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Isolation and Identification of ß-Galactosidase Producing Bacterial Strains and Partial Purification of ß-Galactosidase

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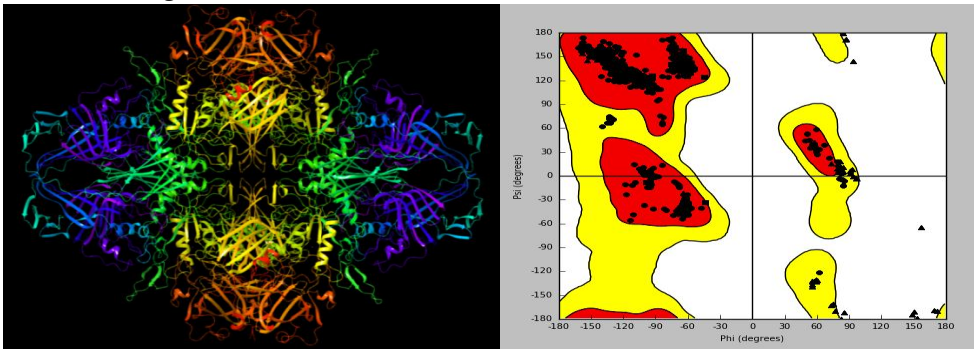
Abstract: ***ß –Galactosidase or Lactase (EC 3.2.1.23) is exoglycosidase which hydrolyzes the ß-glycosidic bond formed between a galactose and its organic moiety, which hydrolyses the ß-1,4-D-galactosidic linkage of lactose, and its related chromogens such as, o-nitro-phenyl-ß-D-galactopyranoside (oNPG), p-nitro-phenyl-ß-D-galactopyranoside (pNPG) and 6-bromo-2-naphthyl-galacto-pyranoside (BNG). ß –Galactosidase is an inducible enzyme and is widely distributed in nature, being found in various types of microorganisms, plant and animal tissues. ß –Galactosidase is also used industrially to obtain the hydrolyzates of lactose from milk and milk whey for utilization in bakery products, ice cream, animal feed and as a sugar source for several fermentation products. This enzyme can be produced by large number of bacteria but Streptococcus thermophilus and Bacillus stearothermophilus are considered as potential bacterial sources. In this study, the bacterial strains producing ß-galactosidase enzyme were isolated, indentified on the basis of morphological, cultural and biochemical characteristics. The 16S rRNA gene sequence of the isolates revealed a close relationship to Bacillus cereus. The activity of ß–Galactosidase was measured in crude extract. Ammonium sulphate precipitation, sample dialysis and inhibition studies were carried out for isolated and partially purified ß –Galactosidase.***

Keywords: ***o-nitro-phenyl-ß-D-galactopyranoside (oNPG), p-nitro-phenyl-ß-D-galactopyranoside (pNPG) and 6-bromo-2-naphthyl-galacto-pyranoside (BNG).***

I. INTRODUCTION

ß-Galactosidase belongs to glycosyl hydrolyses enzyme, which hydrolyzes ß-glycosidic bond formed between a galactose and its organic moiety, as well as chromogens like ortho-nitro-phenyl-β-D-galactopyranoside (oNPG), para-nitrophenyl-β-D-galactopyranoside (pNPG) and 6-bromo-2-naphthyl-galacto-pyranoside (BNG) [1]. ß-Galactosidase catalyzes the reactions with β-D-galatopyranosides with oxygen glycosidic bond [2]. Three enzymatic activities exhibited by ß-Galactosidase, first it cleaves lactose to glucose and galactose, second it converts lactose to allolactose (gratuitous inducer) which binds to lacZ repressors and increases the amount of ß-galactosidase and third the allolactose were cleaved to monosaccharides [3]. ß-Galactosidase is a tetramer of four identical subunits with 1023 amino acids each [4]. 3J7H is the Protein data bank (PDB) structure of *E-coli* ß-galactosidase [5]. The structure 3J7H was downloaded in Maestro 11.2 Schrödinger software and also analysed in Ramachandran plot (Figure 1b) for *E.coli* ß-galactosidase in which most of the torsional angles were in the allowed regions and hence this structure could be used for the purpose of molecular docking.



(A) (B)

Figure 1. (A) Depicting structure and (B) Ramachandran plot for E.coli ß-galactosidase.

ß-galactosidase is industrially used to obtain hydrolyzates of lactose from milk and milk whey for animal feed, dairy products and sugar source for fermentation products. The enzymatic hydrolysis of lactose from milk and milk whey is desirable for lactose-intolerant individuals [6].

A. Reaction Catalysed by β-Galactosidase

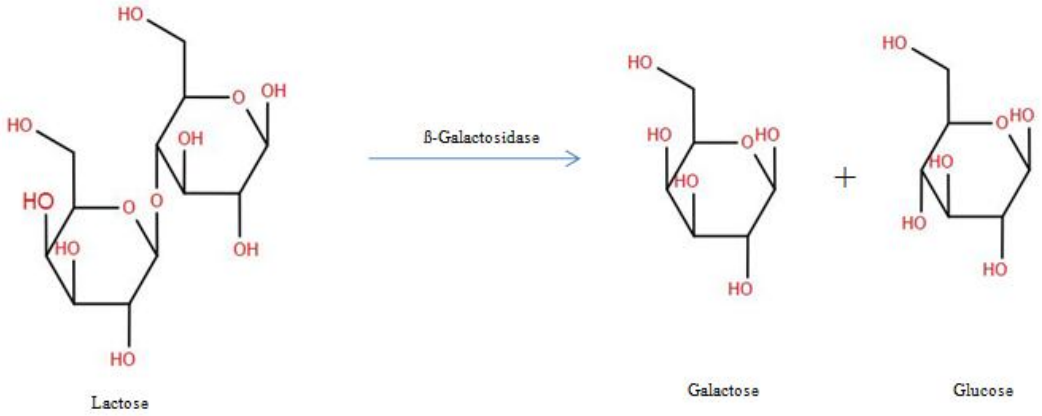


Figure 2. Reaction catalysed by β-galactosidase to form galactose and glucose.

β-galactosidase catalyzes the breakdown of the substrate lactose, a disaccharide sugar found in milk into monosaccharide galactose and glucose. The oxygen bridge connecting the two sides of the lactose molecule is cleaved by the addition of a water molecule.

II. MATERIALS AND METHODS

A. Chemicals

o-nitro-phenyl-β-D-galactopyranoside (oNPG), isopropyl β-D-1-thiogalactopyranoside (IPTG), X-Gal (5-bromo-4-chloro-3-indole-β-galactopyranoside), sodium bicarbonate, Na_2HPO_4 and NaH_2PO_4, bacterial staining reagents, sodium chloride, beef extract, peptone and nutrient agar, ammonium sulphate, bovine serum albumin (BSA), Folin-Ciocalteau reagent (FCR), and all other chemicals used in this study were of analytical grade.

B. Bacterial Cultures and Growth Conditions

The bacteria were isolated from soil by enrichment culture technique, and β-galactosidase expressing bacteria were identified by transferring it to the nutrient agar medium containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (0.01 g) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (0.01 mM). The blue colour expressing bacteria [7] was then used for further studies. Single loop of bacterial culture was inoculated into 50 ml of agar broth in a 250 ml Erlenmeyer flask and incubated at 37 ºC for 48 h, once the isolates were purified, the strains showing high enzyme activity was selected and used for further studies [8].

C. Identification of Bacterial Isolates by 16S rRNA Gene Sequencing

The bacterial 16S rRNA gene was amplified from the total genomic DNA using ITS (Internal transcribed spacer) specific primers 63F (5’ CAG GCC TAA CAC ATG CAA GTC 3’) and 1387 R (5’ GGG CGG AGT GTA CAA GGC 3’) to amplify approximately 600 bp of a consensus 16S rRNA gene. The polymerase chain reaction (PCR) conditions were 35 cycles of 95 °C denaturation for 1 min, annealing at 55 °C for 45 sec and extension at 72 °C for 1 min and in addition one cycle of extension at 72 °C for 10 min.

The PCR product was purified from Qiagen purification kit and was directly sequenced using a Big Dye terminator kit (Applied Bio system) [9], sequence reaction was allowed to run on ABI-PRISM automated DNA sequencer (ABI-3730 DNA analyzer). The nucleotide sequence analysis was carried out in NCBI Source server (www.NCBI.com). The alignment of the sequences was obtained using CLUSTALW program.

The phylogenic tree was constructed using the maximum like hood method using Kimura-2- parameter Model in the MEGA 5.1 software [10]. The sequences of the 16S rRNA gene of the isolate A was 99% similar to *Bacillus* cereus and isolate B was 92% similar to Microbacterium testaceum.

D. Cultural, Morphological and Biochemical Characterization of Bacterial Isolates

The morphological and biochemical characteristics of the isolated bacterial strains were carried out. Cell shape, size, motility, gram staining and endospore staining were carried out as reported in microbiological methods. Biochemical tests like catalase, urease, indole, citrate utilization, gelatin liquefaction, starch hydrolysis, sugar fermentation etc was carried out and depending upon the results obtained bacterial strains were identified.

E. Enzyme Assay

Cell free extracts were obtained by centrifugation of freshly grown cells at 4000xg for 15 min at 4 °C, washed thrice with 50 mM phosphate (pH 6.5) buffer and suspended in 50 ml of buffer. Cell suspensions were sonicated for 5 min in ice bath and then centrifuged at 12000xg for 30 min at 4 °C. The supernatant obtained was used as a crude extract for enzyme assays. β-galactosidase activity was measured according to the method of Miller [11], using the substrate o-nitrophenol-β-D-galactoside (oNPG) which was hydrolyzed by enzyme to form o-nitrophenol and galactose, standard ONP curve was prepared. The enzyme activity was assayed spectrophotometrically at 420 nm, by adding alkali the reaction terminates and develops yellow colour complex. The reaction mixture containing 0.5 ml of 6 mM oNPG in 0.1 M Phosphate buffer, pH 7.0 and 0.5 ml of crude enzyme was incubated for 30 min at 37 °C. The reaction was then stopped by adding 0.5 ml of 1 M Na_2CO_3. The amount of ONP released from oNPG was determined by measuring the absorbance at 420 nm [12]. Protein was determined by the method of Lowry et al. [13], using bovine serum albumin (BSA) as standard. One unit of β-galactosidase activity was defined as the amount of enzyme that liberates 1µmole ONP per minute under standard assay conditions.

F. Ammonium Sulphate Precipitation

The supernatant obtained after sonication and centrifugation at 12000xg for 30 min at 4 °C was treated with solid ammonium sulphate to 30% saturation and precipitated protein was removed by centrifugation. The supernatant was then brought to 70% saturation with ammonium sulphate [14]. The centrifuged precipitate (8000 rpm, 20 min, 4 °C) was redissolved in a small volume of 0.1 M phosphate buffer of pH 7 and then used for further purification.

G. Dialysis

Dialysis was performed overnight for the precipitate obtained from ammonium sulphate precipitation against 0.1 M phosphate buffer (pH 7) solution. Dialysis tubes were previously activated and soaked in 1 M sodium phosphate buffer and dialyzed against the same buffer.

H. Effect of pH

The effect of pH on β-galactosidase activity was studied using three different buffers, 0.05 M citrate buffer (pH 4-6), 0.05 M sodium phosphate buffer (pH 6.5-7.5) and 0.05 M Tris-HCl buffer (pH 8).

I. Effect of temperature

To determine optimum temperature, the β-galactosidase activity of enzyme was measured at different temperatures 30-50 °C.

III. RESULTS

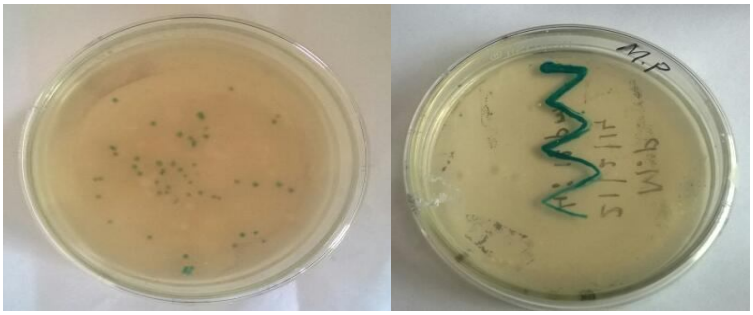
A. Characterization of Organisms

Bacterial consortium which was isolated from soil samples by enrichment culture technique in the absence (control) and presence of X-Gal and IPTG is shown in (Figure 3). The blue colour producing colony was sub cultured and used for further studies. The two different bacterial isolates were characterized by analysing their cultural, morphological and biochemical characterized as shown in (Table 1). Both bacterial isolates were Gram-positive. The optimum pH and temperature of both isolates were 7.0 and 37 ºC respectively as shown in (Figure 6). According to the method of Bergey's Manual of Determinative Bacteriology [15], these both isolates belong to genus Bacillus and Microbactrium sp. The complete sequence of 16S rRNA gene for both isolates were determined and analysis of sequences were done at NCBI, and relevant data bases were downloaded for further analysis [16]. The isolates were further identified by phylogenetic analysis based on 16S rRNA gene sequences as shown in (Figure 5). The isolate A showed 99% similarities with B. cereus and isolate B showed 92% similarities with M. testaceum. Table 2 summarizes the partial purification procedure which was used. These steps were carried out at 0-5 ºC. Isolated bacterial cells were harvested and washed thrice with 50 mM phosphate buffer (pH 6.5), and cells were disrupted by sonication at 4 ºC, and centrifuged at 12000xg for 30 min

at 4 ºC. The supernatant was saturated with solid ammonium sulphate to 70% at 4 ºC and precipitated protein was removed by centrifugation at 8000 rpm for 20 min at 4 ºC and the precipitate was redissolved in a small volume of 0.1 M phosphate buffer (pH 7) and the same was used for further purification and this solution was dialyzed against 0.1 M phosphate buffer (pH 7) solution.

B. Properties of the Enzyme

The partially purified enzyme from *B. cereus* had a specific activity of 5000 µmole/min/mg protein after the dialysis step and partially purified enzyme from *M. testaceum* had a specific activity of 8750 µmole/min/mg protein after dialysis, which was optimally active at pH 7.0 and 37 ºC. The effect of metal ions, chelating agents and sulphydryl agents on the enzyme activity are given in Table 3. Metal ions such as Hg^{2+}, Ag^{+}, Cu^{+} and Fe^{3+} inhibited the beta gal. *o*-Phenanthroline inhibited the enzyme activity. The enzyme was also inactivated by treatment with an oxidizing agent like H_2O_2.



(A) β-galactosidase Positive (B) Subculture

Figure 3. (A) β-galactosidase producing bacteria. (B) Subculture of A.

Table 1. Morphological and biochemical characterization of Bacillus cereus and Microbacterium testaceum.

CHARACTERISTICS	OBSERVATIONS	
	B. cereus	*M. testaceum*
Morphological properties		
Cell shape	Rod	Rod
Gram reaction	+ve	+ve
Motility	+ve	+ve
Endospore	+ve	+ve
Pigment	Colorless	Slight yellow
Biochemical characteristics		
Catalase	+ve	+ve
Urease	-ve	-ve
Citrate utilization	-ve	-ve
Starch hydrolysis	+ve	+ve
Hydrogen sulphide	-ve	-ve
Gelatin liquefaction	+ve	+ve
Indole production	-ve	+ve
Nitrate reduction	+ve	+ve
M R reaction	-ve	-ve
V P reaction	+ve	+ve
Acid produced from		
Glucose	+ve	+ve
Raffinose	-ve	-ve
Mannose	+ve	+ve
Fructose	+ve	+ve
Lactose	+ve	+ve

+ve; present, -ve; absent

C. Identification of Bacterial strains on basis on 16S rRNA Analysis

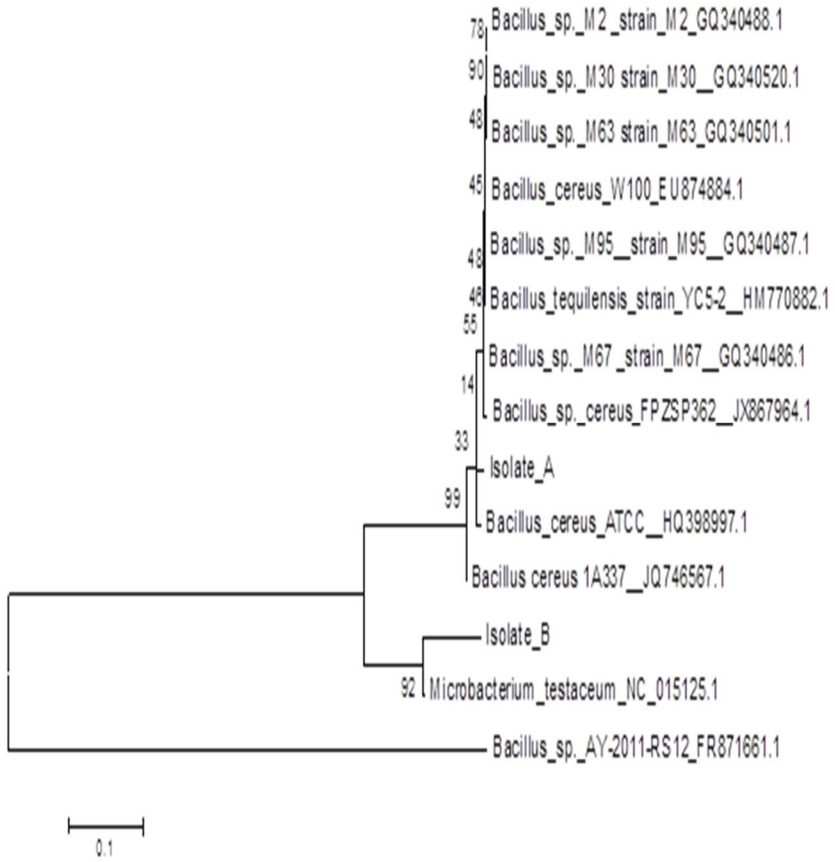
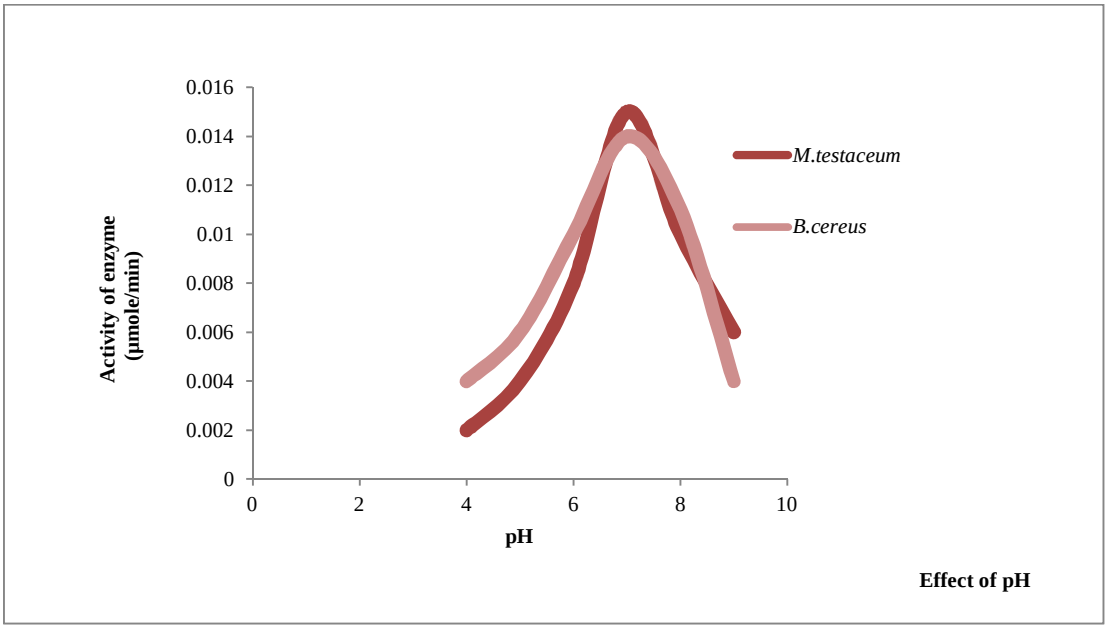


Figure 4. Phylogenic tree describing B. cereus (Isolate A) and M. testaceum (Isolate B)

D. Effect of pH and Temperature

(A)



(B)

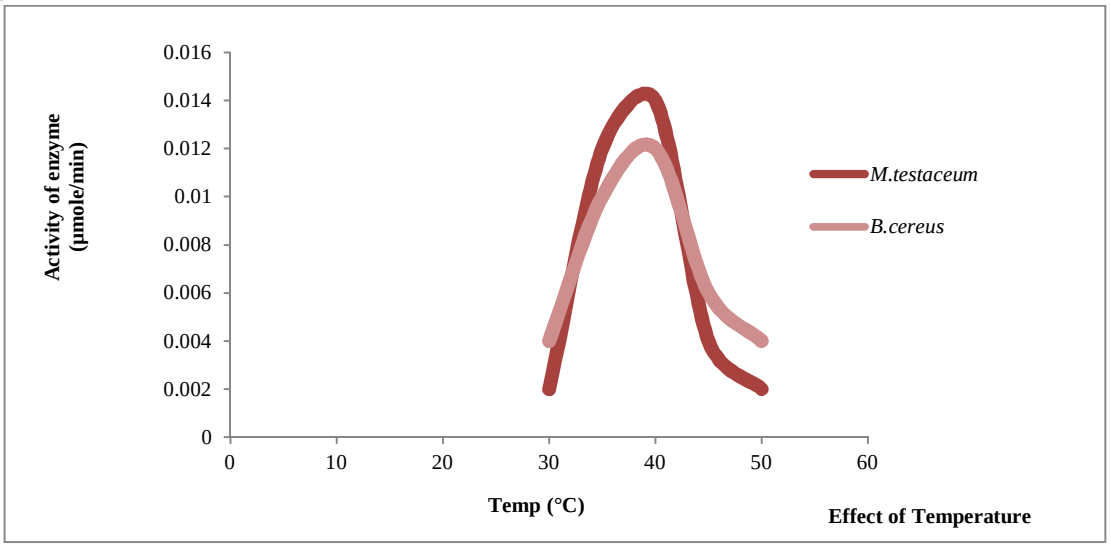


Figure 5. (A) Effect of pH and (B) Effect of temperature of B. cereus and M. testaceum.

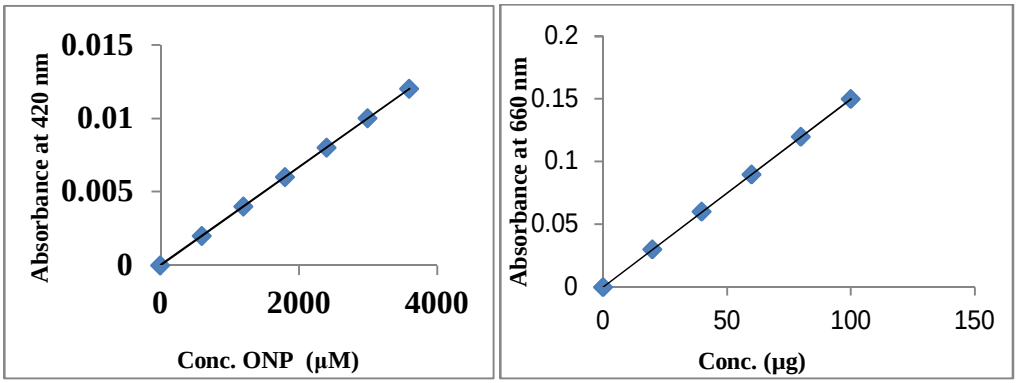


Figure 6. (A) Standard ONP curve and (B) Standard curve for protein.

Table 2. Partial purification of β-galactosidase from Bacillus cereus and Microbacterium testaceum.

	Bacillus cereus						Microbacterium testaceum				
Step	Total Vol (ml)	Total Activity (µmole/ min)	Total Protein (mg)	Specific Activity (µmole/min/mg protein)	Fold purification	Yield (%)	Total Activity (µmole/ min)	Total Protein (mg)	Specific Activity (µmole/min/mg protein)	Fold purification	Yield (%)
Crude extract	15	2925	1.005	2910	-	100	3750	0.78	4807	-	100
$(NH_4)_2SO_4$ 70% fractionation	05	875	0.22	3977	1.366	30	1000	0.16	6250	1.300	26
Dialysis	03	450	0.009	5000	1.257	15	525	0.06	8750	1.4	14

Table 3. Effects of metal ions, chelating agents and sulphydryl agents on β-galactosidase.

INHIBITORS	FINAL CONC. (MM)	INHIBITION (%)
$AgNO_3$	0.1	86
$CuSO_4$	0.1	74
$HgCl_2$	0.1	91
$FeCl_3$	0.1	46
o-Phenanthroline	0.1	88
Glutathione	0.5	0
2-Mercaptoethanol	0.5	0
Dithiothreitol	0.5	0
H_2O_2	0.1	96

IV. CONCLUSION

The 16S rRNA gene sequences of the isolates A and B revealed a close relatedness to Bacillus cereus and Microbacterium testaceum with 99% and 92% similarity. Hence the strains were confirmed as Bacillus cereus (Isolate A) and Microbacterium testaceum (Isolate B). β-galactosidase is an important enzyme in the food and pharmaceutical industry. For example, the removal of lactose from milk for lactose intolerant people and the production of galacto-oligosaccharides for use in probiotic food. It is used in genetics and molecular biology as a marker. Deficiency of β-galactosidase causes Galactosialdosis (Morquiro B syndrome). Attempts were made to increase β-galactosidase production by the addition of the reported β-galactosidase inducer like IPTG to enhance the expression of β-galactosidase gene at the transcription level in the growth media which serves an ideal candidate for hydrolysis of lactose in milk which can be used for lactose intolerant people. Enzyme activity was inhibited by o-Phenanthroline, heavy metal ions and hydrogen peroxide but sulphydryl groups had no effect on enzyme inhibition.

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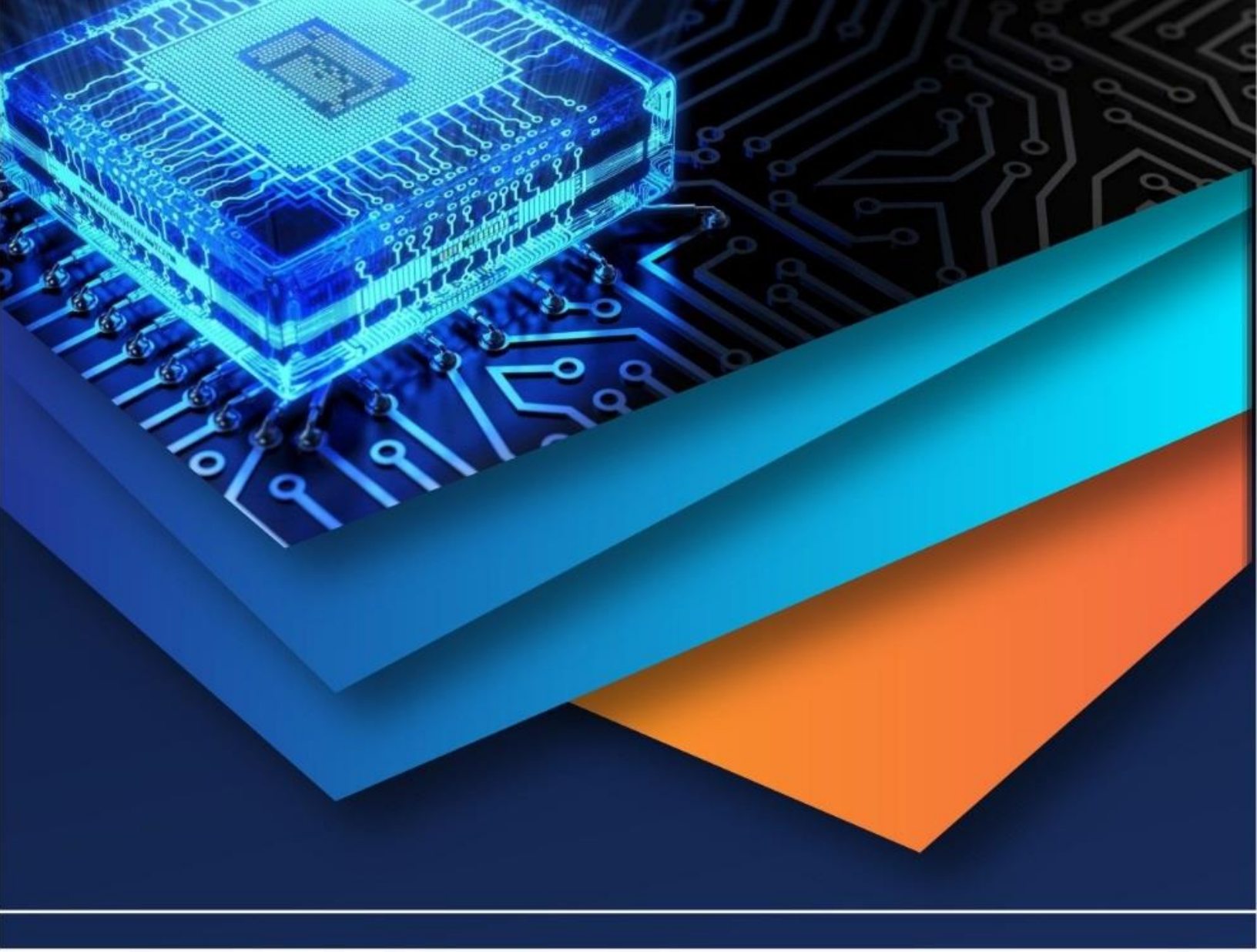

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