Isolation and Characterization of Cellulose-Producing Bacteria from Agricultural Wastes

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DOI: https://doi.org/10.51244/IJRSI.2026.13010233

Received: 04 February 2026; Accepted: 09 February 2026; Published: 18 February 2026

ABSTRACT

The aim of the present study is to demonstrate the isolation, identification and screening of bacteria with high cellulase activity from soil samples. Cellulase degrading bacteria were isolated from soil sample using serial dilution and pour plate method. It indicated that favourable fermentation conditions and the selection of a suitable growth medium played a key role in the production of cellulase from newly isolated Cellulase sp. Due to its particular characteristics this enzyme will be used in saccharification process for bioethanol production from plant biomasses. An accurate and precise method for the assay of cellulase activity in soil was developed. It involves determination of the reducing sugars produced when a soil sample is incubated with acetate buffer, carboxy methyl cellulase (CMC), and toluene.

Results showed that the optimal pH of cellulase activity. Reducing sugars produced from cellulase activity. Reducing sugar values increased significantly when air-dried or field-moist soil samples were incubated as described but without CMC, suggesting a role for cellulase in degradation of the native substrates. Supplementation of glucose, peptone and cysteine to the fermentation medium are favoured enzyme secretion. The optimum pH and temperature for the activity of crude enzyme was 7.5 and 60°C, respectively. Bacteria and fungi were further identified by morphological and biochemical tests and subjected to cellulose production using medium of fermentation period at 35°C with agitation speed of 1000rpm.

Key Words: Cellulose, enzymes, saccharification, Agricultural Wastes.

INTRODUCTION

Cellulases are defined as biological catalysts that mediate the breakdown of cellulase into simpler sugars. These enzymes play a crucial role in the degradation of plant biomass, especially in the context of converting lignocellulosic materials into fermentable sugars, which can then be used for producing biofuels, animal feed, and other bioproducts. Cellulases catalyse the hydrolysis of β-1,4-glycosidic bonds in cellulase, a major structural component of plant cell walls, thereby facilitating its conversion into glucose. This process is essential not only for natural biomass recycling but also for several biotechnological applications in the textile, paper, food, and bioenergy industries.

Enzymatic hydrolysis of cellulase is carried out by a synergistic action of three main classes of cellulases: endoglucanases, which randomly cleave internal bonds in cellulose chains; exoglycanases, which act on chain ends to release cellobiose units; and β-glucosidases, which hydrolyse to glucose. This synergism ensures the complete conversion of insoluble cellulose into soluble glucose molecules.

The discovery and development of cellulase enzymes have a rich scientific history. In the early 19th century, French chemist Henri Braconnot (1819) was among the first to describe cellulase as a distinct component of plant material. However, the enzymatic degradation of cellulose gained significant attention only in the 20th century. One of the key breakthroughs was made by Elwyn T. Reese in the 1940s, who, along with colleagues at the U.S. Department of Agriculture, isolated a fungus, Trichoderma reesei, known for its high cellulase activity. This discovery marked the beginning of extensive research on microbial cellulases.

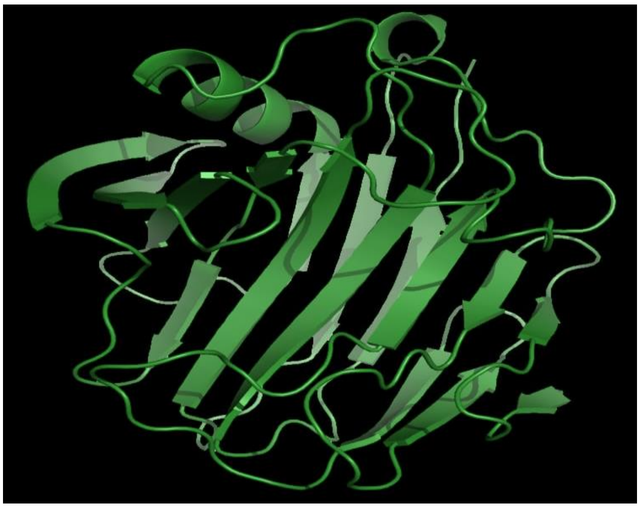

Figure :1 cellulase enzyme

Production of cellulase in microbial cultures is strictly concerned with growth and various factors affect the productivity. Various biomass inducing residues including; lignocellulosic material, paper waste, pulses cereals straw and bagasse's have been widely used as carbon sources for commercial cellulase fermentations. Low yields over prolonged fermentation is the major limiting factor and for the production of cellulases; solid state fermentation (SSF) is gaining popularity being cost effective and equally useful for the bioconversion lignocellulosic material using cellulolytic microorganisms.

In industrial biotechnology, cellulases have emerged as a key group of enzymes due to their capability to convert agricultural residues like corn cobs into fermentable sugars. Corn cobs, a rich lignocellulosic substrate, consist of approximately 35–45% cellulose, making them an ideal feedstock for enzymatic processing. Pretreatment of corn cob powder enhances the surface area and breaks down lignin barriers, making cellulose more accessible to enzymatic action.

Cellulase-producing microorganisms are commonly found in soil, compost, and decaying plant matter. Soil, in particular, harbours a diverse microbial ecosystem that includes fungi, bacteria, and actinomycetes capable of secreting cellulolytic enzymes. Microbial strains like Aspergillus niger, Penicillium spp., and Bacillus subtilis are frequently isolated from soil samples and screened for cellulase production. These organisms are known for their stability under varying pH and temperature conditions, traits that are vital for industrial processing.

Recent advancements in molecular biology and bacteria engineering have enabled the cloning, overexpression, and modification of cellulase genes to improve enzyme efficiency and stability. The integration of cellulases into bioconversion processes supports the global transition toward a bio-based economy by reducing dependence on fossil fuels and minimizing environmental impact.

The *Clostridium thermocellum* endoglucanase Cells contains two different catalytic domains in a polypeptide, i.e., a subfamily E1 catalytic domain and a family J catalytic domain [*J. Bacteriol*., 178, 5732–5740 (1996)]. The family J catalytic domain (CDJ-CelJ) was produced by a recombinant *Escherichia coli* and purified. The purified CDJ-CelJ gave a single band on SDS–polyacrylamide gel electrophoresis and the molecular weight of this enzyme (60,000) was consistent with the value (60,333) calculated from the DNA sequence. CDJ-CelJ hydrolysed various substrates, Xylan, and lichenin but not *p*-nitrophenyl (PNP)-cellobiose, PNP-glucoside, or PNP-Xylo side at all. CDJ-CelJ was active on Avicel, a microcrystalline cellulose, and the specific activity of CDJ-CelJ on Avicel (0.0078 U/mg bacteria) was comparable to that of Cells, which is recognized as the most important catalytic subunit of the *C. thermocellum*, cellulose, suggesting that CelJ is also an important catalytic subunit in the cellulose of this bacterium, in addition to CelS. Mohammad Mainu Volume 61, 1997 - Issue 3

The cellulolytic system from culture filtrates of *Clostridium thermocellum*, when partially purified by preparative electrophoresis, showed a single bacterium and on analytical polyacrylamide gel electrophoresis; however, sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicated that at least five bacteria were present. Carbohydrate was present in the purified fraction and it is suggested that the cellulase was present as a complex. A nonspecific cellobiose phosphorylase from *Clostridium thermocellum*, which has been purified over 100-fold, is active on 10 different glucosyl acceptors, D-glucose, 2-deoxyglucose, 6-deoxyglucose, D-glucosamine, D-mannose, D-altrose, L-galactose, L-fucose, D-arabinose, and D-xylose. Following electrophoresis on polyacrylamide gels, the activity with various acceptors occurs at the location of the major component, indicating that a single enzyme catalyses these reactions.

MATERIAL AND METHODS

SAMPLE:

Sample	Composition	Purpose
Soil (agriculture waste)	2gms	Bacteria cultural preparation
Cone cobs powder	5gms	Invitro – application of biofuel production using cellulose enzyme hydrolysis
Glucose	1mg	Invitro- application of reducing sugar estimation

Materials:

Laboratory equipment : 1. Petri plates, 2. Autoclave, 3. laminar air flow, 4. weighing machine, 5.centrifuge, 6. Eppendorf tubes, 7.Erlenmeyer's flasks, 8. measuring cylinders, 9. spectrophotometer, 10.colorimeter, 11. spatula, 12. Inoculation loop, 13. incubators, 15. microscope, 16. L-shaped spreader, 17. test tubes,18. Microtips, 19.pipettes, 20.glass slides, 21. Magnetic stirrer, 22. pH meter.

Chemicals required:

Nutrient agar media (50ml)

Chemicals required	Composition
Bacteriological peptone	250 milli gram
Yeast extract powder	150 milli gram
Sodium chloride	250 milli grams
Agar powder	800 milli grams
Distilled water	50 ml

CMC AGAR (50 ml) (Carboxymethyl cellulose agar)

Chemicals required	Composition
Carboxymethyl cellulose agar (cmc)	1gm
Peptone	0.05gm
yeast extract	0.025gm
Dipotassium phassium hydrogen osthophosphate	0.05 gm

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue I January 2026

Magnesium sulphate	0.025
Agar	0.75
Distilled water	50ml

CMC broth (50ml)

Chemicals required	**Composition**
Carboxymethyl cellulase agar (cmc)	1gm
Peptone	0.05gm
yeast extract	0.025gm
Dipotassium phassium hydrogen osthophosphate	0.05 gm
Magnesium sulphate	0.025
Distilled water	50ml
Carboxymethyl cellulase agar (cmc)	1gm
Peptone	0.05gm
yeast extract	0.025gm
Dipotassium phassium hydrogen osthophosphate	0.05 gm
Magnesium sulphate	0.025
Agar	0.75
Distilled water	50ml

Chemicals Required For Assay:

Chemicals required	**Composition**
Citrate monohydrate	1gm
Sodium hydroxide	0.032gm
DNS	o. o37gm
Sodium potassium tatarate	1.27gm
Phenol	27microliters
Sodium meta sulphate	0.029gm
Distilled water	50ml

Chemical required	Composition
Methyl red	Few drops
Trypticase	1gm
Sodium chloride	5gm
Beef extract	5gms
Phenol red	Few drops
Dextrose	5.0gm
di-potassium phosphate	5.0gm
Urea	10gm
Agar	15gm
Tryptophan	1.0g/l

METHODOLOGY

Sample collection

Agri waste soil sample was collected from public park in the location of near domlur area and it is transferred to the laboratory.

Sample preparation

1 gm of soil sample is dissolved in 100 ml of distilled water in a conical flask until the soil particles settled down.

Culturing of microorganisms from the soil sample by nutrient agar

Take a conical flask add 250 mg of Bacteriological peptone , 150 mg of Yeast extract powder , 250 mg of Sodium chloride , 800 mg of Agar powder , 50 ml of Distilled water was added and sterilized it in autoclave for 15 – 25 min then make it cool down under the laminar air flow chamber then take a 1000 ml pipette transfer 300 ml soil sample from distilled water in to Petri plate after solidifying the media take a l rod preheat the l rod then spread the entire plate with the soil sample and keep it in incubator for 24 hour at 35° c. After incubation next day bacterial colonies are formed in the Petri plate.

Streaking method of bacterial colonies by using McC nutrient agar

Take a conical flask add 1 gm of CMC , 0.5 gm of peptone , 0.5 gm of yeast extract , 0.5 gm of K_2 HPO_4 , 0.5 GM OF $MGSO_4$, 1.5 gm of agar , 100ml of distilled water and mix it well plug with a cotton tightly wrap with a paper along with these 2 Petri plates also wrapped keep it in autoclave for sterilization , make it cool down under laminar air flow chamber collect some bacterial colony from the previous cultured plates with a inoculation loop streak in to the CMC agar divide the Patri plates into 4 parts keep it in incubation for 24 hour at 35ºc. After incubation bacterial colonies are formed in the streaked Petri plates.

Identification of cellulase enzyme by CMC broth

Take a conical flask add 1gm of CMC agar 0.5 gm of peptone , 0.5 gm of yeast extract , 0.5 gm of K_2HPO_4 , 0.05 gm of $MGSO_4$, 100 ml distilled water was added and mix it well plug it with a cotton tightly wrap it with paper along with these 2 petriplates also wrapped keep it in autoclave for sterilization make it cool down under laminar air flow chamber leave it to solidify collect a single colony from the previous streaked plates with a inoculation loop streak it in to Petri plate keep it in incubator for 24 hours at 37ºc After incubation cellulase enzyme was observed in the Petri plate.

Inoculation of cellulase enzyme in CMC broth

Take a 50 ml conical flask add 1gm of CMC agar 0.5 gm of peptone , 0.5 gm of yeast extract , 0.5 gm of K_2HPO_4 , 0.05 gm of $MGSO_4$, add 25 ml distilled water and mix it well plug it with a cotton tightly and sterilize it in autoclave make it cool down under laminar air flow chamber then collect some enzyme particles from the previous Petri plates with inoculation loop streak it in CMC broth keep it in incubator for 24 hour at 35ºc After incubation we observed production bacteria.

Optimization physical and chemical parameters

PH

pH was CMC broth as one of the physical parameters and check for the cellulase activity to determine the optimal conditions under which cellulsase production and effect is maximum. Five different ranges of pH- 5, 6, 7 and 8 were taken as the parameters. Five flasks were taken to prepare 25ml of cmc broth in each. Each flash contains 25ml of CMC broth consisting of the following components- 500mg of cmc broth, 25mg of bacteriological peptone and 50mg of yeast extract powder and made up to 25ml with distilled water. pH is adjusted in the flasks by adding NaOH to increase the basicity of the solution or HCl to increase the acidity of the solution. The flasks were autoclaved at 121 °C, 15 psi for 15 minutes to ensure its sterility and to get rid of the contaminants. After autoclaving, the flasks are inoculated with bacterial samples from plate 1 which showed maximum absorbance. The flasks marked for different pH values were incubated in 35°C in an incubator for 48 hours.

After 72 hours of incubation, bacterial growth is observed in the flasks which can now be checked for their cellulase activity. Cellulase activity that is conducted for the pure cultures was conducted and absorbance was checked at 540nm.

Temperature

Temperature was CMC broth as one of the physical parameters and check for the cellulase activity and to determine the optimal conditions under which cellulase production and effect is maximum. Temperatures of 25°C,35°C, 45°C and 55°C were taken as the parameters. Four flasks were taken to prepare 25ml of cmc broth in each. Each flash contains 25ml of cmc broth consisting of the following components- 500mg of cmc, 25mg of bacteriological peptone and 50mg of yeast extract powder and made up to 25ml with distilled water. pH is adjusted to 8 in the flasks by adding NaOH to increase the basicity of the solution or HCl to increase the acidity of the solution. The flasks were autoclaved at 121 °C, 15 psi for 15 minutes to ensure its sterility and to get rid of the contaminants. After autoclaving, the flasks are inoculated with bacterial samples from plate 1 which showed maximum absorbance. The flaks marked with temperature are incubated in different incubators at different temperatures for 48 hours.

After 48 hours of incubation, bacterial growth is observed in the flasks which can now be checked for their cellulase activity. Cellulase activity that is conducted for the pure cultures was conducted and absorbance was checked at 540nm.

Purification of cellulase enzyme

Centrifuge of 45^0c culture media in 10,000 rpm (10 to 15mints) and collect the supernatant. supernatant is placed in a beaker contain a bar magnet and place of magnetic stirrer. Measure desired amount of ammonium sulphate and add gently. transfer the solution to tube centrifuge 10,000-15000 at 40^0 c. the supernatant was subjected to 80% ammonium sulphate precipitation by a 480ml of 100% saturated ammonium sulphate with 120ml of supernatant. precipitation of overnight and then centrifuged at 10,000 rpm for 10 mints. Pellet collected and redissolved with small amount of ice cold 0.05 m citrate buffer ph 4.0. dissolve salt in 15ml of distilled water. adjust ph to 7.4 using HCL or NaOH and make a final volume to 20 ml filtration (0.22 µm). after measuring the ph value the pellet will take and add 2ml of phosphate buffer. And transfer into a jar keep into refrigerator.

Review of applications

Review of application studies are highly focused, enabling to deduce mechanisms of actions and to control many confounding variables. However, weakness of this type of studies is the uncertainty that the effects observed at cell level would occur in the 'real world' of the complex living organism. in this experiment, we have explored the different lab level review of applications of cellulase enzyme using various biological substances like corn cobs powder, glucose.

1. Biofuel production using cellulase enzyme hydrolysis process:

Corn cob obtained from the waste bin near a grains market was washed in water to remove dust and sun dried for two days. The cobs were broken with the aid of a wooden mortar and pestle, ground in an electric grinder and sieved through a mesh sieve (pore size, 100 µm). The resulting powder was treated with alkali solution to swell the cellulose and make it available for enzyme hydrolysis following a method described elsewhere the dried powder obtained was stored in sealed polythene bags at room temperature till required.

Prepare ethanol standard (0.1%,0.2%,0.5%,1.0%) ethanol in water. Take a1ml of each ethanol standard in a test tube. add 3ml of potassium dichromate reagent. incubate at 60 ^{0}c for 30 mints. Cool the test tubes at room temperature. measure absorbance at 600nm using a spectrophotometer. Plot standard curve (absorbance v/s ethanol concentration).

Sample analysis

Take 1ml the supernatant (fermented sample) in a test tube. add 3ml of potassium dichromate reagent. Incubate at 60^{0}c for 20 mints. Cool the tubes and measure absorbance at 600nm.delermine ethanol concentration using the standard curve.

Reducing sugar estimation (DNS assay):

Take 2gm of corn cob powder then 2ml of enzyme sample and add 5ml of sterilized distill water and mix it well and keep into incubate for 24hours. After incubation take the hydrolyzed cone cob solution and centrifuge on it 10,000 rpm for 10 mints. and take Surprenant.

Dissolve 1g DNS in 20ml of 2m NaOH. add 30g sodium potassium tartar are in 50ml distill water. Mix and make up the volume to 100ml with distill water. And add a few drops of phenol and sodium meta sulfite for stability.

Prepare a stock solution of glucose (1mg/ml). prepare a series of dilution for the standard curve. (0.2,0.4,0.6,0.8,1.0).

Bio chemical test for cellulase:

Urease test:

Prepare the urea agar slant portion and streak on the surface of the agar in test tube inoculate with 1-2 loops. And incubate at 37^{0}c for 3-4 days.

Methyl red solution:

Prepare the urea agar test tube and add 0.1 g of methyl red in 300ml of ethanol (95%) add 200ml of distilled water.

Indole test:

Incubate the organism's bacillus, inoculate at 37^{0}c for 24 hours. after 24 hours add 0.5ml kovacs reagent to the broth.

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue I January 2026

Citrate utilization test

Prepare the agar slant portion and streak on the slant bacillus organism, incubate at 37^0c for 2 days.

RESULTS

Soil sample collection and preparation of bacterial cultures

Figure 2: soil sample for bacterial culture

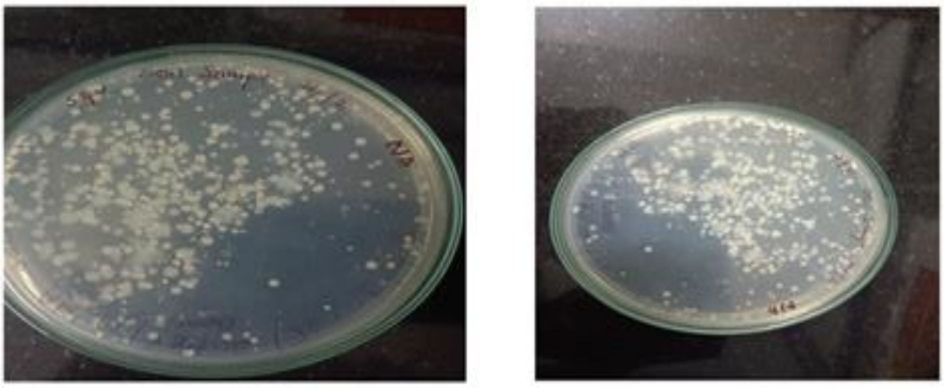

Figure 3: Growth of bacteria in soil sample in nutrient agar plate

Identification of cell morphology and colony characteristics

Plate number	Shape and number of colonies	Types of colonies observed	Margins	Texture	Pigmentation of the colonies	elevation
1	Round, irregular, punctiform. More than 60 colonies	Fungi, bacteria	Smooth, lobate	Smooth, concentric	Dull White, yellow	Flat
2	Round, irregular, punctiform. more than 100 colonies	Fungal, bacteria, actinomycetes	Smooth, lobate, undulate	Smooth, powdery	Dull red, dull white	Convex, flat
3	Round, punctiform. More than 20 colonies.	Bacteria	Concentric, smooth	powdery	Dull red and white	flat

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue I January 2026

4	Irregular, punctiform. More than 40 colonies	Bacteria	undulate	powdery	Dull white	Flat
5	Round, punctiform,	Bacteria, actinomycetes	Smooth, concentric	Smooth	Red, dull white	Flat
6	Round, irregular, punctiform, more than 20 colonies	Fungi, Bacteria	Smooth, concentric	Powdery	Dull white	Raised
7	Round, punctiform. More than 20 colonies	Bacteria, actinomycetes	Smooth, concentric	Smooth	Red, dull white	Raised, convex
8	Round, irregular. More than 20	Bacteria	Smooth, irregular	Powdery	Dull white	Flat

Table 1: Cell morphology and colony characteristics

Clear zones are formed around the inoculated area. Four out the eight plates that show clear zone are chosen to be checked for cellulase activity. Plate 1, Plate 2, have showed a clear zone after pure culture that indicates the presence of a cellulase activity. These plates have been inoculated into casein broths to assess their bacterial growth and it is checked for its cellulase activity by cellulase assay process of pure cultures.

Screening for cellulase enzyme from soil isolates

Figure 4: Pure culture preparation from soil sample and nutrient agar plates

Bio chemical test for cellulase:

Urease test: Prepare the urea agar slant portion and streak on the surface of the agar in test tube inoculate with 1-2 loops. And incubate at 37^0c for 3-4 days.

Positive: yellow color indication

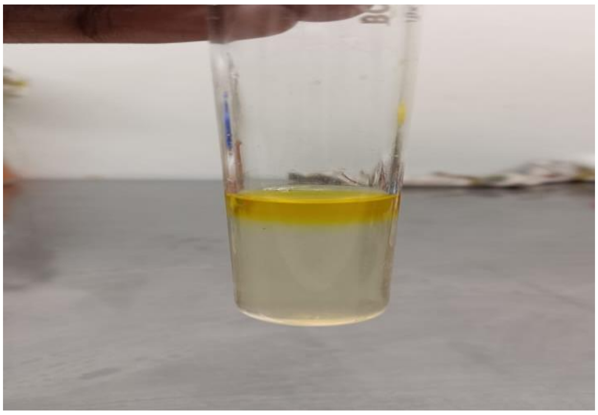

Figure :5 urea test

Methyl red solution: Prepare the urea's agar test tube and add 0.1 g of methyl red in 300ml of ethanol (95%) add 200ml of distilled water.

Positive: lit red color indication

Indole test: Incubate the organism's bacillus, inoculate at 37^0c for 24 hours. after 24 hours add 0.5ml kovacs reagent to the broth.

Positive: cherry red

Citrate utilization test: Prepare the agar slant portion and streak on the slant bacillus organism, incubate at 37^0c for 2 days.

Positive: change the dark blue color

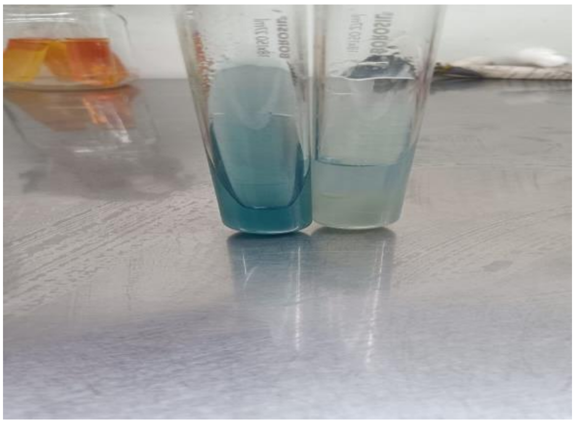

Figure: 6 Citrate utilization test

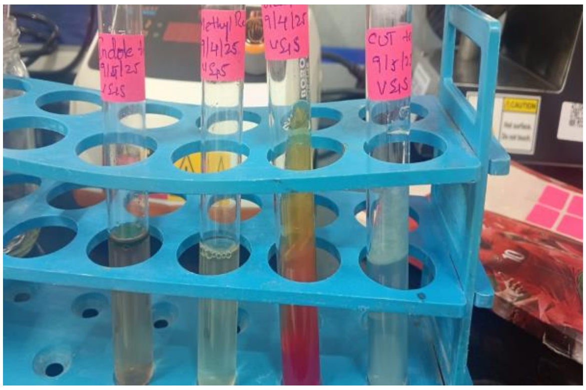

Figure: 7 Biochemical tests

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Optimization of physical parameters-

Temperature:

After 24 hours of incubation, clear zones were observed on isolate from plates -1 that have been spot inoculated with the selected colonies from the bacterial cultures of and soil sample. Clear zones are important when checking plates for enzyme activity because they indicate enzyme degradation. In enzyme activity assays, plates are typically embedded with a bacteria substrate (e.g. nutrient agar cmc agar). When enzyme-producing microbes grow, they secrete enzymes that hydrolyse the surrounding enzymes. This creates clear, transparent zones around the colonies, contrasting with the opaque, undigested enzyme areas. The presence, size, and intensity of the clear zones reflect the enzyme activity and efficiency, making it easier to identify and compare enzyme-producing strains.

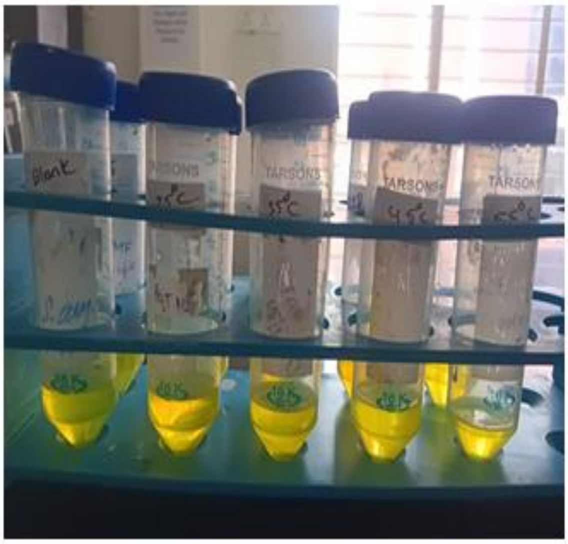

Figure: 8 Centrifuge of enzyme assay

Absorbance values for CMC broth inoculated at different temperatures incubated at the same pH range for 24 hours	
Temperatures	**Absorbance (660nm)**
25^0c	0.025
35^0c	0.028
45^0c	0.045
55^0c	0.004

Table 2: Absorbance values for enzyme assay of pure cultures

The tubes in the above image were taken for indicating cellulase activity exhibited by plates 1, These plates which showed a clear zone were selected for enzyme activity assay procedure. After adding DNS reagent agent, the CMC broth inoculated bacterial cultures from the plate 1, respectively have shown absorbance values at 540nm as given below in the table. The absorbance values above indicate 45^0C was chosen because it displayed higher absorbance values, indicating greater Enzyme activity. Higher absorbance reflects a higher concentration of hydrolysed fragments, suggesting that the enzyme was more effective at breaking down the substrate. This plate likely contained a more active or abundant cellulase-producing strain, making it the optimal choice for further analysis and applications.

Optimization of physical parameters

Ph:

The above flasks consist of CMC broth which has been inoculated with bacterial sample and incubated with five different pH values at the same temperature at 35 °C for 24 hours to initiate bacterial growth. The growth of bacteria in the CMC broth can be identified with the increase in turbidity of the cell. The bacterial growth in flasks of pH- 5 and pH-6 were minimal but flasks of pH- 7, pH-8 and pH-9 has shown turbidity and a good amount of bacterial growth.

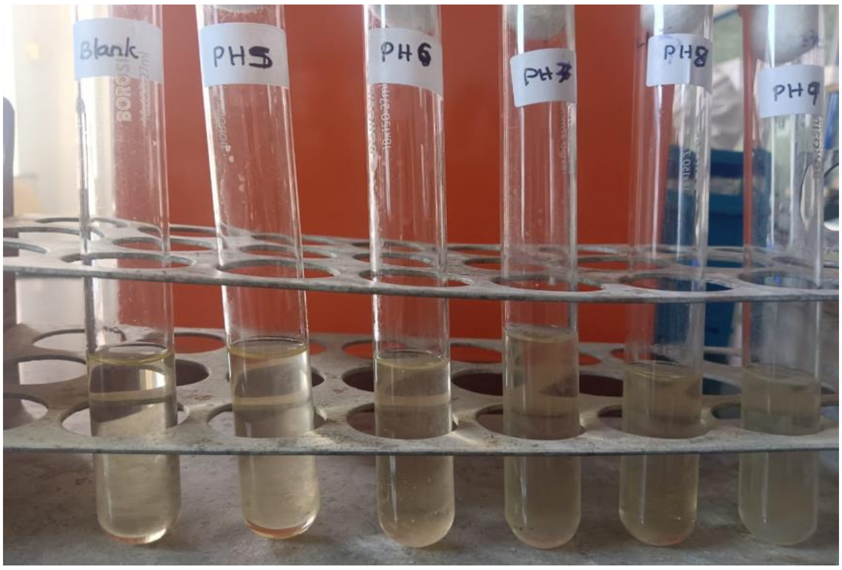

Figure :9 Screening for cellulase activity for pH

Absorbance values for Optimization parameters- pH	
pH ranges	**Absorbance (660nm)**
pH-5	0.034nm
pH-6	0.051nm
pH-7	0.022 nm
pH-8	0.038 nm
pH-9	0.043 nm

Table 3: Absorbance values for cellulase assay for pH

pH- 6 has shown optimal growth of bacteria in CMC broth that has steadily increased the absorbance values. This is an indicated that basic pH levels are required for the growth of a bacteria culture that produces a good amount of cellulase enzyme. However, the flask with pH-6 has shown a greater absorbance value but growth of the bacteria was low due to the addition of HCl while adjusting the pH values.

The above figure is adapted from cellulase enzyme Purification: Principles and Practice 2nd edition (1987) Scope RK which explains the amount of ammonium sulphate to be added to a certain amount of solution for a certain level of saturation to obtain an enzyme. By using the ammonium sulphate table, the amount of ammonium sulphate to be used for salting out the precipitate that contains the enzyme cellulase. The enzyme which has been resuspended in the phosphate buffer will serve as a base for invitro applications.

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Figure :10 purificatin of cellulase enzyme in the magnetic stirrer

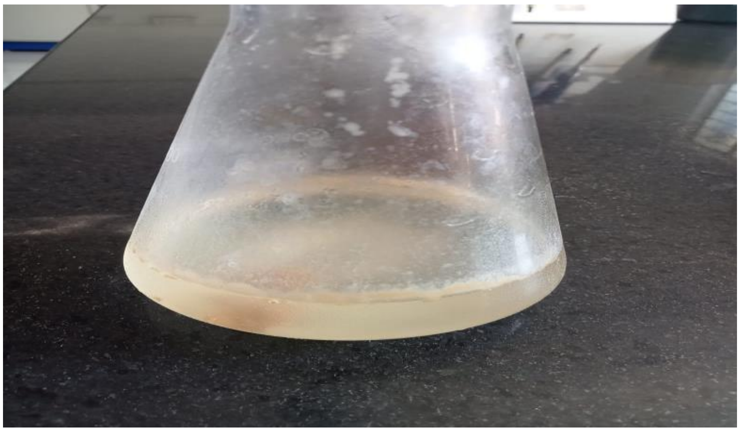


Figure 11: Purification of enzyme by ammonium sulphate precipitation

Purification of cellulase enzyme:

Table 1. Ammonium Sulfate Fractionation Table showing the mass (in grams) of solid ammonium sulfate to add per liter of solution at 20 ºC.   This table is adapted from *Protein Purification: Principles and Practice* 2nd Ed. (1987) Scope RK.

	Final Percent Saturation to be Obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting % saturation	Amount of ammonium sulfate to add (grams) per liter of solution at 20 ºC																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	357	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	292	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	227	265	304	344	386	429	475	522	571
30			0	30	61	92	126	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	97	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	266
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

Figure 4: Ammonium sulphate fractionation table (65)

At low salt concentrations (below 0.15M), adding more salt generally increases solubility. This happens because the salt ions reduce the repulsive forces between bacteria molecules, allowing them to dissolve more easily—a process called salting-in. However, when the salt concentration becomes too high, it starts to reduce enzyme solubility, known as salting-out. In this case, the salt ions compete with the enzyme for water molecules. With fewer water molecules available, the surface tension of water increases, causing enzyme to fold more tightly. As the enzyme's surface area decreases, there are fewer enzyme-water interactions, leading to stronger hydrophobic interactions between bacteria molecules. This causes the enzyme to clump together and eventually precipitate out of the solution. The CMC broth which has been precipitated with ammonium sulphate has obtained a pellet that has been resuspended in phosphate buffer in order to get a final enzyme.

The cellulase enzyme has been assayed for its enzyme activity and the absorbance was measured using a spectrophotometer, and the resulting values obatined indicated that the enzyme obtained by purification of the sample is has a good amount of cellulase.

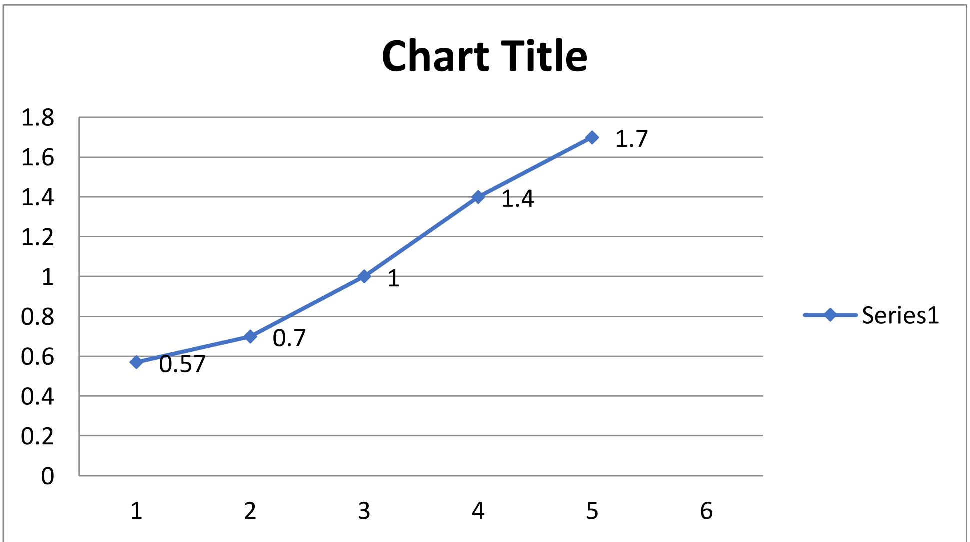
Review of applications:

In application studies are highly focused, enabling to deduce mechanisms of actions and to control many confounding variables. However, weakness of this type of studies is the uncertainty that the effects observed at cell level would occur in the 'real world' of the complex living organism. in this experiment, we have explored the different lab level applications of cellulase enzyme using various biological substances like cone cobs powder, yeast extract powder and chemical substances like CMC.

1. **Biofuel production using cellulase enzyme hydrolysis process:**

Take a 5 test tubes and add each test tube 10ml of distilled water. And add 1%,1.5%,2.00%,2.50% of ethanol for test tubes. And add 3ml of potassium dichromate each test tube. And water both at 65^{0}c at 15 mints. take a reading will taking 600nm.

Absorbance values for assay using different concentrations of substrate	
Concentration	**Absorbance (600nm)**
1.0%	0.57nm
1.5%	0.7nm
2.00%	1.0nm
2.50%	1.4nm
Sample	1.7nm



Step 1: Organize the data

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From your table:

Step 2: Use the linear regression formula

We want to find the equation:

$y = mx + c$

Where:

Is absorbance

is concentration (in %)

is the slope

is the y-intercept

Step 4: Use formulas to calculate slope and intercept

Slope (m):

$$m = \frac{n\sum(xy) - (\sum x) (\sum y)} {n\sum(x^2) - (\sum x) ^2}$$

$$m = \frac {5(12.22) - (10) (5.37)} {5(22.5) - (10) ^2}$$

$$= \frac {61.1 - 53.7} {112.5 - 100}$$

$$= \frac {7.4} {12.5} = 0.592$$

Intercept (c)

$$c = \frac {\sum y - m\sum x} {n}$$

$$= \frac {5.37 - 0.592(10)}{5}$$

$$= \frac {5.37 - 5.92}{5}$$

$$= \frac {-0.55}{5} = -0.11$$

Final Equation:

$$\text {Absorbance} = 0.592 \times \text {Concentration (\%)} - 0.11$$

Figure 12: cellulase enzyme assay

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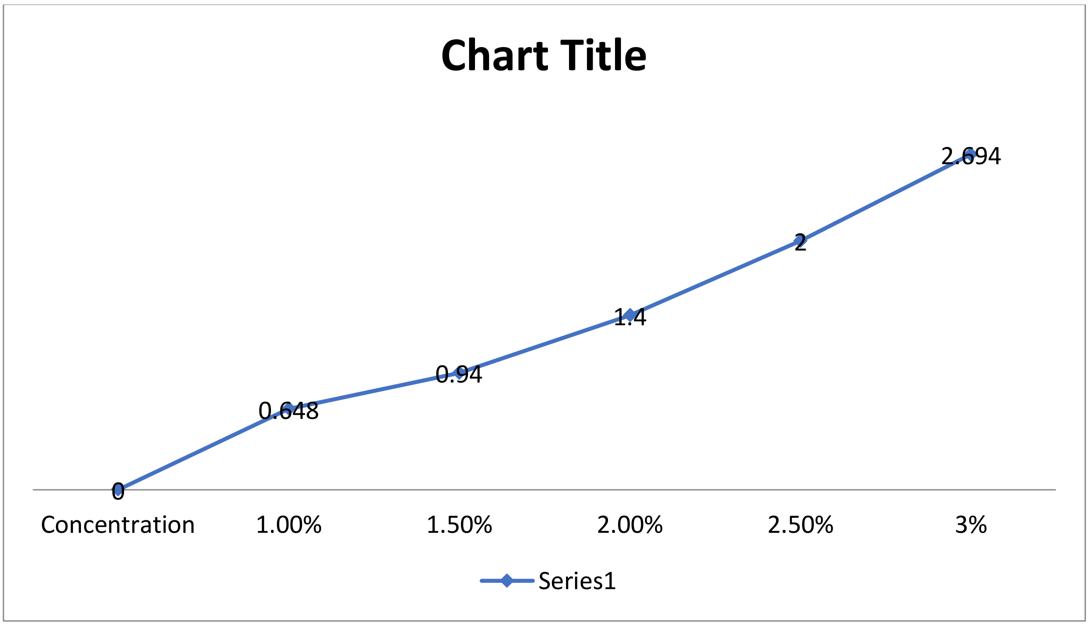
2. **Reducing sugar estimation (DNS assay):**

Prepare standard glucose solutions (0.2–1.0 mg/mL). Mix 1 mL of sample or standard with 1 mL of DNS reagent. Heat the mixture in a boiling water bath for 5–10 minutes. Cool the tubes and add 10 mL distilled water to dilute. Measure absorbance at **540 nm. Plot** a standard curve (absorbance vs. sugar concentration) to quantify unknowns. DNA absorbs UV light maximally at **660 nm**. The concentration of DNA can be determined using UV spectrophotometry based on the Beer- Ambert law:

Absorbance values for assay using different concentrations of substrate	
Concentration of	**Absorbance (540nm)**
1.00%	0.648
1.50%	0.94
2.00%	1.4
2.50%	2
sample	2.694

Table 5: reducing sugar estimation (DNS assay)



Organize the Data

Use the Linear Regression Formula

We want the equation:

y = mx + c

Where:

is absorbance

is concentration (in %)

INTERNATIONAL JOURNAL OF RESEARCH AND SCIENTIFIC INNOVATION (IJRSI)
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is the slope

is the y-intercept

Calculate Slope (m)

m = \frac{n\sum(xy) - (\sum x) (\sum y)} {n\sum(x^2) - (\sum x) ^2}

m = \frac {4(9.858) - (7) (4.988)} {4(13.5) - (7) ^2}

= \frac {39.432 - 34.916} {54 - 49}

= \frac 4.516}{5} = 0.9032

Calculate Intercept (c)

c = \frac {\sum y - m\sum x} {n}

= \frac {4.988 - 0.9032(7)}{4}

= \frac {4.988 - 6.3224}{4}

= \frac {-1.3344}{4} = -0.3336

Final Equation

\text {Absorbance} = 0.9032 \times \text {Concentration (%)} - 0.3336

Calculate Unknown Concentration

You have a sample absorbance of 2.694, so:

2.694 = 0.9032 \times x - 0.3336

Add 0.3336 to both sides:

2.694 + 0.3336 = 0.9032x

Right arrow 3.0276 = 0.9032x

Now divide:

x = \frac {3.0276} {0.9032} \approx. 3.35\%

Final Answer:

The concentration of the sample is 3.35%.

Mix 1 mL of DNA sample with 2 mL of DNA reagent. Incubate the mixture at 95°C in a water bath for 10 minutes. Cool and measure absorbance at **590 nm. Use** a standard curve prepared from known DNA concentrations to calculate sample DNA content.

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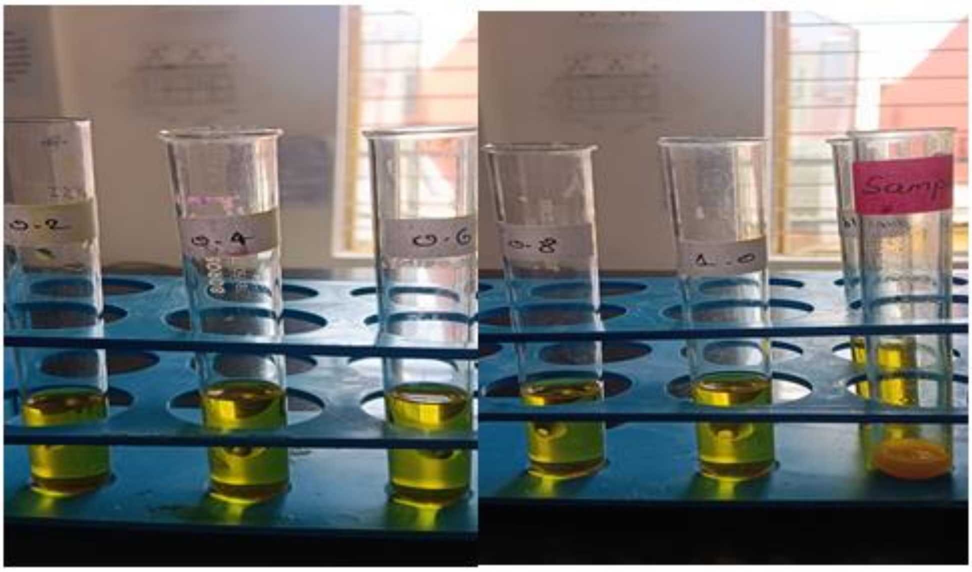

Figure :13 Reducing Sugar Estimation

DISCUSSIONS

The study on enzymatic hydrolysis using cellulase enzyme focuses on the breakdown of lignocellulosic agricultural waste, particularly corn cob powder and soil organic matter who emphasized that pretreatment enhances cellulase efficiency by removing lignin and hemicellulose. Corn cobs, being rich in cellulase, served as an ideal substrate. The experiment was designed by optimizing key parameters such as pH, temperature, and incubation time, similar to the method used by Kumar et al. (2018), who demonstrated increased glucose yield under controlled conditions. The enzyme cellulase was applied to both soil samples and corn cob powder to evaluate sugar release.

Soil samples showed relatively lower sugar yield due to matrix complexity and inhibitory compounds. However, corn cobs yielded significant amounts of reducing sugars after enzymatic treatment, confirming their potential as a bioresource for renewable energy. The experiment also followed the screening and optimization, where cellulase activity was confirmed using DNS assay and absorbance was measured at 540 nm. Overall, the study supports the utilization of agro-waste in eco-friendly bioconversion processes.

CONCLUSION

In conclusion of the experiment, the enzymatic hydrolysis of agricultural waste such as soil samples and corn cob powder using cellulase enzyme has demonstrated promising potential for bioconversion processes in sustainable biotechnology. Through the application of cellulase, the cellulase-rich substrates were effectively broken down into fermentable sugars, showcasing the efficiency of enzymatic treatment in converting biomass into valuable bioproducts. Strains from soil samples and corn cob material were subjected to controlled enzymatic treatment, with optimization of parameters including pH, temperature, and incubation time.

The analysis revealed a higher sugar yield from corn cob powder, likely due to its higher cellulase content and more accessible structure compared to complex organic matter in soil. The hydrolysis efficiency reflects the enzymatic specificity and supports previous research on the suitability of agricultural waste in bio processing industries. The DNS assay conducted confirmed the enzymatic activity, and the optimized conditions significantly enhanced the sugar conversion rate.

This study reinforces the relevance of using cellulase in eco-friendly applications, particularly in the production of bioethanol and other biofuels. The findings support the potential of utilizing agro-waste as a renewable feedstock, thereby reducing environmental load while promoting circular economy principles. With growing demand for sustainable solutions, the use of microbial enzymes like cellulase derived from soil ecosystems or agricultural residues represents a cost-effective and green alternative. Overall, the study contributes to the

ISSN No. 2321-2705 | DOI: 10.51244/IJRSI |Volume XIII Issue I January 2026

ongoing efforts in biotechnological innovation aimed at sustainable resource management and energy production.

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