Isolation and screening of cellulose degrading bacteria and optimization of their cellulase activity.

[1]Gayatri Kishor Shete, [2]Sunil R. Jagiasi

[1]PG Student, 2Associate Professor
Department of Microbiology
Seva Sadan's R.K. Talreja College of Arts
Science and commerce, Ulhasnagar-421003, District– Thane, India.

Abstract*- Cellulose, the most abundant organic molecule on Earth and the most abundant constituent of all plant materials, is a linear biopolymer of glucose molecules linked by beta-1,4-glycosidic bonds. Enzymatic hydrolysis of cellulose necessitates the use of synergistic mixtures of hydrolytic enzymes such as endoglucanases, exoglucanases (cellobiohydrolases), and glucosidases. Microorganisms with cellulolytic activity are being considered as potential candidates for biotechnological applications. The study was designed to isolate and screen potent cellulase producing bacteria. For screening purpose congo red agar media was used. Among all isolates, ten isolates showed cellulase activity. On further screening quantitatively by DNSA method, the potent isolate was screened out. Enzyme activity of that isolate was observed as 106mcg/ml/min at 540nm. By optimization Maximum growth of cellulose degradation bacteria was recorded at 37°C and pH 7.0. Among the carbon sources tested, maximum growth was observed in glucose also potassium nitrate was good nitrogen sources for better survival of CDB isolates. And lastly identification of potent isolate was done by 16s rRNA sequence analysis. And it was resulted that the isolate was *Brevundimonas diminuta.

***Index Terms*- Cellulase, Exoglucanases Enzymes, Polysaccharides, Optimization.**

I. INTRODUCTION

Cellulose is a complex polymer that is an essential component of the Earth's carbon cycle, accounting for between 35-70% of plant biomass [1]. It is also the most abundant organic compound on the planet, and it is most commonly found in plant biomass, though some bacteria can produce cellulose as well. The cellulose structure is made up of glucose monomers linked by beta-1,4-linkages that are often arranged crystallinely, making it difficult to depolymerize into mono- or di-saccharide subunits. Cellulose is also the abundant organic compound on the earth. Plants are widely recognised as the most abundant source of renewable carbon and energy on the earth. It is also regarded as one of the important carbon sources on the planet, with annual biosynthesis by both land plants also marine organisms amounting to 0.8510 11 tones per year [1]. Cellulosic biomass is widely available As agricultural waste around the world, and because of its high sugar content for potential fermentation, cellulose degradation has received a lot of attention as a feedstock for biofuels. Microbial processes have been at the forefront of cellulosic ethanol production research since biofuels gained popularity. In biomass, cellulose exists in two forms: crystalline and amorphous. The majority of cellulose is crystalline cellulose, with a small amount of cellulose being organised amorphous.

Cellobiose is the repeat unit formed by this linkage, and it is what makes up cellulose chains. Long cellulose chains are stabilised in plant cell walls by strong hydrogen and van der walls bonds, resulting in microfibrils. Cellulose microfibrils contain a high proportion of crystalline regions and a low proportion of amorphous region [2]. One of the chemical way in degradation of cellulose is ionising radiations, in this decomposition of the pyranose ring leads to the formation of compounds with carbonyl and carboxy groups, as well as the formation of hydrogen, carbon dioxide, and carbon monoxide [3]. Which not efficient to degrade it into its soluble form. So microbial Cellulose degradation is more effective than the other methods. Cellulose degradation and subsequent uses are critical for global carbon sources. The value of cellulose as a renewable energy source has sparked intense research and industrial interest in cellulose hydrolysis. Microbial cellulose utilisation is responsible for one of the biosphere's largest material flows [4].

Cellulases are one of the most extensively studied multicomponent enzyme systems due to their ability to decompose cellulosic biomass into glucose, which can then be converted into other valuable chemicals and energy [5]. As a result, treatment of cellulose with cellulolytic enzymes for practical purposes has piqued the interest of several biotechnology researchers [6][7]. Cellulase production is generally influenced by a variety of growth parameters such as inoculum size, pH value, temperature, the presence of inducers, medium additives, aeration, growth, and time [8]. Cellulase enzyme system consists of three soluble extracellular enzyme classes: beta 1,4-Endoglucanase, beta 1,4—exoglucanase, and beta-glucosidase (-D-glucoside glucohydrolase or cellobiase). Organisms with this ability use a number of enzymes that work synergistically to break down plant polysaccharides in order to effectively degrade crystalline polymers like cellulose.

Studies on microbial cellulase production have primarily focused on fungi, but there is growing interest in bacteria [9]. The majority of cellulose degradation in nature is caused by microorganisms. They overcome this obstacle with the help of a multi-enzyme system. Aerobic bacteria produced a large number of individuals and extracellular enzymes with binding modules for various cellulose conformations, whereas anaerobic bacteria have a distinct extracellular multienzyme complex known as cellulase [10]. Aerobic and anaerobic hemophilic bacteria, with filamentous fungi, basidiomycetes, thermophilic bacteria, and actinomycetes are the most common cellulose consuming species.Cellulase is generally secreted by microorganisms that can dissolve cellulose, such as fungi, bacteria, and actinomycetes. In comparison to fungi, researchers have considered various bacteria that produce cellulases due to their

exponential growth, production of a multi-enzyme complex, and susceptibility to harsh conditions. Cellulomonas, Pseudomonas sp., and Micrococcus, Cellvibrio, and Bacillus are among the bacteria with cellulolytic properties [11].

2. MATERIALS AND METHODS:

2.1. Sample collection:

Soil sample was collected from the local farm yard, with the help of sterile spatula it was transferred to non contaminated plastic bag and transported to the laboratory.

2.2. Enrichment of sample:

Soil sample which was collected from local farm yard from that 1g was added in Macbeth broth with filter paper strips as a source of cellulose and incubated at 28°C for 2-3 days. After incubation period of 3 days, 10 ml of above Macbeth broth was taken and again added in Cellulose Congo Red medium broth i.e. 200ml of medium in 500ml flask and incubated at 28°C for 5 days for better enrichment.

2.3. Isolation and screening of cellulose degrading bacteria:

From enriched broth, 10 fold dilutions were made with the help of sterile saline. Then the dilutions were spread plated on cellulose congo red agar media plates, followed by incubation at RT for 48 hours. The media used in isolation for cellulose degrading bacteria is Cellulose congo red agar media, in which congo red in this agar medium act as cellulose degrading indicator, which gives groundwork for a quick and sensitive screening test for cellulolytic bacteria [12]. After incubation, clearance around the colonies was considered and taken as positive for cellulose degrader. The zone diameter of the selected colonies was noted. And the colonies were maintained on NA agar slants at 4°C for further identification and cellulase enzymes production [13]. The Cellulose-degrading potential of the positive isolates was also qualitatively estimated by calculating ratio of zone size to colony diameter, which was recorded as HC value i.e., Hydrolysis capacity [14].

2.4. Cellulase enzyme production by Submerged fermentation:

Cellulolytic bacteria can produce the enzyme cellulase, which breaks down cellulose into simple sugar. Submerged fermentation (SmF) has traditionally been used to obtain industrially important enzymes due to the ease of handling and greater control over environmental factors such as temperature and pH. It improves the yield and reduces the cost of enzyme production [15]. Selected colonies were inoculated in cellulose congo red broth in different tubes. Then the tubes were incubated at RT for 48 hours, followed by centrifugation to collect the supernatant. 0.5 ml of supernatant and 0.5 ml of substrate solution (cellulose) were added to the respective tubes, and tubes were kept for 5-10 minutes for reaction. It was then used as an unknown in an assay for estimating cellulase enzyme activity.

2.5.Cellulase enzyme activity estimation:

The estimation of cellulase enzyme activity was carried out according to the DNSA method [16]. 3, 5-Dinitrosalicylic acid (DNSA) is widely used in biochemistry to estimate reducing sugars. Depending on the concentration of reducing sugar present, the colour of the reagent changes from yellow to orange or red. 3,5-Dinitrosalicylic acid (DNS or DNSA) is an aromatic compound that reacts with reducing sugars and other reducing molecules to form 3-amino-5-nitrosalicylic acid. By standard assay, glucose concentrations of 200 mcg/ml to 1000 mcg/ml were used. And the unknown obtained from enzyme production were used. 1 ml DNSA reagent was added to all the tubes (UK and UK (1:2)). The tubes were kept in a boiling water bath for 10 minutes. Followed by adding 8 ml of distilled water was added in each tube, after it get cooled. And lastly, the absorbance at 540nm was taken and recorded. The potent isolate was selected on the basis of higher readings.

2.6. Identification of isolate:

2.6.1. Morphological identification:

For morphological identification, standard Gram staining was done and isolate's Gram nature and morphology was observed.

2.6.2. 16S rRNA gene sequence method:

For genomic DNA isolation, Biobee Spin EXpure Microbial DNA isolation kit developed by Bogar Bio Bee stores Pvt Ltd., was used for selected microbial sample. For that first cell lysis or cell homogenization was done followed by using three buffers binding, washing and elusion buffer and by following standard procedure DNA concentrations were measured by Qubit flurometer 3.0 or 1% Agarose Gel Electrophoresis. Followed by PCR, for that 20 base of sequence 5' AGAGTTTGATCTGGCTCAG 3' named as 27F was used as forward primer and another 20 bases sequence 5' TACGGTACCTTGTTACGACTT 3' named 1492R was used as reverse primee. In that denaturation, annealing and extension was done, for 6 different stages different PCR conditions were maintained. In which for initial denaturation temperature was 95˚C and time was 2 min. Likewise for final extension temperature 72˚C and time was 10 min. For purification ABI PRISM® BigDyeTM Terminator Cycle Sequencing kits with AmpliTaq DNA polymerase was used. Single-pass sequencing was performed on each template using below 16s rRNA universal primers. The 16s rRNA sequence was blast using NCBI blast similarity search tool. The program MUSCLE 3.7 was used for multiple alignments of sequences. And the resulting aligned sequences were cured using the program Gblocks 0.91b.This Gblocks eliminates poorly aligned positions and divergent regions. Finally, the program PhyML 3.0 aLRTwas used for phylogeny analysis and HKY85 as Substitution model.

2.7. Optimization of environmental and nutritional factors affecting enzyme production:

2.7.1. Optimization of pH on enzyme activity:

Each enzyme has a preferred pH. The optimal pH of an enzyme is the ideal pH value at which the enzyme works best, or the value of the pH at which the enzymatic activity is highest [17]. Optimization of pH on enzyme activity was done with respect to time. (5 minutes to 30 minutes). 100 ml of phosphate buffer was made, in that substrate cellulose was added. 20 ml of above mixture was divided into 5 flasks. Then 10 ml of the above mixture was pipetted out in 5 different tubes named as 5, 6, 7, 8, 9 pH. With the help of pH paper, the pH of the solution was maintained in the given range. 10 ml of enzyme solution were added to each tube. So each

tube contained buffer, cellulose as substrate, and enzyme. 1 ml of reaction mixture was taken and 1 ml of DNSA reagent was added with respect to time to all 5 pH tubes after 5 minutes of enzyme addition. It was kept in a boiling water bath for 10 minutes, followed by the addition of 6 ml of distilled water. Likewise, after every 5 minutes of interval, 1 ml of mixture and 1ml of reagent were added, and the same procedure was followed as above. Lastly, absorbance at 540nm was taken of all the tubes.

2.7.2. Optimization of temperature on enzyme activity:

Temperature is also an important environmental factor that affects enzyme activity. Reactions also have an optimum temperature at which enzyme works more effectively [18]. In 5 tubes named 0, 10, 28/RT, 37 and 55°C, substrate solution with 2 ml of phosphate buffer was added to each tube. Followed by adding 0.5ml of enzyme solution to each tube. All the tubes were incubated at their respective temperatures for 10 minutes. After the incubation period, the reaction was terminated by adding 1 ml of DNSA reagent in all the tubes. The tubes were kept in a boiling water bath for 10 minutes. 6 ml distilled water were added in all. Enzyme activity was checked by taking OD at 540nm.

2.7.3. Utilization of Carbon Source and Nitrogen Sources:

The cellulase enzyme production by cellulolytic microorganisms is also greatly influenced by media components, especially carbon and nitrogen sources, being the main energy and also the growth promoting factors. The selection of cost-effective nutrients like carbon and nitrogen is of utmost desire for the optimum production of enzymes [19].

Carbon source:

Cellulose congo red broth was prepared and added to 10 ml in 10 different tubes. (5 tubes for control). Each carbon source, i.e., glucose, sucrose, lactose, starch and fructose was added to all the respective test tubes. The pH was adjusted to 7.0. Test tubes were autoclaved at 121°C for 15 minutes for sterilization of the broth. Then the test culture was inoculated in the Test test tubes not in control tubes. The tubes were incubated at 37°C for 48 hours. After incubation, tubes were centrifuged, and the supernatant was collected. 1 ml of DNSA reagent was added to each tube and kept in boiling water bath for 10 minutes. 6 ml distilled water was added in all the tubes. Activity was measured by taking OD.

Nitrogen source:

It was performed same as carbon source just instead of carbon sources nitrogen sources were added. Cellulose congo red broth was prepared and added 10 ml in different 10 tubes. (5 tubes for control). Each nitrogen source, i.e., Ammonium sulphate, Sodium nitrate, Potassium nitrate, Ammonium chloride, Urea was added in the respective test tubes. pH was adjusted at 7.0. Test tubes were autoclaved at 121°C for 15 minutes for sterilization of the broth. Then the test culture was inoculated in the Test test tubes not in control tubes. The tubes were incubated at 37°C for 48 hours. After incubation, tubes were centrifuged, and the supernatant was collected. 1ml of DNSA reagent was added in each tube and kept in boiling water bath for 10 minutes. 6 ml distilled water was added to all the tubes. Activity was measured by taking OD.

2.7.4: Effect of metal ions on enzyme activity:

Metal ions are essential in the biological function of many enzymes. Metal-, ligand-, and enzyme-bridge complexes are examples of metal-protein interactions [20]. Thus, which is also an important environmental factor that is influenced by enzyme activity in this study. Metal ions activity was checked in 3 concentrations, i.e. 1 mM, 5 mM and 10 mM. For that, test tubes were labelled with the names of the metal ions and their respective concentrations in mM. For each concentration, 10 ml of buffer was added in test tubes with substrate, and 0.1 g of metal ion was added in 1mM concentration; 0.5 g of metal was added in 5 mM concentration and lastly, 1 g of metal was added in 10 mM concentration. The mixture was mixed, and 0.5 ml of the mixture was taken and added to a fresh labelled tube. 0.5 ml of enzyme was then added and all the tubes were incubated at RT for 30 minutes. 1 ml of DNSA reagent was added in each tube and kept in a boiling water bath for 10 minutes. 6 ml of distilled water was added in each tube. And its residual activity was checked by taking OD at 540 nm.

3. RESULTS AND DISCUSSION:

3.1. Isolation and Screening of Cellulose degrading bacteria:

Well isolated colonies with zone of hydrolysis were observed on Cellulose congo red agar media plates after 48 hours of incubation at RT. 10 isolates were selected on the basis of their large zone size compared to others. Their zone size and colony size were noted. And named as Isolate 1-10. The ratio of zone size to colony diameter was recorded as HC value i.e., hydrolysis capacity of selected 10 isolates. The details are given in **Table 1.**

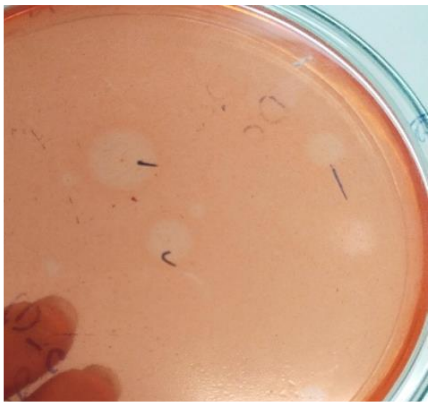

Figure 1. Colonies showing zone of hydrolysis on Cellulose congo red agar media plate.

Table 1. Size of zone of hydrolysis and HC values

Sr.No.	Isolates	Colony size(cm)	Zone size (cm)	HC value (Z/C)
1.	Isolate 1	0.4	0.7	1.75
2.	Isolate 2	Pinpoint	0.3	-
3.	Isolate 3	0.2	0.4	2
4.	Isolate 4	0.4	0.6	1.5
5.	Isolate 5	0.3	0.5	1.6
6.	Isolate 6	0.3	0.7	2.3
7.	Isolate 7	Pinpoint	0.3	-
8.	Isolate 8	0.3	0.9	**3**
9.	Isolate 9	Pinpoint	0.2	-
10.	Isolate 10	0.2	0.5	1.6

As seen in in table 1, the clear zone ranged from 2mm to 9mm i.e., 0.2cm to 0.9cm and the HC value (ratio of zone size to colony diameter) ranged from pinpoint colonies or 1.5 to 3. These results are nearly similar to findings reported by [21] who also found hydrolytic values between 1.25 to 2.5. Whereas [13] observed the HC values between 1.33 to 2.87.

3.2. Enzyme Production:

10 selected isolates were inoculated in the cellulose congo red broth and incubated at RT for 48 hours. After incubation, tubes were centrifuged and collected supernatant was used for cellulose enzyme activity determination as a crude enzyme source.

3.3. Cellulase enzyme activity estimation:

The estimation of cellulase enzyme activity was carried out according to the DNSA method. By standard assay, glucose concentrations of 200 mcg/ml to 1000 mcg/ml were used. The supernatant of all 10 selected isolates was used as unknown (Uk1 to Uk10). 1 ml DNSA was added in all the tubes. The tubes were kept in a boiling water bath for 10 minutes. And distilled water was added in each tube, and their activity was checked by taking OD at 540 nm. The potent isolate was selected on the basis of the isolate giving the highest activity. The activities of all the isolates are given below.

Table 2. Cellulase enzyme activity of isolates

Isolates	OD at 540nm	Enzyme activity (mcg/ml/min)
Isolate 1	0.05	10
Isolate 2	0.10	18
Isolate 3	0.24	44
Isolate 4	0.05	10
Isolate 5	0.09	16
Isolate 6	0.14	26
Isolate 7	0.17	32
Isolate 8	0.57	**106**
Isolate 9	0.10	18
Isolate 10	0.10	18

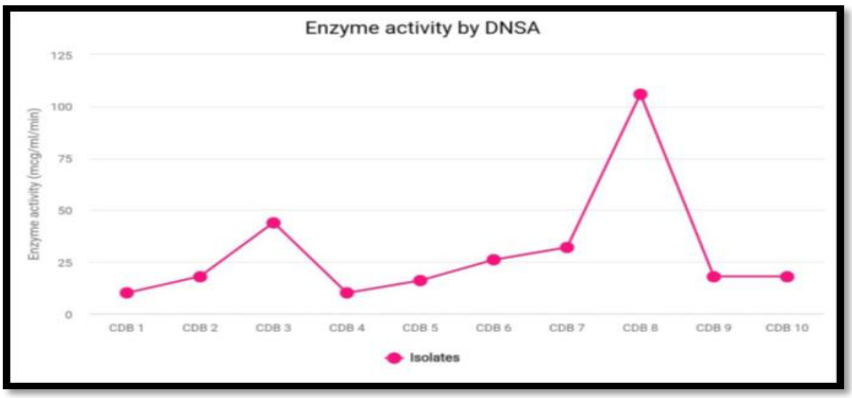

Figure 2. Graph representing enzyme activity of 10 isolates

Cellulase enzyme activity was seen ranging from 10 mcg/ml/min to 106 mcg/ml/min. Isolate 1 and isolate 4 showed the lowest activity, which is 10 mcg/ml/min, and isoalte 8 showed the highest enzyme activity, i.e., 106 mcg/ml/min. [22] observed the highest enzyme activity of 489 mcg/ml, and the result showed that cellulase enzyme activity was high in blotting paper. Whereas [12] reported that cellulase activity on filter paper was found to be highest for CDB 10 with 0.194 IU/ml.
So isolate 8 was selected on the basis of its qualitative and quantitative assay.

3.4. Identification of isolate:

3.4.1. Morphological identification:

By Gram staining it was found out that the potent isolate was Gram negative bacilli.

3.4.2. 16S rRNA gene sequence method:

For 16s rRNA gene sequence method,Biobee Spin EXpure Microbial DNA isolation kit manufactured by Bogar Bio Bee stores Pvt Ltd., forward Primer 27F (5'AGAGTTTGATCTGGCTCAG3') and reversed primer 1492R (5' TACGGTACCTTGTTACGACTT 3'), the chosen potent isolate was subjected to 16S rRNA gene sequencing to identify the strain. *Brevundimonas diminuta* was identified as the query sequence in a phylogenetic study of the 16S rRNA sequence using the NCBI blast similarity search tool with accession number OQ538166.1 The program MUSCLE 3.7 was used for multiple alignments of sequences. And the resulting aligned sequences were cured using the program Gblocks 0.91b.This Gblocks eliminates poorly aligned positions and divergent regions. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model.

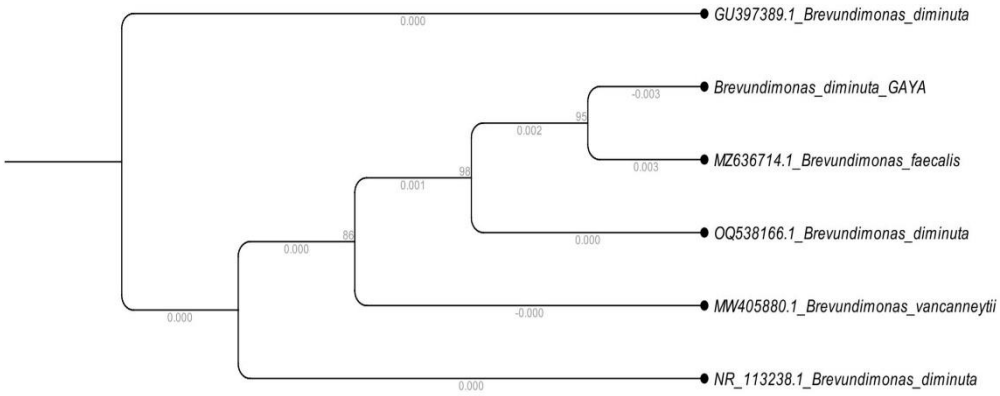

Figure 3. Phylogenetic tree for analysis of strain.

3.5. Optimization of environmental and nutritional factors affecting enzyme production:

3.5.1. Optimization of pH on enzyme activity:

Optimization of pH on enzyme activity was done with respect to time, i.e., from 5 minutes to 30 minutes. That is to check at which pH and at what time the activity is higher. For that, a standard pH range from acidic to basic was selected from 5 to 9. In phosphate buffer with the help of KH_2PO_4 and K_2HPO_4. And standard procedure was followed, but in relation to time, i.e., in a 5 minutes interval. And OD was checked at 540nm.
Thus, it was resulted that at a time of 30 minutes, i.e., an incubation of 30 minutes at pH 7, the enzyme activity was comparatively higher than the others and which was observed 0.42. The lowest activity was observed 0.23 which was at pH 7 at 10 minutes and at pH 9 at 5 minutes. It was also observed that the enzyme activity had been reduced by increasing the pH. So the isolate or the enzyme works best at a neutral pH. But, at all the selected pH, isolate survived and gave enzyme activity. Although these cellulose-degrading organisms prefer a neutral pH range, they have adapted to survive in slightly basic and acidic pH environments.

Table 3. Optimization of pH with respect to time on enzyme activity.

pH / Time	5 (OD at 540nm)	6 (OD at 540nm)	7 (OD at 540nm)	8 (OD at 540nm)	9 (OD at 540nm)
5 minutes	0.27	0.26	0.24	0.24	0.23
10 minutes	0.30	0.28	0.23	0.28	0.29
15 minutes	0.28	0.26	0.26	0.24	0.31
20 minutes	0.25	0.31	0.28	0.26	0.28
25 minutes	0.34	0.33	0.26	0.33	0.36
30 minutes	0.34	0.34	**0.42**	0.33	0.36

The results were found to be similar to [22] i.e they also got the neutral pH. Also [14] observed highest enzyme activity at pH 7.0. indicating neutral pH. Whereas [16] observed highest enzyme activity at alkaline pH which was at pH 9.0. also [23] observed pH 8 as the optimum pH in their study. This may be due to the bacteria being stable at alkaline pH.

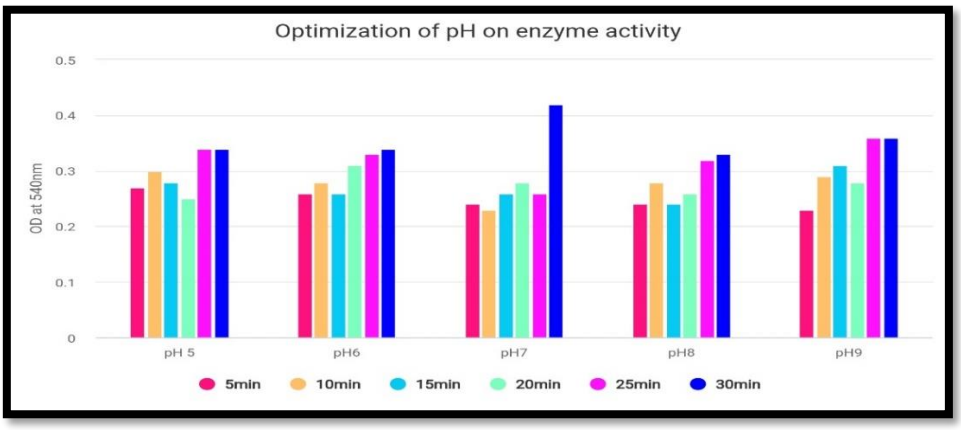

Figure 4. Optimization of pH on enzyme activity

3.5.2. Optimization of temperature on enzyme activity:

Temperature is also an important environmental factor which affects enzyme activity. Reactions also have the optimum temperature where the enzyme works more effectively. 5 temperatures were selected (0, 10, 28, 37, 55^0 C). After adding substrate solution, the buffer and enzyme tubes were incubated at respective temperatures for minutes. And the optical density was checked.

Table 4. Enzyme activity at various temperatures.

Sr.No.	Temperature	OD at 540nm
1.	0^0 C	0.11
2.	10^0C	0.09
3.	28^0C	0.08
4.	**37^0C**	**0.16**
5.	55^0C	0.09

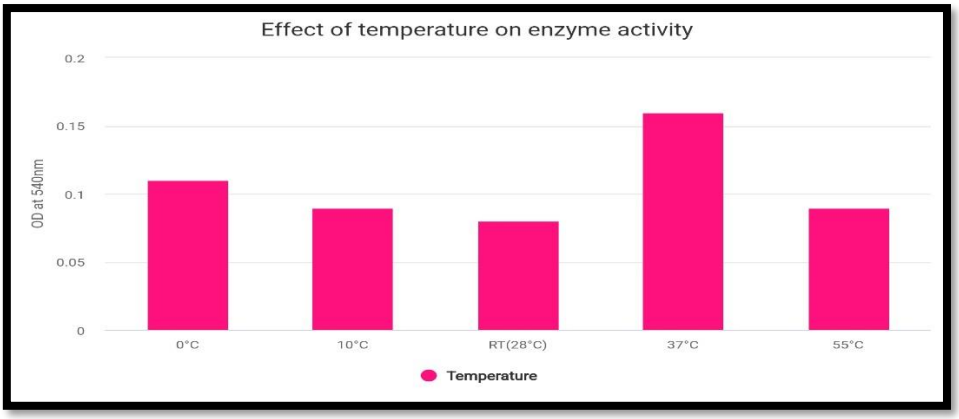

Figure 5. Effect of temperature on enzyme activity

Figure 4 clearly shows that the highest enzyme activity of a potent isolate was observed at 37^0C. And the lowest enzyme activity was observed at RT. These results are close to those of [24], who found out that the cellulase enzyme produced by the strain *Pseudomonas fluorescens*, showed an optimum temperature 40^0C. Due to changes in microbial protein structure and properties caused by temperature variations, incubation temperature is critical for optimal enzyme production. Metabolic activities are reduced at temperatures below or above the optimum, resulting in inhibition of growth and enzyme synthesis. Also, [25] reported that the highest temperature was 40^0C. Whereas [23] concluded enzyme activity at 50-60^0C was the highest and 30^0C was the lowest of *Paenibacillus terrae* ME27-1.

3.5.3 Utilization of Carbon Source and Nitrogen Sources:

Carbon source:

It was observed that glucose was yielded the highest activity. And starch was the least efficient in utilizing carbon sources. The values of all the alternative carbon sources are given in table 5. The results showed that all of the organisms could grow in a variety of nutritional sources. Glucose was the highest, followed by sucrose and lactose, as shown in figure 6. The cellulose degrading bacteria tested used glucose as a basic carbon source and then used other sugar sources sparingly. [22] also obtained glucose as the highest source of carbon yielding, highest enzyme activity followed by fructose and maltose. Results reported by [24] showed that glucose brought the highest cellulase production compared to other carbon sources at 24 h incubation. Lactose and fructose also showed high cellulase production at 24 h of incubation. And [14] concluded that fructose produced high cellulase only for *E.coli* spp., and Glucose showed highest activity by another strain in his study.

Table 5. Utilization of Carbon Source

Sr.No.	Carbon source	(OD at 540nm)
1.	**Glucose**	**1.57**
2.	Lactose	1.17
3.	Starch	0.74
4.	Fructose	0.95
5.	Sucrose	1.44

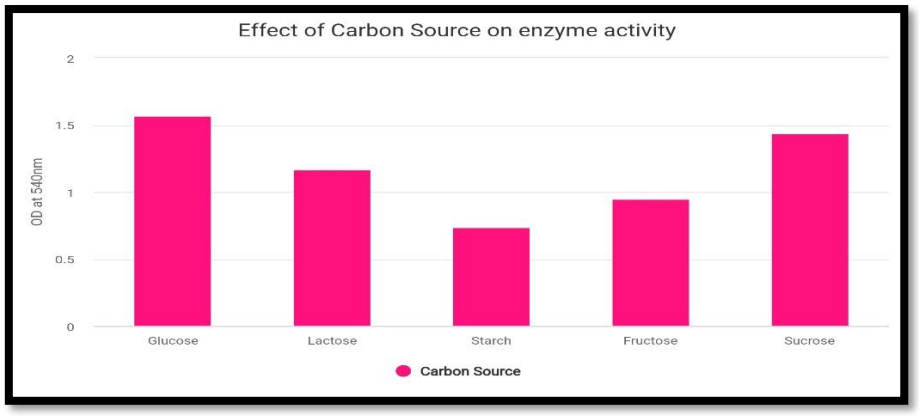

Figure 6. Utilization of Carbon Source

Nitrogen source:

The nitrogen sources like ammonium sulphate, Sodium nitrate, potassium nitrate, ammonium chloride and urea were used for checking the nitrogen source, which yields more activity by the isolate. Highest enzyme activity was observed in potassium nitrate. Ammonium chloride was also seen to be good nitrogen sources in this case. Nitrogen is a major cell protein, and stimulation of

cellulase activity by potassium nitrate may be due to direct entry into protein synthesizing enzymes. So the effect of potassium nitrate was more than the other sources of nitrogen.

Table 6. Utilization of nitrogen sources

Sr.No.	Nitrogen sources	(OD at 540nm)
1.	Ammonium sulphate	0.08
2.	Sodium nitrate	0.08
3.	**Potassium nitrate**	**0.18**
4.	Ammonium chloride	0.14
5.	Urea	0.04

A work earlier done by [22] also identified that ammonium sulphate, ammonium nitrate, and potassium nitrate as good sources of nitrogen. Whereas [14] reported ammonium chloride as a good source of nitrogen, yielding the highest enzyme activity, followed by urea. Also, ammonium sulphate was found to be the best nitrogen source in a work done by [24].

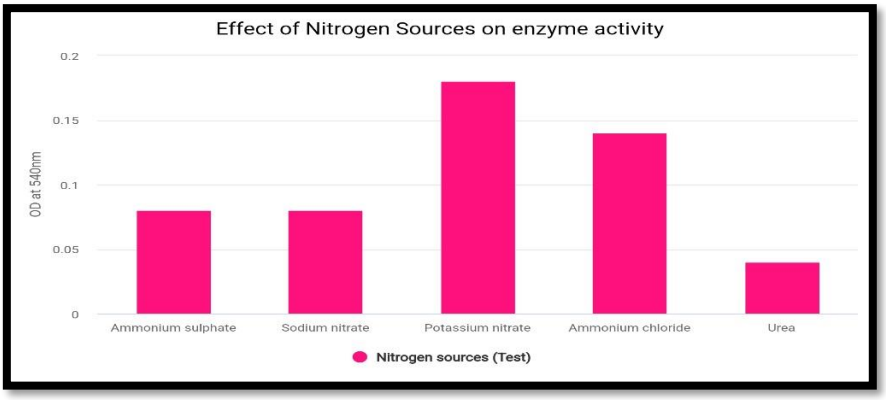

Figure 7. Utilization of nitrogen sources

3.5.4. Effect of metal ions on enzyme activity:

Metal ions such as zinc, manganese sulphate, cobalt chloride, cadmium chloride, and mercuric chloride were used. They were added in tris HCl buffer with enzyme and substrate solutions. All the metal ions were taken in three concentrations, such as 1 mM, 5 mM and 10 mM. From table 7, it can be observed that manganese sulphate at 10mM of concentration gives more activity 0.43 nm and in 5 mM, its 0.37 and at 1 mM of manganese sulphate, it is 0.06. Also, it can be noted that the lowest activity was observed at 1mM of zinc and 1 mM and 5 mM of cobalt chloride. And the readings were increased by an increase in the molar concentration in the cases of zinc, manganese sulphate, and cadmium chloride. In this case, changing or increasing the molar concentration of metal ions increased their activity.

Table 7. Effect of metal ions on enzyme activity

Sr No.	Metals	1mM (OD at 540nm)	5mM (OD at 540nm)	10mM (OD at 540nm)
1.	Zinc	0.02	0.06	0.12
2.	Manganese sulphate	0.06	0.37	**0.43**
3.	Cobalt chloride	0.03	0.03	0.05
4.	Cadmium chloride	0.09	0.06	0.18
5.	Mercuric chloride	0.21	0.20	0.22

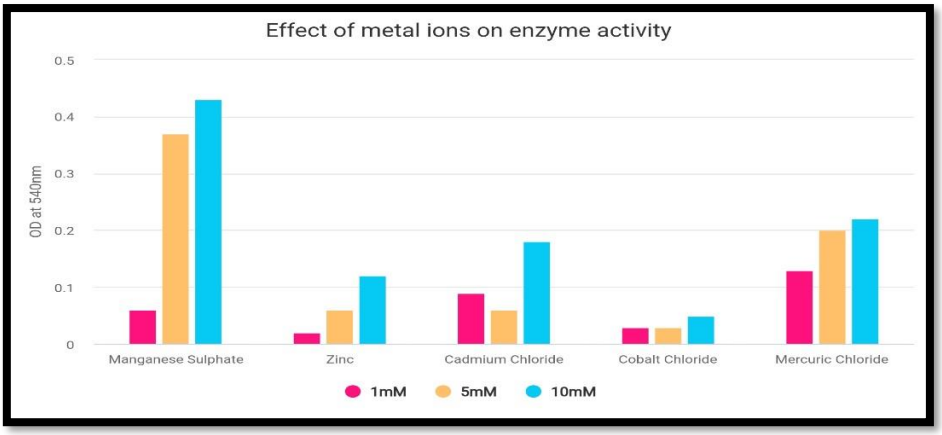

Figure 8. Effect of metal ions on enzyme activity

[26] observed that potassium showed highest activity where as SDS was seen to be lowest. Also, Most metal ions, including Ca^{2+}, Mg^{2+}, Cd^{2+}, Pb^{2+}, and Zn^{2+}, had a minor inhibitory effect on enzyme activity, whereas K^{+} and Mn^{2+} increased cellulase activity by 10%. And [16] resulted that cobalt yields high activity.

4. CONCLUSION:

The purpose of this study was to isolate potential cellulase-producing bacterial isolates from soil samples. Specific isolates were chosen based on their ability to degrade cellulose and cellulolytic properties. Isolate 1 to isolate 10, i.e., 10 isolates, were chosen for possible cellulase activity determination because their colony diameter to clear zone diameter ratio was greater than others. CBD 8 was presumptively identified as *Brevundimonas diminuta*, based on morphological, cultural, and 16s rRNA sequence analysis. The production of cellulase from each isolate was done by submerged fermentation process, from which it was found that the highest enzyme activity was 106 mcg/ml/min by isolate 8. So it was further selected for optimization of environmental and nutritional factors, or it was optimized to determine optimal parameters for stability and to ensure improved enzyme production and activity. In which it was observed that the cellulase enzyme produced by the potent isolate, isolate 8, had an optimal incubation temperature of 37^{0}C. And pH 7 was found to be highest. Also glucose and potassium nitrate was observed to be carbon and nitrogen sources, showing the highest readings at 540 nm respectively. And lastly 10 mM concentration of manganese sulphate was found to be a metal ion showing highest enzyme activity. These optimized requirements are visible in a variety of industrial sectors. Mutagenesis and protein engineering techniques require additional large-scale culture studies to optimize other parameters such as inoculum concentration, medium additives, and inducer presence.

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