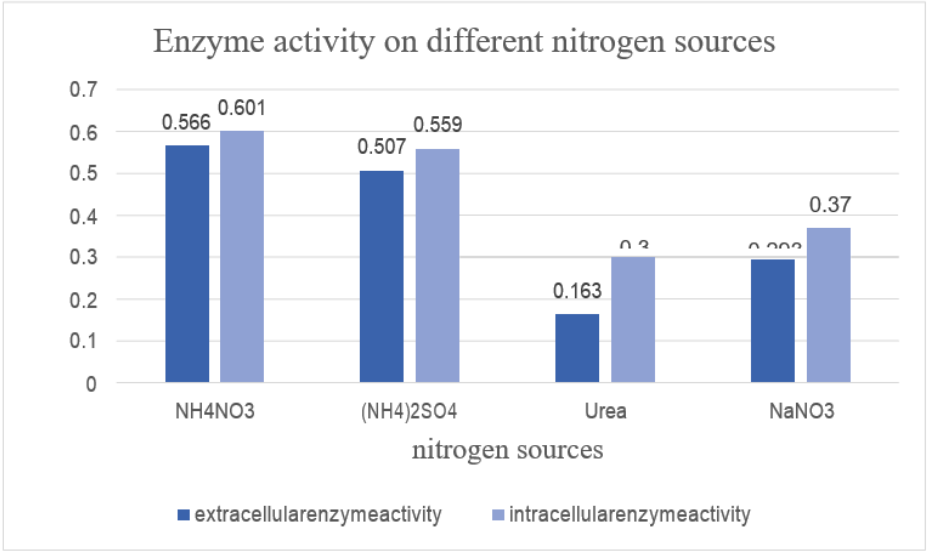


Table 8: Effects of different metal ions on extracellular and intracellular on extracellular and intracellular cell growth at 420nm and beta-galactosidase activity U/ml

Metal ions	OD at 420nm (extracellular)	Extracellular enzyme activity (U/ml)	OD at 420nm (intracellular)	Intracellular enzyme activity (U/ml)
$MgSO_4$	0.628	0.418	1.104	0.736
K_2SO_4	0.480	0.320	0.520	0.346
$FeSO_4$	0.550	0.366	0.72	0.480

The production of β-galactosidase was increased when the production medium was supplemented with $MgSO_4$. This indicated the necessary of $Mg2+$ for the stabilization of the enzyme.

Figure 8: Graphical representation of effect of metal ions on cell growth at 420nm of extracellular and intracellular and lactase enzyme growth after 24 hours at 37°C in shaking incubator and enzyme assay with ONPG as a substrate.

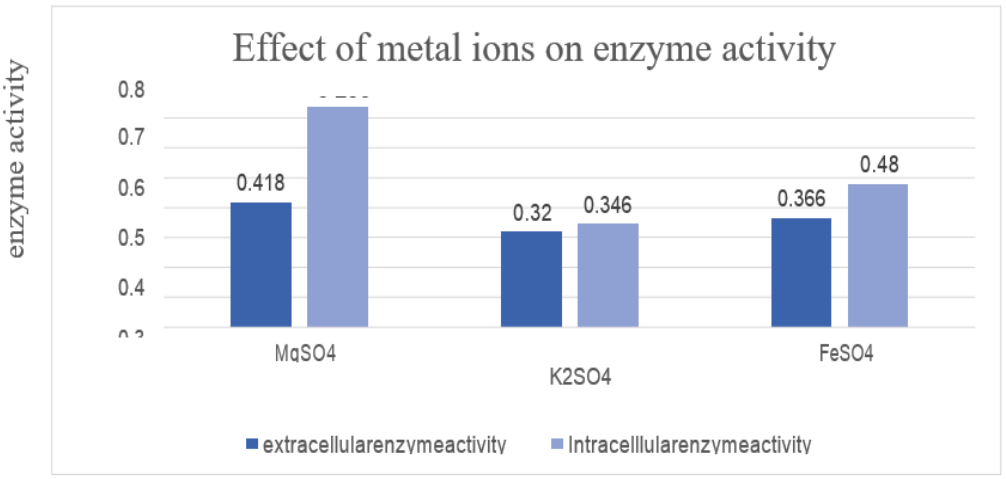


Table 9: Effect of natural sources on extracellular and intracellular on extracellular and intracellular cell growth at 420nm and beta-galactosidase activity U/ml

Natural sources	OD at 420nm (extracellular)	Extracellular enzyme activity (U/ml)	OD at 420nm (intracellular)	Intracellular enzyme activity (U/ml)
Wheat flour	0.680	0.453	1.181	0.787
Corn flour	0.230	0.153	1.042	0.694

Figure 9: Graphical representation of effect of natural sources on cell growth at 420nm of extracellular and intracellular and lactase enzyme growth after 24 hours at 37°C in shaking incubator and enzyme assay with ONPG as a substrate.

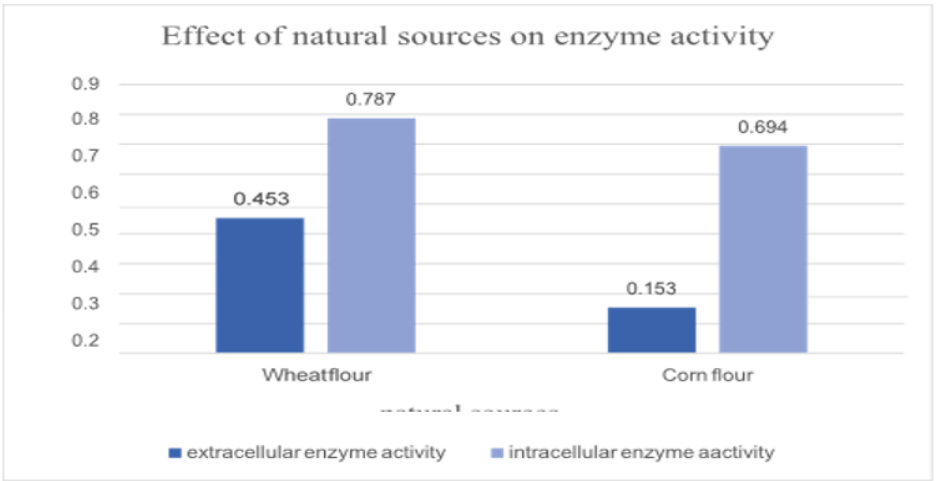


Figure 10: Activity of extracellular beta-galactosidase from 2-13 pH after centrifugation at 6000 rpm for 10min and then incubation for 15min using ONPG as substrate and Na2CO3 as reaction stopper. The yellow color shows the reaction of beta-galactosidase withONPG.

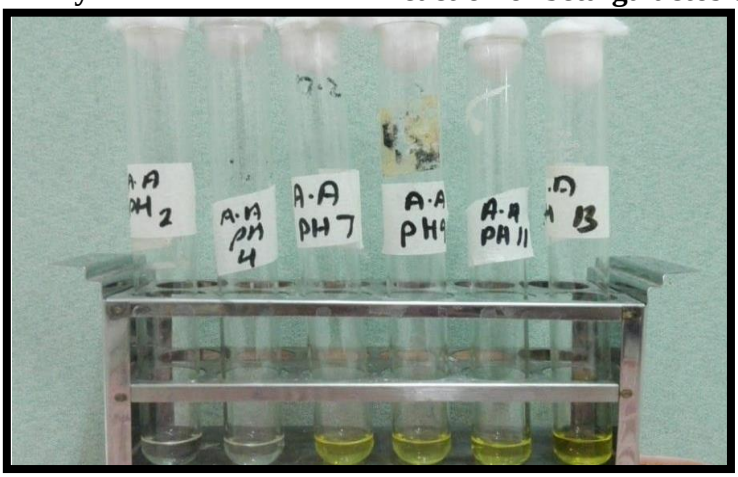
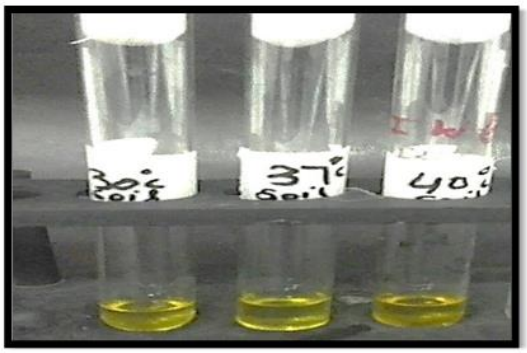
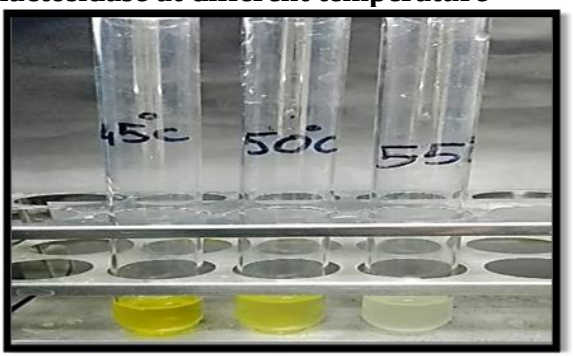


Figure 11: Activity of extracellular beta-galactosidase at different temperature





(a) **(b)**

(a) 30-40°C and (b) 45-55 °C after centrifugation at 6000 rpm for 10min and then incubation for 15min using ONPG as substrate and Na2CO3 as reaction stopper. ONPG reaction with beta-galactosidase is indicated by yellow coloration.

Figure 12: Activity of extracellular beta-galactosidase at different incubation period (20, 24, 48 & 72 hours) after centrifugation at 6000 rpm for 10min and then incubation for 15min using ONPG as substrate and Na2CO3 as reaction stopper. ONPG reaction with beta-galactosidase is indicated by yellow coloration

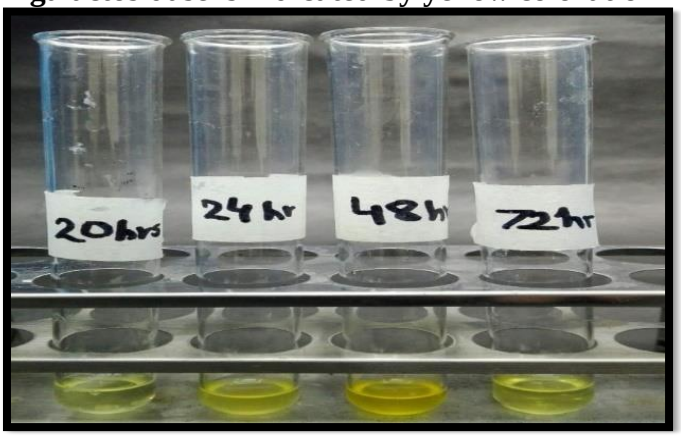


Fig 13: Beta-galactosidase activity at different carbon sources (glucose, ribose, sucrose, maltose dextrose and lactose) after centrifugation at 6000 rpm for 10min and then incubation for 15min using ONPG as substrate and Na2CO3 as reaction stopper. ONPG reaction with beta-galactosidase is indicated by yellow coloration

Figure 14: Activity of extracellular beta-galactosidase at different nitrogen sources after centrifugation at 6000 rpm for 10min and then incubation for 15min using ONPG as substrate and Na2CO3 as reaction stopper. The yellow color shows the reaction of beta-galactosidase with ONPG

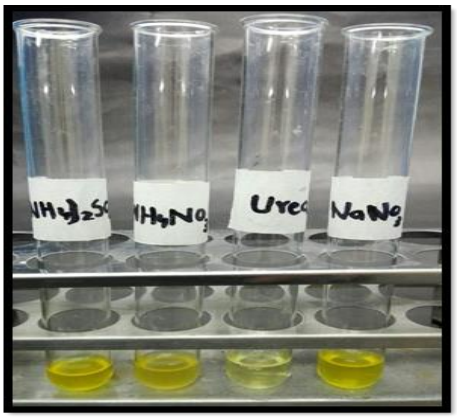
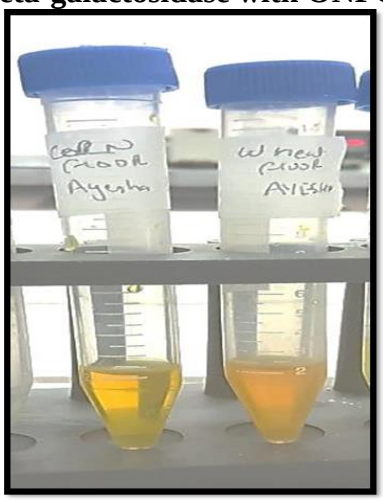


Figure 15: Activity of extracellular beta-galactosidase at different metal ions after centrifugation at 6000 rpm for 10min and then incubation for 15min using ONPG as substrate and Na2CO3 as reaction stopper. The yellow color shows the reaction of beta-galactosidase with ONPG.

Figure 16: Activity of extracellular beta-galactosidase at natural resources corn flour and wheat flour after centrifugation at 6000 rpm for 10min and then incubation for 15min using ONPG as substrate and Na2CO3 as reaction stopper. The yellow color shows the reaction of beta-galactosidase with ONPG.



DISCUSSION

Beta-galactosidasehydrolyzes lactose present in milk or milk products. This enzyme is also known as lactase. Lactose is a disaccharide milk sugar, which is broken down into simple monosaccharide units glucose and galactose by lactase enzyme. Lack of lactase enzyme in body causes a digestive disorder known as lactose intolerance. Beta-galactosidase catalyzes lactose in small intestine of animals and humans. Its presence in small intestine is mainly in fingerlike projection, villus of jejunum. In some cases human infant lacks certain amount of lactase for digestion of lactose, as it is not observed in embryo until last stage of maturation. Its peak activity is after birth. So the humans unable to digest milk or having lactase deficiency develop this condition called lactose intolerance (Sharma and Singh, 2014).The optimal growth conditions of beta-galactosidase in results are temperature at 37°C, pH at 7 and maximum activity at incubation period 48 was observed. These results are in contrary to (Carević*et al*., 2015) the lactic acid bacteria in fermented camel milk made by mixed cells of *S. thermophilus*, *L. acidophilus* and *L. delbrueckii ssp. bulgaricus*had maximum activity at temperature 37.6-40.5°C at the end of 10 hours and optimal pH from 6.5-7.5.

These results also agree with those of who reported that β-gal enzyme activity was found to be higher at pH 6.5 to 7.5 at 37˚C from *B. animalis*, but it appeared to be deletrious effect as enzyme is rapidly loose its activity at lower and higher of this range. Various workers reported that β-gal activity was affected by metallic ions Moreover also reported that the highest enzyme activity was observed in the pH range of 6.7 to 7.5. Lactic acid bacteria consists of heterogeneous group of gram-positive bacteria, whose main fermentation product from carbohydrate is lactate. The group comprises cocci (*streptococcus*, *pediococcus*, *leuconostoc*) and rods (*lactobacillus* and *bifdobacterium*), which are either exclusively (homofermentative) or at least 50% (heterofermentative) lactate producers (Maity*et al*., 2013).

In this study *Lactobacillus* spp was identified to produce beta-galactosidase from soil collected near dairy farm. *Lactobacillus* spp are gram positive, rod shaped, facultative anaerobic, non-motile usually, non-endospore forming, catalase and oxidase negative lactic acid bacteria. This bacterial species are found in dairy products, fermented products, gastro-intestinal tract and soil (Goldstein *et al*.,2015).

Use of substrate Ortho-Nitrophenyl-β-galactoside in enzyme assay was able to detect activity of β-galactosidase. The conversion of substrate into product is observed with this enzyme assay. Maximum activity of beta-galactosidase is observed at glucose, sucrose and ammonium sulfate among carbon and nitrogen sources. Ammonium sulphate and wheat four among metal ions and natural sources.So major function of *Lactobacillus* spp producing beta-galactosidase is break down of milk sugar lactose. And lactose intolerance individuals take different lactase supplements and

artificial lactase products (e.g. milk, cheese, yogurt, ice cream). Consumption of lactose above certain level results in abdominal pain, cramps, diarrhea, nausea, flatulence or gas and watery stools reported by (Swagerty*et al*., 2002). β-galactosidase is an essential enzyme in food and dairy industry because of its tremendous benefits. It is manufactured in dairy industry for the improvement of industrial properties of non-fermented milk products and the development of galacto-oligosaccharide goods. Also extensively used in food industry to boost sweetness, digestibility, solubility, flavor of dairy product, commercial utilization of by product whey, used as prebiotics as it has many beneficial effects on intestinal microflora (Huber,2001).

CONCLUSION

It was concluded that the highest biocatalytic potential was achieved by using *Lactobacillus* spp. Lactose media containing X-gal is the best method of blue-white screening. In view of the greater efficiency of β-galactosidase identification, optimization of the relevant cultivation factors (incubation time, carbon source, nitrogen sources, metal ions and natural sources) was conducted. Maximal enzyme activity was achieved by shake flask culture fermentation after 48 h. Accomplished β- galactosidase activities were significantly increased in continuance, by optimizing at different temperature and pH. Based on the results reported in many papers, β- galactosidase extracted and purified from *Lactobacillus* spp has potential application in the food industry especially in the field of milk and sweet whey hydrolysis, as well as in GOS production.

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