

ISOLATION AND CHARACTERIZATION OF B-GALACTOSIDASE PRODUCING
BACILLUS SUBSTILIS ISOLATED FROM RAW MILKRashika Yousaf¹, Farheen Aslam^{*2}, Hafsa amat ul Rasool³ and Saima Iftikhar⁴^{*1}Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan.²School of Biological Sciences, Quid-e-Azam Campus, University of the Punjab, Lahore, Pakistan.

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ABSTRACT

This research is based on production of beta-galactosidase from bacillus strains of bacteria. Beta-galactosidase is important enzyme that can be produced commercially by using bacterial strains. This enzyme hydrolyze lactose into products glucose and galactose. Beta-galactosidase has various industrial applications like treatment of lactose mal absorption and lactose hydrolyzed milk. In present study, beta-galactosidase was isolated from raw milk source by using repeated streaking method. By using 16S ribosomal sequencing, *Bacillus subtilis* was detected that produce beta-galactosidase enzyme in nature. This bacterium showed best activity at 37°C optimized temperature, optimized time of incubation was found to be 48 hours, optimum pH with best activity was around 6 and 7 and optimum substrate concentration was 1.5% lactose. Beta-galactosidase enzyme was extracted by using different saturation levels of ammonium sulphate and then dialysis was performed. For determining the size of protein, SDS PAGE was performed. Present research gives valuable insight regarding β -galactosidase production by *Bacillus subtilis* present in raw milk.

KEYWORD: β -galactosidase, *Bacillus subtilis*., Lactase intolerance.**INTRODUCTION**

β -galactosidase enzyme is associated with carbohydrate degradation and synthesis. The second name of β -galactosidase is lactase enzyme.^[1] This enzyme is prominently found in many microorganisms, animals and plants. They can be extracted from different bacterial types and fungi. β -galactosidase have many pH ranges for different bacterial and fungal sources.^[2] β -galactosidase from fungal source have pH range from (2.5-5.4), whereas this enzyme has neutral pH for bacterial source.^[3]

Some people do not have capability to produce β -galactosidase enzyme so, so they are unable to digest lactose and become lactose-intolerant. They cannot consume food that has sufficient lactose quantity in them. A great number of oligosaccharides can also be synthesized by β -galactosidase. Currently Transglycosylation activity of beta-galactosidase is very prominent for generating galacto-oligosaccharides (GOS) prebiotics. GOS increase the intestinal bacterial microflora and act as non-digestible prebiotics. They increase the growth of *Lactobacillus* and *Bifidobacterium* species that are very beneficial for human health. The biological significance of oligosaccharides comprise modifying the liver function, anticarcinogenic effect, cholesterol lowering effect through binding the cholesterol in the small intestine. GOS also prevents the gastrointestinal tract from pathogens by acting as trap

receptor.^[4] In prebiotic foods beta-galactosidases are used and high level of GOS make them more efficient to be used.

Recent studies have highlighted new uses for β -galactosidase, such as extracting biologically active oligosaccharides from milk. This enzyme is recognized for its transgalactosylation capability. While lactose hydrolysis is more significant at lower concentrations of lactose, the production of oligosaccharides via transgalactosylation enhances as lactose concentration rises.

Beta-galactosidases have broad applications in industries especially food industry. They are used in the production of beverages, breads, soft drinks and milk products. In present research, β -galactosidase production by *Bacillus subtilis* present in raw milk was characterized and optimized.^[5]

MATERIALS AND METHODS**Isolation of Lactose Producing Bacteria**

Raw milk sample was used to check the abundance of beta-galactosidase enzyme by making different dilutions. The lactose agar media containing Lactose 5g, yeast extract 10g, pH of the medium (7.0) was used and growth of bacterial colonies was observed after 24hrs of incubation at 37°C. Individual colonies from culture plates were picked by a sterilized loop and streaking was

done on new lactose agar media. The pure culture was obtained after repeated streaking method. The morphological characteristics of pure isolate was observed.^[6] Lactose producing bacteria can be seen on lactose agar plates with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) and colonies can be identified with greenish blue colour.^[7]

Determination of β -galactosidase activity

β -galactosidase activity was observed by growing bacteria in LB broth media with 24h of incubation in shaking incubator. After growing bacterial culture extracellular and intracellular activity of enzyme was observed with ortho-nitrophenyl- β -D-galactopyranoside (ONPG) as substrate and Na₂CO₃ as inhibitor in enzyme assay. The color change to yellow showed the activity of enzyme and the O.D was taken at 420nm. One unit of β -galactosidase activity (U) was defined as the amount of enzyme that liberates 1 μ mole ONP per minute under assay conditions. Enzyme activity was calculated.^[8]

Identification of Bacterial Species

The identification of bacteria was done by using 16S rRNA sequencing. Evolutionary analyses were performed using MEGA software. Vasi Bioinformatic analysis was applied on the obtained sequence and 3D structure was predicted.

Enzyme Characterization

Enzyme characterization Agar plates that was prepared by adding X-Gal shows blue colonies. These greenish blue colonies indicate the presence of enzyme beta-galactosidase. This colony is chosen for further enzyme characterization. Enzyme characterization was done by

using different parameters; different time, incubation period, carbon sources, nitrogen sources, pH and metal ions. By using these parameters, we determined the highest enzyme activity.^[9]

Preparation of crude enzyme

The precipitation of crude β -galactosidase was performed using varying concentrations of ammonium sulfate ((NH₄)₂SO₄), as outlined by Batra *et al.* (2002). Briefly, cells were harvested via centrifugation at 4,000 \times g and resuspended in a final volume of 50 ml. The cell suspensions were then sonicated on ice. The cell extract was then precipitated with ammonium sulfate at 4°C (10–90%). The precipitated protein was resuspended in a defined amount of Z-buffer^[10] and stored at 4°C as a stock enzyme until further use.

SDS-PAGE Analysis

8% Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed to identify the size of beta-galactosidase protein. The size of this protein varies from 110kDa to 120kDa.^[11]

RESULTS

Bacterial isolation and screening

Bacteria that produce beta-galactosidase are present in milk and milk products. In this study bacteria were isolated from raw milk sample by growing them on lactose agar media containing X-Gal as shown in figure 1(A). Due to presence of X-Gal greenish colonies were formed on agar plates. These blue colonies indicated production of beta-galactosidase producing bacteria.

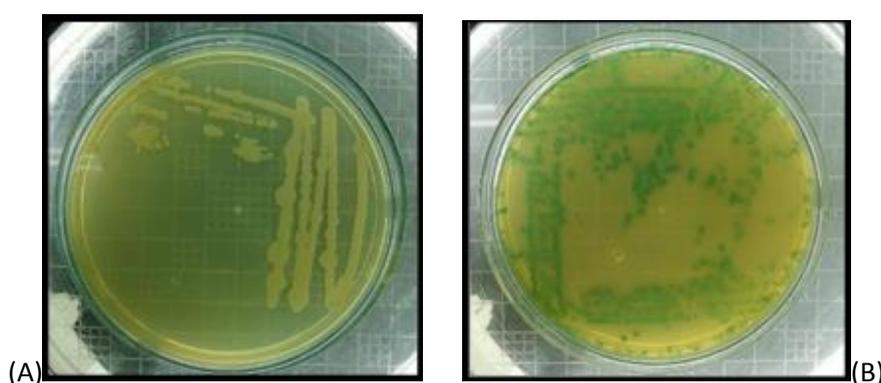


Figure 1: (A) Bacterial colonies produced on lactose agar plate after 24h of incubation. (B) Bacterial pure isolates after 24h of incubation by streaking.

16S rRNA sequencing results

The bacterial sample was sent for 16s Rrna Sequencing which proves that the identified bacteria is *Bacillus subtilis*.

Enzyme characterization

Greenish blue colonies that produce enzyme beta-galactosidase were inoculated in LB broth media and placed for overnight incubation at 37°C. Then after that

it was checked for enzyme production by performing enzyme assay on it by centrifugation, pellet that is intracellular enzyme was separated and ONPG that substrate was added, the enzyme was characterized and beta-galactosidase activity was optimized at different parameters; temperature, pH, nitrogen sources, carbon sources, incubation time and substrate concentration. The O.D was measured at 420nm.

Effect of different time of incubation in β -galactosidase activity

Different time of incubation 20h, 24h, 48h and 72hours was used. Extracellular and intracellular O.D was

measured at 420nm. Enzyme activity was measured by using enzyme activity formula. Extracellular and intracellular enzyme activity values are shown below.

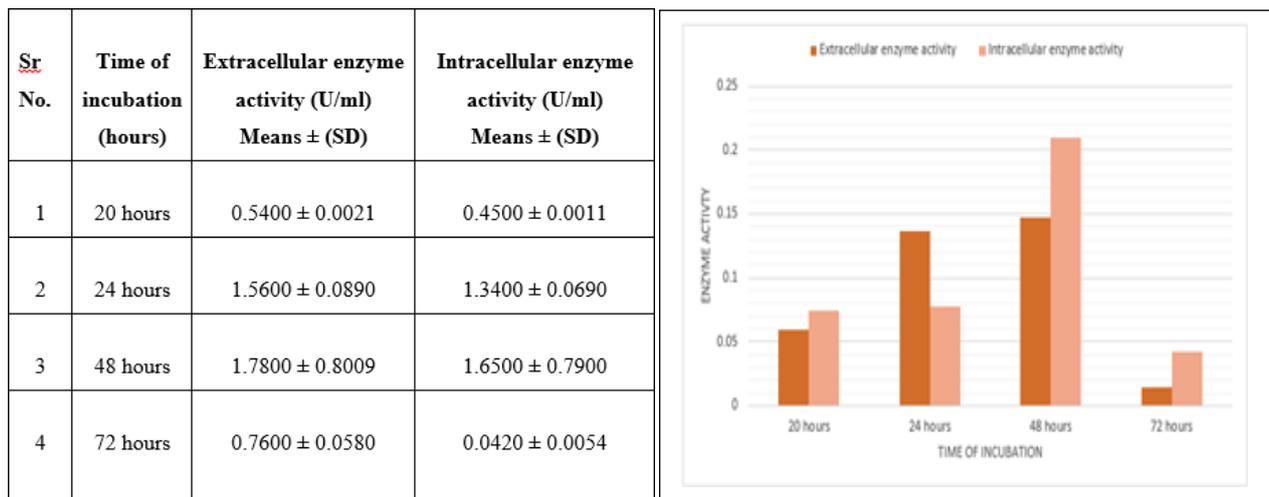


Figure 2: Effect of different time of incubation in β -galactosidase activity.

Effect of different carbon sources in β -galactosidase activity

Different carbon sources was used to measure enzyme activity. Extracellular and intracellular O.D was

measured at 420nm. Enzyme activity was measured by using enzyme activity formula. Extracellular and intracellular enzyme activity values are shown below.

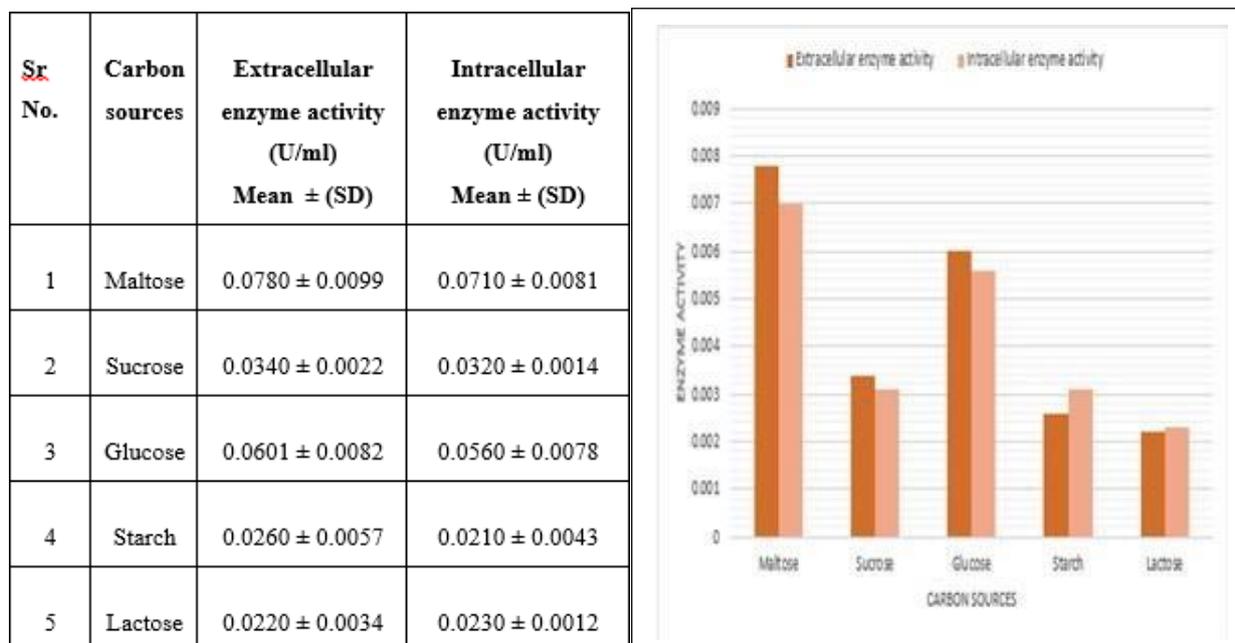


Figure 3: Effect of different carbon sources in β -galactosidase activity.

Effect of different temperature in β -galactosidase activity

Different temperature categories 25°C, 30°C, 37°C and 40°C was measured. Extracellular and intracellular

O.D was measured at 420nm. Enzyme activity was measured by using formula. Extracellular and intracellular enzyme activity values are shown below.

Sr No.	Temperature	Extracellular enzyme activity (U/ml) Mean ± (SD)	Intracellular enzyme activity (U/ml) Mean ± (SD)
1	25 °C	0.0390 ± 0.0023	0.0680 ± 0.0012
2	30 °C	0.0570 ± 0.0040	0.0048 ± 0.0032
3	37 °C	0.5200 ± 0.0560	0.0280 ± 0.0034
4	40 °C	1.0450 ± 0.0670	0.9707 ± 0.0430

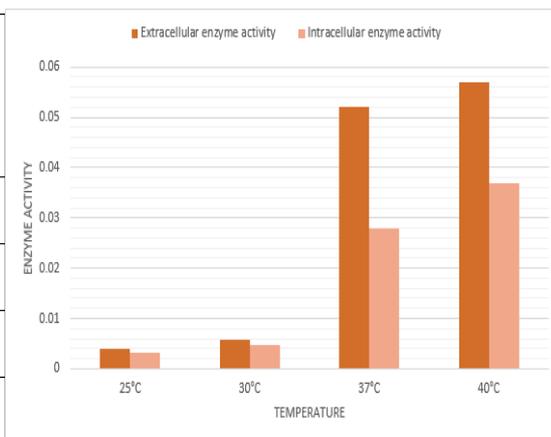


Figure 4: Effect of different temperature in β-galactosidase activity.

Effect of different substrate concentration in β-galactosidase activity

Different substrate concentration was used to measure

enzyme activity. Extracellular and intracellular O.D was measured at 420nm. Enzyme activity was measured by using enzyme activity formula.

Sr No.	Substrate concentration	Extracellular enzyme activity (U/ml) Mean ± (SD)	Intracellular enzyme activity (U/ml) Mean ± (SD)
1	0.5%	0.0330 ± 0.0012	0.0210 ± 0.0010
2	1%	0.0890 ± 0.0015	0.0780 ± 0.0014
3	1.5%	0.2170 ± 0.0170	0.0870 ± 0.0021
4	2%	0.2980 ± 0.0530	0.1902 ± 0.0420
5	2.5%	0.2110 ± 0.0590	0.1310 ± 0.0520

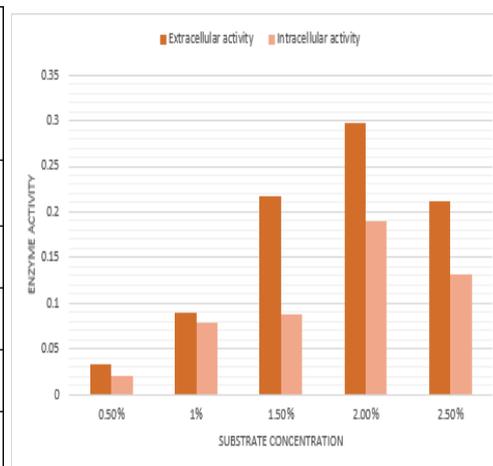


Figure 5. Enzyme activity at different substrate concentration.

Effect of different pH conditions in β-galactosidase activity

Sr No.	pH	Extracellular enzyme activity (U/ml) Mean ± (SD)	Intracellular enzyme activity (U/ml) Mean ± (SD)
1	pH 5	0.0140 ± 0.0032	0.0092 ± 0.0021
2	pH 6	0.0190 ± 0.0024	0.0096 ± 0.0019
3	pH 7	0.0320 ± 0.0041	0.0270 ± 0.0036
4	pH 8	0.0301 ± 0.0034	0.0170 ± 0.0021

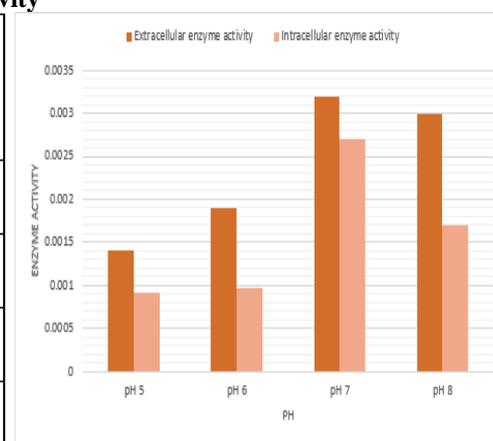


Figure 6: Effect of different pH conditions in β-galactosidase activity.

Effect of different nitrogen sources in β-galactosidase activity

Different nitrogen sources was used to measure enzyme activity. Extracellular and intracellular O.D was

measured at 420nm. Enzyme activity was measured by using enzyme activity formula. Extracellular and intracellular enzyme activity values are shown below.

Sr No.	Nitrogen sources	Extracellular enzyme activity (U/ml) Mean ± (SD)	Intracellular enzyme activity (U/ml) Mean ± (SD)
1	Ammonium sulfate	0.0170 ± 0.0031	0.0180 ± 0.0021
2	Ammonium oxalate	0.0120 ± 0.0019	0.0122 ± 0.0014
3	NaCl	0.0310 ± 0.0052	0.0280 ± 0.0045
4	Peptone	0.0260 ± 0.0079	0.0190 ± 0.0041
5	Beef extract	0.0370 ± 0.0083	0.0200 ± 0.0014
6	Yeast extract	0.0320 ± 0.0076	0.0260 ± 0.0021

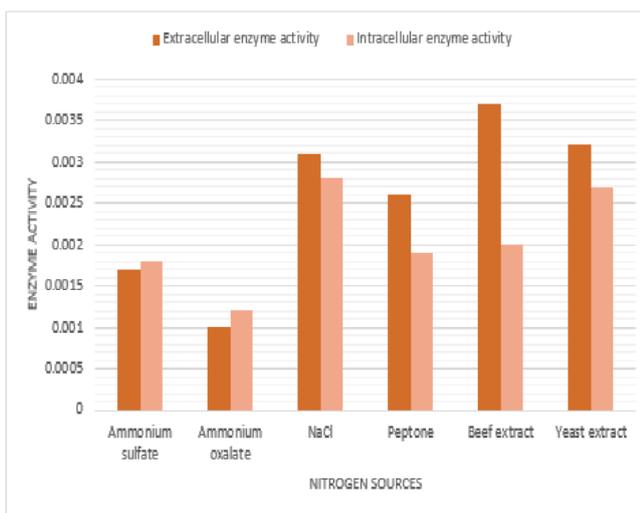


Figure 7: Effect of different nitrogen sources in β-galactosidase activity

Precipitation of crude enzyme β-galactosidase

Beta-galactosidase purification was done by ammonium

sulphate precipitation and dialysis. Enzyme total volume, total activity and specific activity was measured.

Purification	Total volume (ml)	Total activity (U/ml)	Total protein (mg)	Specific activity (U/mg)
Crude enzyme	100	197	30	6.56
Ammonium Sulphate (70%)	50	98.5	15	6.56
Dialysis	40	78.8	12	6.56

Table 1: Representation of total protein content and specific activity of enzyme.

SDS-PAGE result

After performing SDS-PAGE desired protein of enzyme

was separated on gel and its size comes out to be 120kDa that is the size of beta-galactosidase.

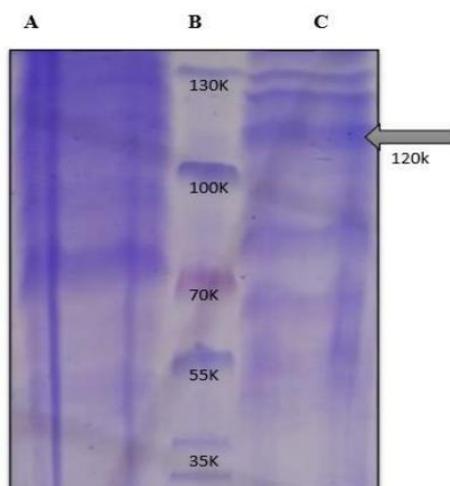


Figure 8: 8% gel image of SDS PAGE shows clear bands of protein that indicates the size of protein about 120kD that is the size of enzyme beta-galactosidase. A indicates the pellet, B indicates the ladder size and C

indicates the supernatant sample. Protein bands appeared in supernatant. This shows the enzyme presence in supernatant.

3D Structure Prediction

Enzyme three dimensional structure predicted by Phyre 2.

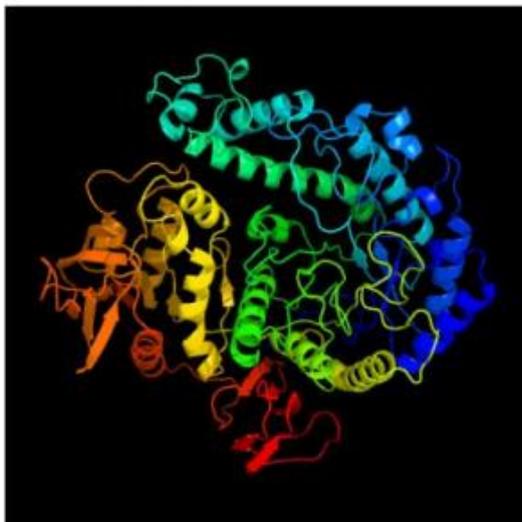


Figure 9: 3D image of beta-gal from phyre2.

This image shows a PDB molecule of beta-galactosidase. 3D structure of the e323a catalytic mutant of ganA gene from bacillus subtilis shows 682 residues (99% of sequence) have been modelled with 100.0% confidence. Detailed structure predicted the percentage of alpha helix and beta strands. Alpha helix are 25% and beta strands are 20% in sequence.

DISCUSSION

For research purpose enzyme beta-galactosidase was isolated from raw milk that is the best source of this enzyme. On lactose agar plate, a pure colony of bacteria that can produce beta-galactosidase was created and cultured for 24 and 48 hours. Moderate growth of bacteria was seen at 24 hours and higher growth was seen at 48 hours of incubation. The bacterial colonies were purified by streaking method. The streaking was done in sterile environment under the laminar air flow. After the streaking method the plates were sealed perfectly to avoid any type of contamination.^[12] After the plate streaking, different morphological tests were done to their appearance and type.

Characterization and partial purification were carried out in this research. During characterization phase beta-galactosidase activity was observed under variety of circumstances including temperature, incubation time, pH, carbon sources, nitrogen sources and metal ions. Beta-galactosidase was characterized by various researchers as well.^[13] assessed the activity of beta-galactosidase by using MRS media with temperature range from 30°C to 45°C. The pH range was 5 to 7. Beta-galactosidase activity was best observed at 37°C and 45°C with incubation period of 12 and 24 hours. The carbon source that promoted enzyme activity was lactose and glucose inhibits the expression of beta-galactosidase.^[14]

The greatest enzyme activity was observed at pH 5 and

temperature 35°C. The best nitrogen source that promote beta-galactosidase activity was yeast extract according to.^[15] Beta-galactosidase activity was also observed by.^[16] Enzyme beta-galactosidase performed its best activity at pH 6.0 and found to be stable at 5.8 to 7.0 pH. In this pH range enzyme showed almost 97% of its activity. This enzyme was active at temperature above 40°C. Enzyme beta-galactosidase shows its best activity at incubation time of 48 hours with temperature 37°C. This enzyme shows its activity at pH 11. The best carbon sources that promotes the activity of beta-galactosidase was lactose and peptone. These sources enhance the production rate and activity of beta-galactosidase according to.^[17] Enzyme beta-galactosidase was purified by SDS-PAGE with size range 116-120kD.

After studying different researches beta-galactosidase activity under different conditions was different and they showed different condition for enzyme activity. In my research enzyme beta-galactosidase that was extracted from raw milk source was shown the maximum activity under different conditions as compare to other researches. The enzyme activity was observed was observed on different parameters which includes carbon sources, nitrogen sources, metal ion sources, time of incubation, pH ranges and temperatures. Enzyme shows best activity with carbon sources maltose with 24 and 48 hours of time of incubation and neutral pH for maximum growth rate. Best nitrogen sources was found to be beef extract and yeast extract enhance the activity of beta-galactosidase. Among metal ions CaCl₂ showed slight increase in beta-galactosidase activity. The best temperature at which enzyme was found to be more active was 37°C.

CONCLUSION

Bacteria are important part of life because they present everywhere but some bacteria are beneficial for life and some are harmful to life. The beta-galactosidase producing

bacteria were isolated from raw milk because it is the major source of beta-galactosidase. Beta-galactosidase can be found in variety of biological entities. Beginning with beta-galactosidase lactose digestion takes place. It breaks down lactose into smaller units; glucose and galactose. In this research, the bacteria which produce beta-galactosidase were isolated and grown on lactose agar plates. Their pure colonies after the streaking method were used for morphological test and characterization. The enzyme activity was observed on different parameters which include nitrogen sources, carbon sources, metal ions, lactose concentration, temperature, time of incubation and different pH ranges. Maltose as carbon source, peptone as nitrogen source, 48 hours of incubation, 37°C temperature and pH 6 to 7 were the conditions that best allowed for observation of intracellular and extracellular activities. The enzyme was purified by ammonium sulphate precipitation and yield was 78 percent.

Statement of conflict

The authors declare that there is no conflict of interests regarding the publication of this article.

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