Journal of Advanced Zoology
ISSN: 0253-7214
Volume 44 S. Issue-5 Year 2023 Page 01:30

Isolation And Characterization Of Cellulase Producing Bacteria From Soil

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Article History

Received:
Revised:
Accepted:

Abstract

The present investigation was undertaken to isolate and Screen the Cellulase Producing Bacteria from Soil. Bacterial cultures were isolated from the soil sample collected from Botanical garden, Karnatak University Campus, Karnataka, India. Four different substrates like *Acacia arabica* pod, *Bauhinia forficata* pod, Cassia *surattensis* pod and *Peltophorum pterocarpum* pods (as cellulose substrate) were used in the submerged production medium. A Total of 57 bacterial cultures were isolated based on Morphology and Biochemical characterization. Among all isolated strains, the three cellulolytic bacterial strains, maximum enzyme activity were showed in *Bacillus cereus* (0.440 IU/ml/min and 0.410 IU/ml/min), followed by *Bacillus subtilius* (0.357 IU/ml/min) and *Bacillus thuringiensis* (0.334 IU/ml/min) to the *Acacia arabica* pod. *Acacia arabica* pod showed maximum enzyme activity comparatively other pods.

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Keywords: *Bacillus* Species, CMC-Agar, Submerged fermentation, Substrates.

Introduction

Enzymes are delicate protein molecules necessary for life. Cellulose is the most abundant biomass on the earth (Venkata *et al.,* 2013) Plant biomass contains cellulose as the major component. Cellulose accounts for 50% of the dry weight of plant biomass and approximately 50% of the dry weight of secondary sources of biomass such as agricultural wastes (Haruta *et al.,* 2003). Presently huge amount of agricultural and industrial cellulosic wastes have been accumulating in environment. Cellulose has attracted worldwide attention as a renewable resource that can be converted into bio- based products and bioenergy (Xing-hua *et al.,* 2009). Celluloses are observed as the most important renewable resource for bioconversion. It has been become the economic interest to develop an effective method to hydrolyze the cellulosic biomass (Saraswati *et al.,* 2012).
Cellulose is commonly degraded by an enzyme called cellulase. This enzyme is produced by several microorganisms, commonly by bacteria and fungi

(Immanuel *et al.,* 2006). Cellulase is an important and essential kind of enzyme for carrying out the depolymerization of
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cellulose into fermentable sugar (Xing-hua *et al.,* 2009). Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on Cellulosic matters (Lee and Koo, 2001). Cellulose degrading microorganisms can convert cellulose into soluble sugars either by acid and enzymatic hydrolysis. Thus, microbial cellulose utilization is responsible for one of the largest material flows in the biosphere (Lynd *et al.,* 2002). Increasing knowledge of mode of action of Cellulase; they were used in enzymatic hydrolysis of cellulosic substances (Kubicek *et al.,* 1993). Despite a worldwide and enormous utilization of natural cellulosic sources, there are still abundant quantities of cellulosic sources, cellulose containing raw materials and waste products that are not exploited or which could be used more efficiently (Sonia *et al.,* 2013). Cellulases are used in the textile industry for cotton softening and denim finishing, in laundry detergents for colour care, cleaning, in the food industry for mashing, in the pulp and paper industries for drainage improvement and fibre modification, and they are even used for pharmaceutical applications. Over all the cellulose enzymes will be commonly used in many industrial applications and the demands for more stable, highly active and specific enzymes will also grow rapidly (Cherry *et al.,* 2003) Cellulases form bacteria are also more effective catalysts. They may also be less inhibited by the presence of material that has already been hydrolyzed. The greatest potential importance is the ease with which bacteria can be genetically engineered (Arifin *et al.,* 2006). Bacteria has high growth rate as compared to fungi has good potential to be used in cellulose production. Some bacterial species viz., Cellulomonas species, *Pseudomonas* species, *Bacillus* species and *Micrococcus* have cellulolytic property (Nakamura and Kappamura, 1982). A large number of microorganisms are capable of degrading cellulose, only a few of them produces significant quantities of cell-free bioactive compounds capable of completely hydrolyzing crystalline cellulose *in vitro*. Numerous investigations have reported the degradation of cellulosic materials, but few studies have examined which microorganisms had met the industrial requirement (Lee and Koo, 2001). Bacteria which have high growth rate as compared to fungi have good potential to be used in cellulase production (Sonia *et al.,* 2013). Among bacteria, Bacillus species produce a number of extracellular enzyme including amylases, proteinases, and polysaccharide hydrolases (Mawadza *et al.,* 2000).

For understanding the mechanism of cellulose degradation by cellulase, it is necessary to isolate, purify and characterize this enzyme. Therefore, the present investigation was designed to isolate and Screen the Cellulase Producing Bacteria from Soil.

Materials and Methods

Isolation of Bacteria

Bacteria were isolated from the soil sample collected from Botanical garden, Karnatak University Campus, Karnataka, India. Traditional serial dilution agar plating method was used for the isolation of cellulolytic bacteria. The medium used for cellulolytic bacteria contains 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and 0.2 % gelatin at pH 7. The Plates were incubated for 48 hours at 30C.

Screening of Bacteria

The incubated CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. 1M NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having clear zone were selected for identification and cellulase production. Further bacterial strains were purified by repeated streaking. The purified colonies were preserved at 4°C.

Screening for cellulase enzyme

Development of Inoculum

The selected bacterial cultures were individually maintained on CMC agar slants at 4C. The selected bacterial cultures were inoculated in broth medium containing 0.03 % $MgSO_4$, 0.2 % K_2HPO_4, 1 % glucose, 0.25 % $(NH_4)_2SO_4$ and 1 % peptone at pH 7 for 24 Hrs of incubation period. After the incubation period these bacterial cells were used as inoculum.

Cellulase enzyme production by Submerged Fermentation Process

The isolated Bacterial strains were screened for cellulase enzyme production in submerged fermentation process. Fermentation medium was prepared by using powders of 1% *Acacia arabica* pod, *Bauhinia forficata* pod, Cassia *surattensis* pod and *Peltophorum pterocarpum* pod (as cellulose substrate), 0.2 % K_2HPO_4, 0.03 % $MgSO_4$, 1 % peptone, 0.25 % $(NH_4)_2SO_4$ and autoclaved at 121ºC for 15min. After autoclave, the medium was inoculated with 1 ml of bacterial isolates and incubated in a rotary shaker at 35C for 24 hrs of fermentation period with agitation speed of 140 rpm. After fermentation the broth was centrifuged at 14000 × g for 10 min at 4C. The supernatant obtained after centrifugation served as crude enzyme source.

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Estimation of Cellulase enzyme

Estimation of Cellulase enzyme activity was assayed using Dinitrosalisic acid (DNS) reagent (Miller, 1959) by estimation of reducing sugars released from CMC. Crude enzyme was added to 0.5 ml of 1 % CMC in 0.05 M phosphate buffer and incubated at 50C for 30 min. After incubation, the reaction was stopped by the addition of 1.5ml of DNS reagent and boiled at 100C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose calibration curve (Shoham *et al.,* 1999). One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1µmol of glucose per minute under standard assay conditions (Muhammad *et al.,* 2012)

Morphological and biochemical characterization

The bacterial strains which produce cellulose enzyme were further subjected to morphological and MR VP test, Citrate utilization test, Starch hydrolysis test, Gelatin hydrolysis test, Nitrate reduction test, Catalase test, Oxidase test, Glucose fermentation test, Lactose fermentation test, Indole test, Urea hydrolysis test, H_2S production test.

Molecular identification of cellulolytic bacteria

The strain which show maximum cellulase activity was further subjected to molecular identification by analysing 16S r RNA
sequence.

Isolation of genomic DNA

2 ml of overnight grown Nutrient broth culture was centrifuged at 10,000 rpm at 4^0C for 10 minutes. The pellet was re suspended in 10 min 10mM Tris, 100 mM Sodium chloride solution and centrifuged at 10,000 rpm 4^0C for 10 minutes. After discarding the supernatant, the pellet was re suspended in 100 µl of $T_{50}E_{20}$ buffer containing 20µl of lysozyme (50mg/ml) and incubated at 37^0C for 20 min, in that solution 1µl of RNase (10 mg/ml) was added and incubated at room temperature for 20 minutes. To this mixture 100µl of SDS (2% in $T_{50}E_{20}$) was added and incubated at 50^0C for 45 min with proper mixing. 2µl of Proteinase K (20mg/ml) was added and incubated at 55^0C for 30 min. The sample was extracted in same volume phenol, Chloroform and Iso-amyl alcohol (25:24:1) and DNA was precipitated with one volume of isopropanol and 0.1 volume of 3M of Sodium acetate. The pellet was washed with 70% Ethanol, dried and dissolved in 100 µl of $T_{10}E_1$ buffer and stored at -20^0C for further use. Concentration of DNA was determined using UV-1800 spectrophotometer (Schimadzu
Corporation). The DNA was stored at 20ºC for further use (Modified method of Sadashiv and Kaliwal, 2013)

Identification of bacteria by sequencing of the 16s rRNA

PCR amplification was performed using Applied Biosystem verti thermal cycler.
The primers for PCR amplification were obtained from Sigma-Aldrich.
Universal Primer (Lane, 1991)
27 forward 5
AGAGTTTCCTGGCTCAG 3
1492 reverse 5
ACGGCTACCTTGTTACGATT 3
The PCR was performed in 20µl reaction mixture containing 2µl of 10X assay buffer, 1µl dNTP mix of 2.5 mM, 0.5µl of mgcl2, 1µl each of forward and reverse primer (5pmol), 0.5µl of Taq polymerase, 1µl of template DNA and 13.5µl of HPLC grade water with the following amplification for 16s rRNA initial denaturation at 95ºC for 4 min followed by 38 cycles of denaturation, annealing and extension (94ºC for 1 min, 59.9ºC for 2 min and 72ºC for 2 min) and final extension at 72ºC for 20 min followed by hold for infinity at 4ºC. The presence of PCR products was determined by 2.5% agarose gel electrophoresis and to analyse the size of

amplified PCR product DNA markers of 100bp was used which was provided by the Puregene. The amplified product was sent for sequencing to SciGenom Labs Pvt Ltd, Cochin, Kerala.

Construction of phylogenetic tree

By using the sequence the bacteria were identified and constructed phylogenetic tree by using NCBI(http://blast.ncbi. nlm.nih.gov/Blast.cgi?PROGRAM=blastn &BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAU LTS=on&LINK_LOC=blasthome) and MEGA 5 Software.

Results and Discussion

A Total of 57 bacterial cultures were isolated based on Morphology and Biochemical characterization. The strains were subjected to Cellulase enzyme production by Submerged Fermentation Process by providing different powders of *Acacia arabica* pod, *Bauhinia forficata* pod, *Cassia surattensis* pod and *Peltophorum pterocarpum* pods (as cellulose substrate). Among all 57 tested bacterial strains B7 (0.440 IU/ml/min) showed maximum enzyme activity, followed by B20 (0. 0.357 IU/ml/min), B37 (0.410 IU/ml/min) and B49 (0.334IU/ml/min) to the *Acacia arabica* pod comparatively other pods (Table 1) All the 57 strains (B1 to B57) were Gram +ve and showed positive for Methyl red test, Voges Proskauer test, Citrate utilization test, Starch hydrolysis test, Gelatin hydrolysis test, Nitrate reduction test, Catalase test, Oxidase test, Glucose fermentation test, Lactose fermentation test and Negative to Indole test, Urea hydrolysis test, H_2 S production test. The highest cellulatic enzyme production strains (B7, B20, B37 and B49) were further subjected to 16S rRNA. The partial amplification of 16S rRNA confirmed on the agarose gel electrophoresis. (Fig.1). By using NCBI and neighbour joining method in MEGA5 the strains were identified as *Bacillus cereus* (B7, B37) (Fig. 2), *Bacillus subtilis* (B20) (Fig. 3) and
Bacillus thuringiensis (B49) (Fig. 4).

Cellulose is converted into fermentable sugars by the enzyme cellulase, and cellulase based bio-refinery technologies are versatile and flexible because they utilize cheaper substrates for enzyme synthesis (Mane *et al.,* 2007). The ability to degrade cellulose is a character distributed among a wide variety of aerobic, facultative aerobic, anaerobic bacteria. Efforts are going on throughout the world to enhance the production and purity of bacterial cellulases (Sreeja *et al.,* 2013). Studying on cellulolytic activity has isolated various bacteria from different environmental sources. (Hatami *et al.,* 2008).

Different Substrates are used in the present study as a carbon source to produce good yield of cellulase enzyme. *Acacia arabica* pod shows maximum enzyme activity comparatively other pods. Similar attempts have been done by many researchers. Ashish Vyas *et al.,* (2005) used groundnut shell, Shuchi Singh *et al.,* (2013) used Rhinoceros Dung, Atchara Sudto *et al.,* (2008) used Agricultural waste for the production of cellulase enzyme. It has been reported that, physico ‗ chemical factors influence the growth of the organisms and also the Cellulase agro - residues by microorganisms depend on many factors, chemical Composition of the agro-residues (cellulose, hemicellulose, lignin, nitrogen, and minerals), presence of an activator or an inhibitor in the agro-residues, diffusion of the catabolite, and type of organisms for fermentation (Chinn *et al.,* 2006). Several microorganisms have been discovered fordecades which have capacity to convert cellulose into simple sugars (Perez *et al.,* 2002).

Many efforts were taken to generate microorganisms with high ability to produce cellulase that can degrade native cellulose (Aristidou and Penttila, 2000). From the present study among all isolated strains, the three cellulolytic bacterial strains the maximum enzyme activity were showed in *Bacillus cereus* (0.440 IU/ml and 0.410 IU/ml), followed by *Bacillus subtilius* (0.357 IU/ml), and *Bacillus thuringiensis* (0.334 IU/ml) to the *Acacia arabica* pod. Similarly Afza *et al.,* (2012) reported 45.42 U/mg cellulase production, Mukesh Kumar *et al.,* (2012) reported cellulase activity 66 U/ml from *Bacillus* cereus which showed more activity when compared to our study and in both studies the strain was confirmed by 16s rDNA method. Venkata *et al.,* (2013) also concluded the *Bacillus cereus* is the promising bacteria to produce cellulase. Bacillus cereus was found to produce the endoglucanase type cellulase (Afza *et al.,* (2012) and most of the isolated *B. cereus* / *B. thuringiensis* strains were found to produce extracellular enzymes (Celenk *et al.,* 2009).

In the present study *Bacillus subtilis* also has been isolated and showed cellulase activity. Similarly Yu-Kyoung Kim, *et al.,* (2012), Ramalingam and Ramasamy, 2013 also reported the cellulase activity of 0.9 unit/mL and 0.140 U/ml respectively, which have high growth rate as compared to fungi, good potential to be used in cellulose production. However, the application of bacteria in producing cellulase is not widely used. (Sonia *et al.,* 2013).

Molecular methods being highly sensitive and selective currently used to identify microorganisms. Environmental conditions may have intense impact on morphological and physiological characteristics, hence

the accurate identification of isolates turned out to be more difficult (Bakri *et al.,* 2010). The molecular techniques are more significant for the characterization of the new isolates, allowing grouping the strains. Furthermore, complex studies (microbiological, biochemical and molecular) are essential, when the identification of new isolate is the purpose of the investigation (Rahna *et al.,* 2013). Species-specific DNA sequences can be used for the identification of bacterial species. The 16s-23s rRNA has proven useful for identification of strains and species (Gurtler & Stanisich, 1996). In the present study the selected three different cellulolytic bacteria such as *Bacillus cereus*, *Bacillus subtilis* and *Bacillus thuringiensis* have been identified based on biochemical and 16s rRNA sequencing. The 16s rRNA sequencing makes it possible to identify and distinguish closely related bacterial species. 16s rRNA method was also used by Shuchi *et al.,* (2013) Where they isolated cellulatic bacteria *Bacillus amyloliquefaciens* from Rhinoceros Dung. Rahna *et al.,* (2013) isolated *Bacillus subtilis* using cellulosic waste as carbon source. Therefore present molecular identification work suggest that, the 16s rRNA sequencing is more accurate for the species identification.

Enzyme production is closely controlled in microorganisms and for improving its productivity, these controls can be improved. Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size, pH value, temperature, presence of inducers, medium additives, aeration, growth time, and so forth (Immanuel *et al.,* 2006). In enzyme fermentation process, the crude extracts contain different mixtures of proteins and undesirable products as organic acids and other metabolites. So that purification of the required favourable product must be take place by different purification methods. (Mukesh Kumar *et al.,* 2012). Optimization of different physicochemical parameter of the production medium is required to get the maximum yield of the enzyme. Further studies were in progress to get high yield production, purification and application of cellulase.

Table.1 Enzyme activity by different strains to different substrates

Sl. No	Strain No	Enzyme activity (IU/ml/minute)			
		Acacia arabica	***Bauhinia forficate***	***Cassia surattensis***	***Peltophorum pterocarpum***
1	B7 (*Bacillus cereus*)	**0.440**	0.213	0.187	0.190
2	B20 (*Bacillus subtilis*)	**0.357**	0.201	0.189	0.178
3	B37 (*Bacillus cereus*)	**0.410**	0.217	0.203	0.187
4	B49 (*Bacillus thuringiensis*)	**0.334**	0.219	0.203	0.193

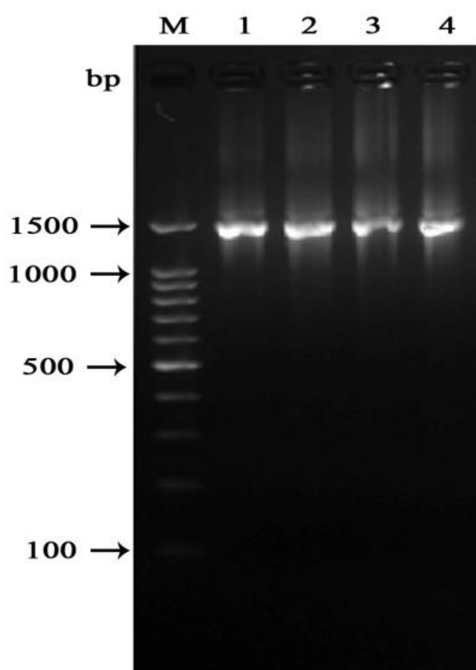


Fig.1 Agarose gel electrophoresis to PCR amplified DNA.

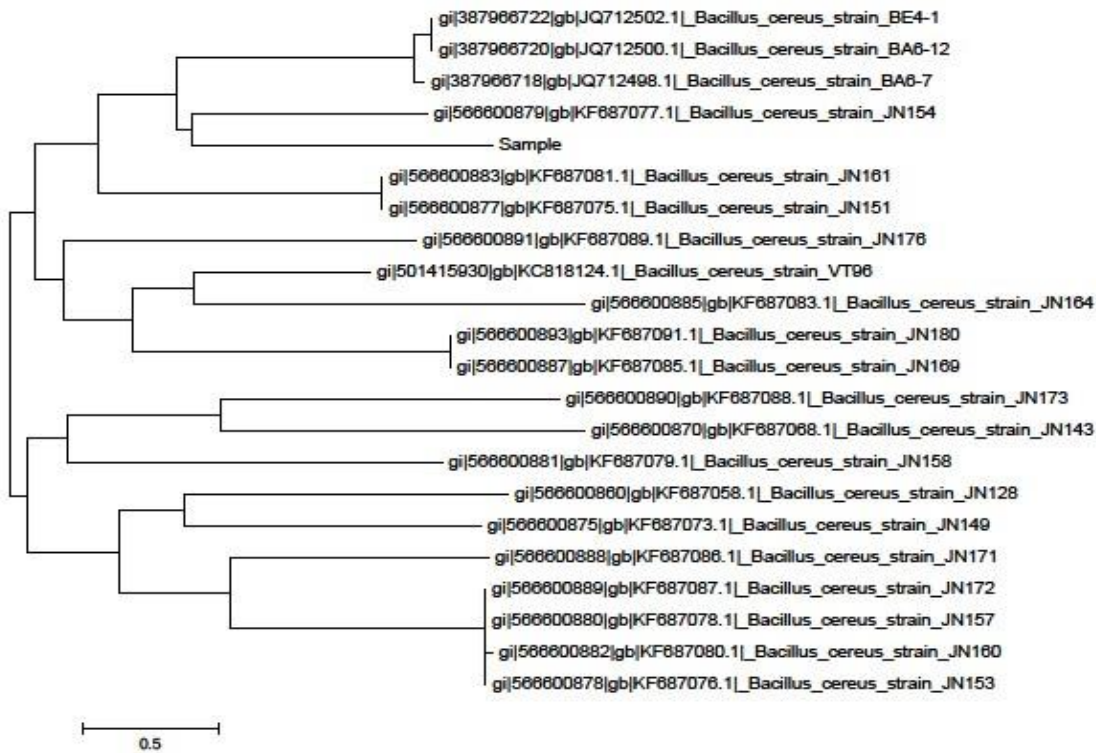


Fig.2 Phylogenetic tree of *Bacillus cereus*

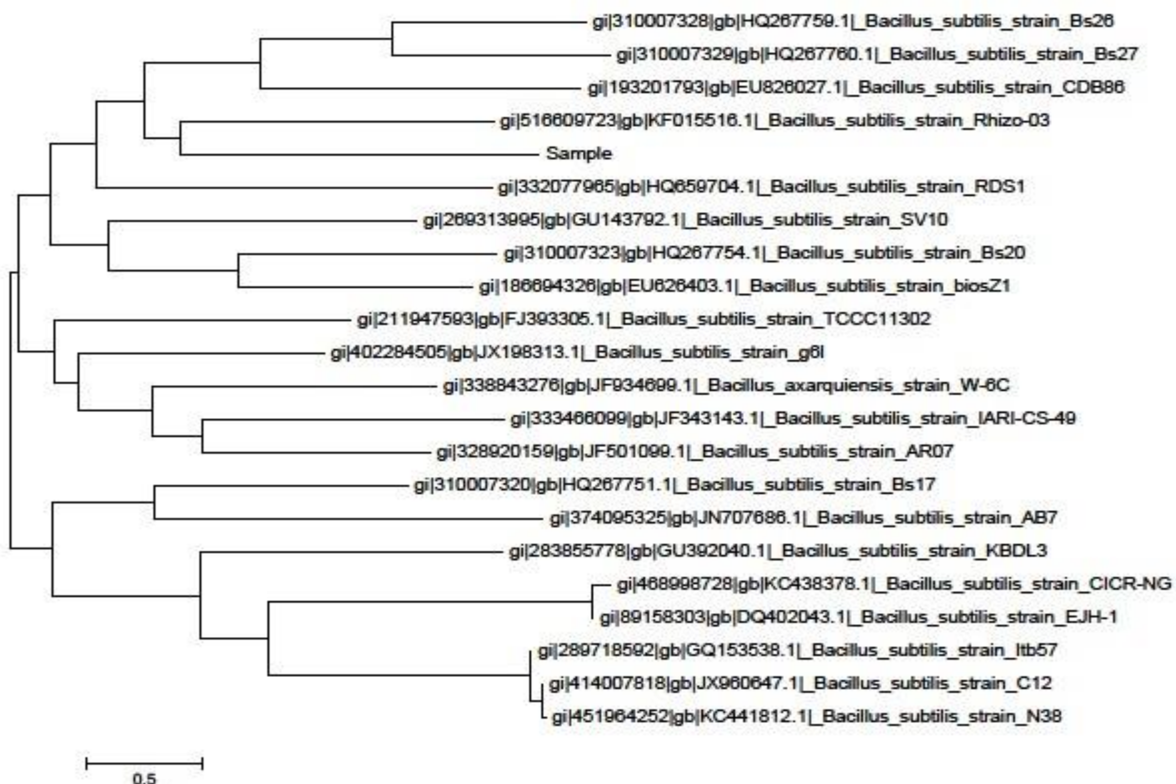


Fig.3 Phylogenetic tree of *Bacillus subtilis*

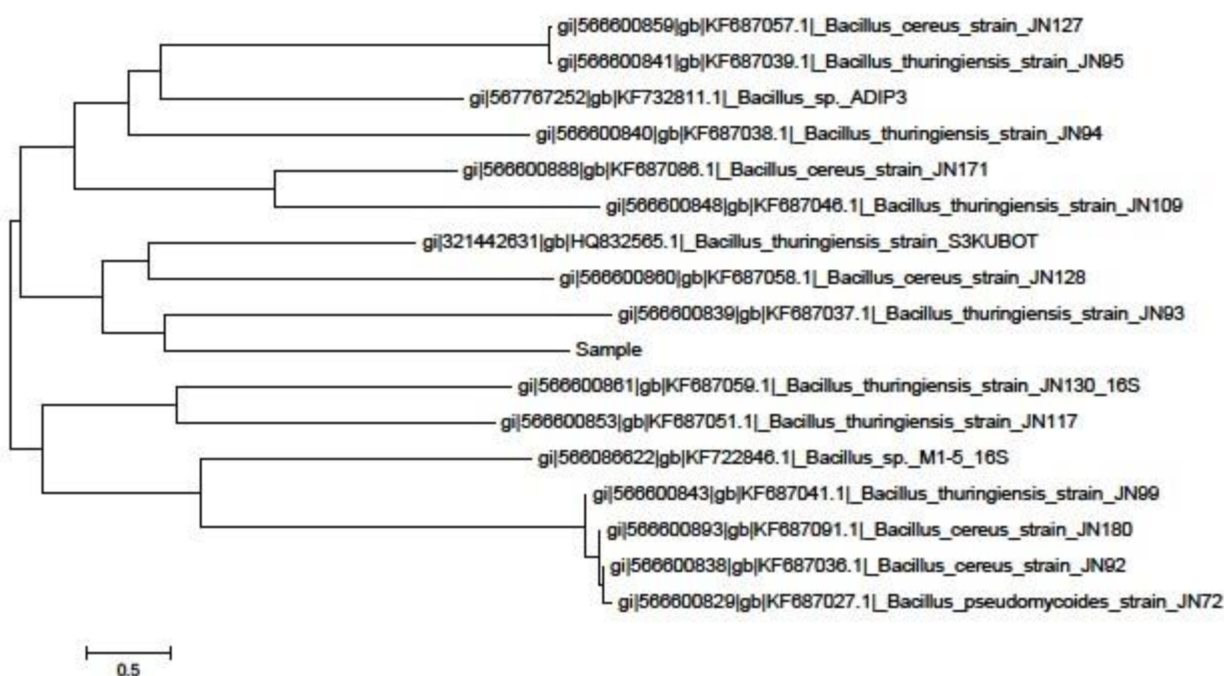


Fig.4 Phylogenetic tree of *Bacillus thuringiensis*

The purified cellulase can be used for various purposes in detergent industries, food industries, and pharmaceutical industries.
In conclusion the three different cellulolytic bacteria such as *Bacillus cereus*, *Bacillus subtilis* and *Bacillus thuringiensis* have been isolated. *Bacillus cereus* showed maximum cellulolytic activity compared to other two isolated bacteria. *Acacia arabica* pod shows maximum enzyme activity comparatively other pods. Optimization of different physico-chemical parameter of the production medium is required to get the maximum yield of the enzyme. Further studies were in progress to get high yield production, purification and application of cellulase.

Acknowledgement

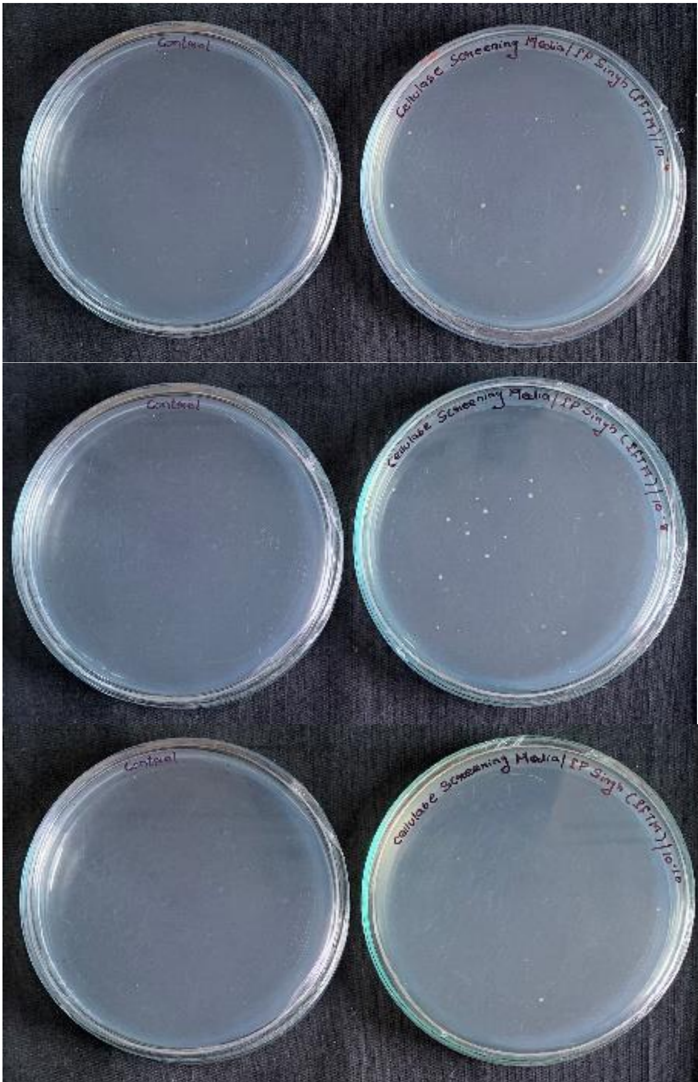
The authors are grateful to the Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India, New Delhi, for funding the Interdisciplinary Program for Life Science Project (BT/PR/4555/INF/22/126/2010 dated 30-09-2010), Bioinformatics Infrastructure Facility Project (BT/BI/25/001/2006 VOL II dt 05-03-2012), UGC-UPE Fellowship(KU/Sch/UGC-UPE/2013-14/1097 Dated21-11-2013) and P. G Departments of Microbiology and Biotechnology Karnatak University, Dharwad for providing the facilities.

TEST REPORT
Test name: Isolation and Screening of Cellulase producing Media, Pure Culture of Positive Isolates, Morphology, Biochemical Test: Catalase Test, Citrate utilization test, Gelatin Hydrolysis Test, Starch Hydrolysis Test, Carbohydrate Utilization, Methyl Red and Voges Proskauer Test
Test Sample: CL002 and CL005

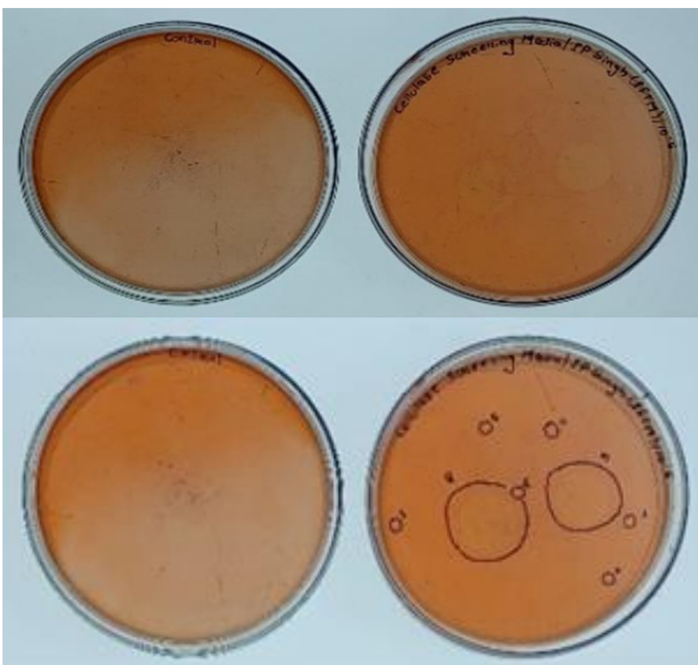
TEST METHOD
Isolation And Screening of Cellulase Producing Bacteria
Procedure:

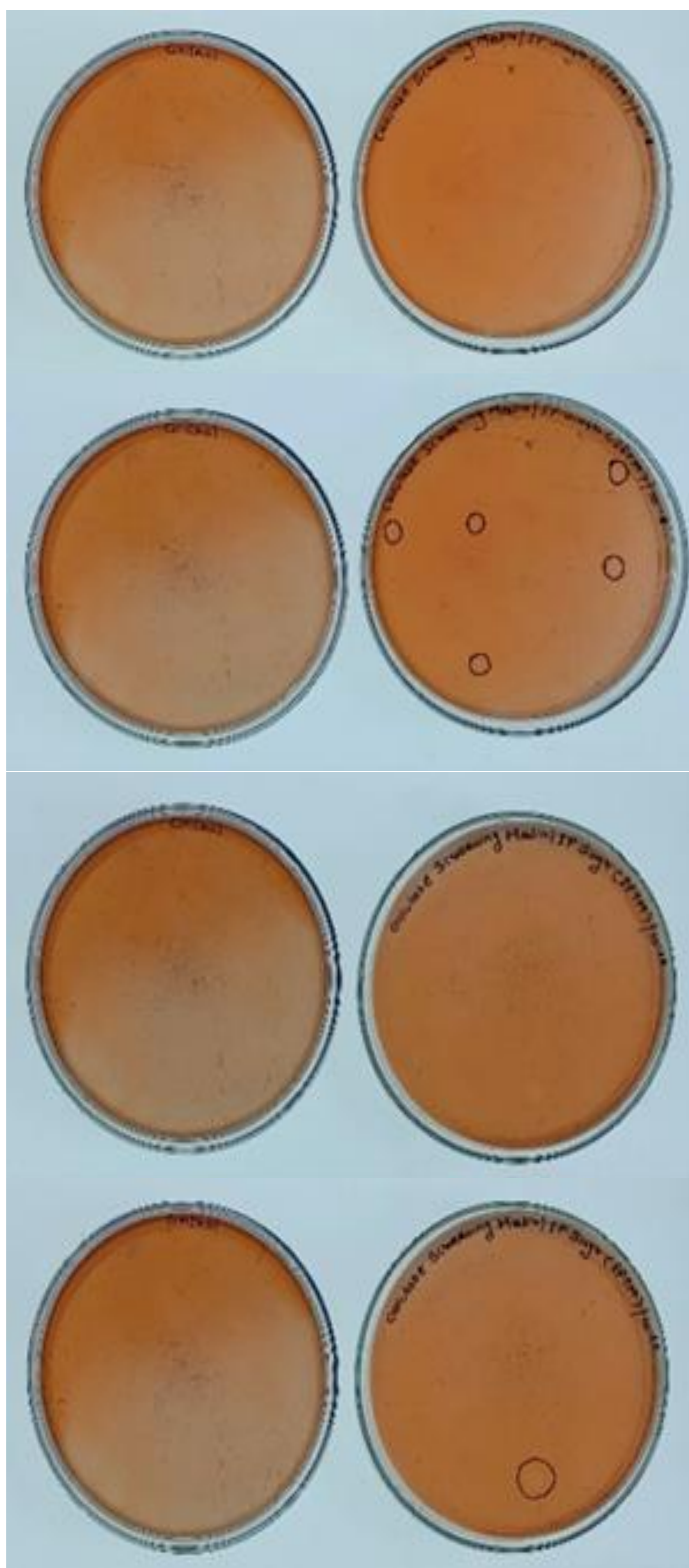
- Soil was collected from UPSIDC Industrial Area, located in Dist-Barabanki
- Collected sample (1 g) was led to serial dilution.
- The sample was diluted up to 10^{-11}
- 100 µl of the solution from concentration 10^{-6}, 10^{-8} and and10^{-10} was transferred into Petri dishes containing carboxymethyl cellulose (CMC) agar media plates containing 0.5 g KH2PO4, 0.25 g
- MgSO4, 0.25 g cellulose and 2 g gelatin for the enhancement of the bacterial activity.
- Petri dishes were then incubated at 37 °C for overnight and preserved at 4 °C.

- Enzyme activity was confirmed by Congo red method.
- Bacterial isolates were inoculated in a basal salt medium containing filter paper for their cellulytic activity test.



Isolation of Cellulase producing





Screening of Cellulase producing

Observation and result:

Morphology and Biochemical results of Isolate 1 : CL002

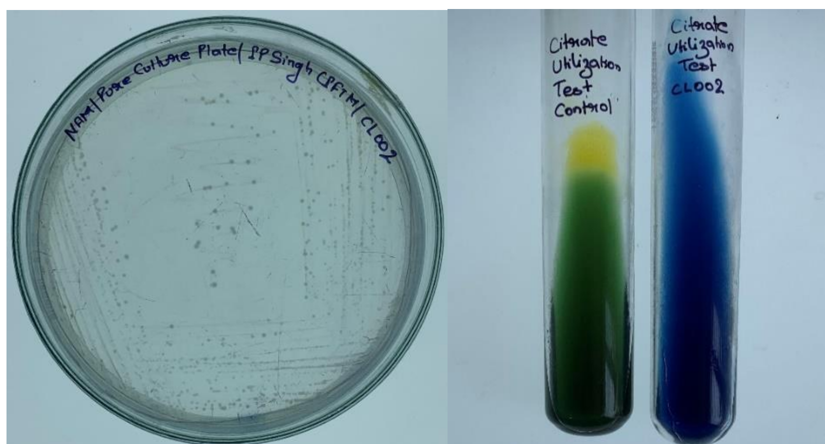
Table 1: Colony appearance of isolate 1 (CL002)

S.No	Shape	Size	Structure	Texture	Appearance	Color	Code
1	Round	Small	Round	Smooth	Creamy	White	CL002

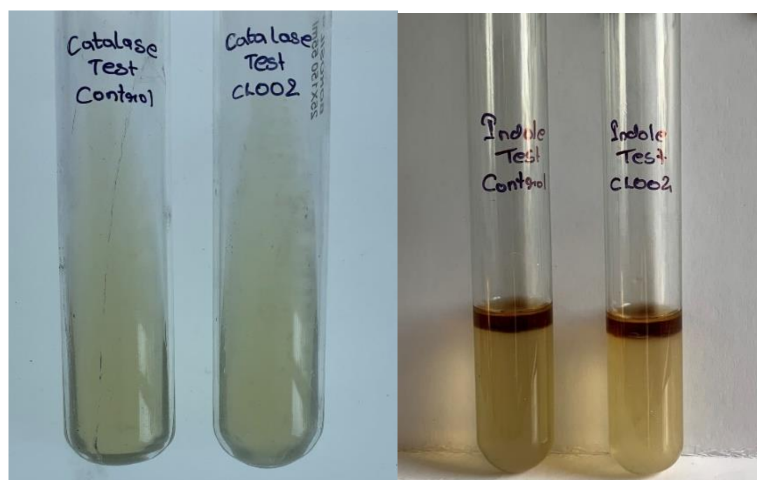
Table 2: Observation and Inference of Biochemical tests of isolate 1 (CL002)

S.No	Test	Observation	Test Result	Inference
1	**Catalase test**	No Bubble formation	-ve	Not a Catalase producing Bacteria
2	**Citrate utilization test**	Color change	+ve	Capable of Fermenting Citrate
3	**Gelatin Hydrolysis Test**	Liquefaction of media	-ve	Not a Gelatinases producing bacteria
4	**Starch Hydrolysis Test**	halo zone	+ve	Amylase producing
5	**Indole Test**	Brown ring	-ve	Unable to decompose tryptophane to indole
6	**Gram's staining**	Purple Colour/round shape	+ve	Gram +ve; coccus
7	**Carbohydrate Utilization Test**	Glucose- Pink to orange colour change	(A/NG)* Weak +ve	Glucose fermenting
		Lactose- Pink to orange	(A/NG)* Weak +ve	Lactose fermenting
		Sucrose- Pink to yellow	(A/NG)* Strong +ve	Sucrose fermenting
8	**Methyl Red**	Red colour ring appearance	+ve	Glucose fermenting with mixed acid formation
9	**Voges Proskauer Test**	No cherry red ring appearance	-ve	No acetyl methyl carbinol

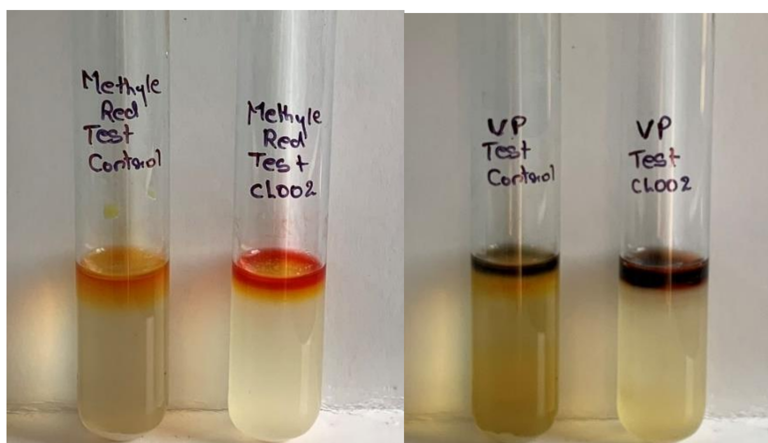
**A : Acid producing ; NG: Non-gas producing*



Pure Culture *Citrate Utilization Test*

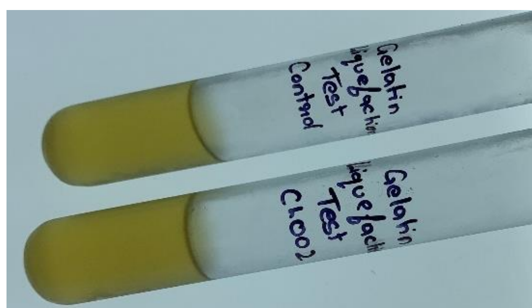


Catalase Test *Indole Test*

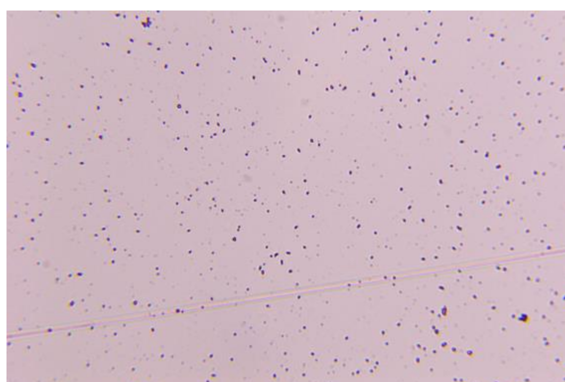


Methyl Red Test *VP Test*

Sugar Fermentation Test *Starch Hydrolysis Test*



Gelatin Liquefaction Test



Gram's Staining

Morphology and Biochemical results of Isolate 1 : CL005

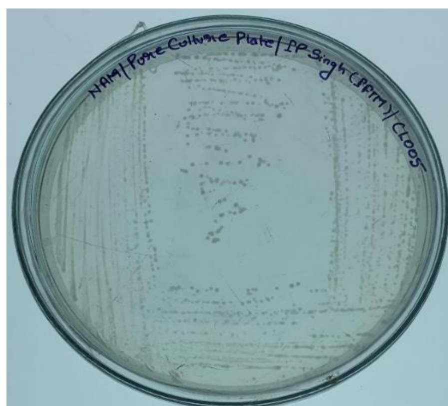
Table 3: Colony appearance of isolate 1 (CL005)

S.No	Shape	Size	Structure	Texture	Appearance	Color	Code
2	Round	Small	Round	Smooth	Creamy	White	CL005

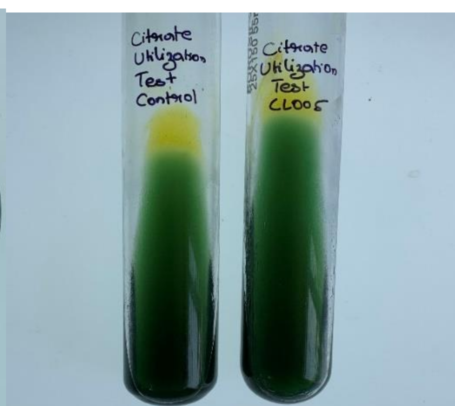
Table 4: Observation and Inference of Biochemical tests of isolate 1 (CL005)

S. No	Test	Observation	Test Result	Inference
1	**Catalase test**	Bubble formation	+ve	Catalase producing
2	**Citrate utilization test**	No Colour change	-ve	Non-Fermentative
3	**Gelatin Hydrolysis Test**	No Liquefaction of media	-ve	Not a Gelatinases producing bacteria
4	**Starch Hydrolysis Test**	Halo zone	+ve	can hydrolyze starch
5	**Indole Test**	Cherry red ring	+ve	Has ability to decompose tryptophane to indole
6	**Gram's staining**	Purple colour/ round shape	+ve	Gram positive; Streptococcus
7	**Carbohydrate Utilization Test**	Glucose- Pink to yellow	(A/NG)* Strong +ve	Weak Glucose fermenting
		Lactose- Pink to yellow	(A/G)* Strong +ve	Weak Lactose fermenting
		Sucrose- No colour change	(NA/NG)* -ve	Non sucrose fermenting
8	**Methyl Red**	Red colour ring appearance	+ve	Glucose fermenting with mixed acid formation
9	**Voges Proskauer Test**	No cherry red ring appearance	-ve	No acetylmethyl carbinol

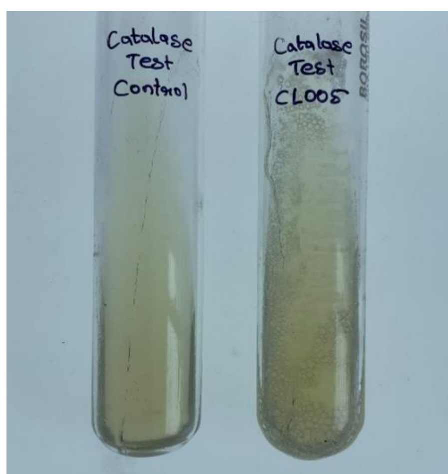
*(A/NG): Acid producing/Non-gas producing
(A/G) : Acid producing/Gas producing
(NA/NG) : Non acid producing/Non-gas producing



Pure Culture

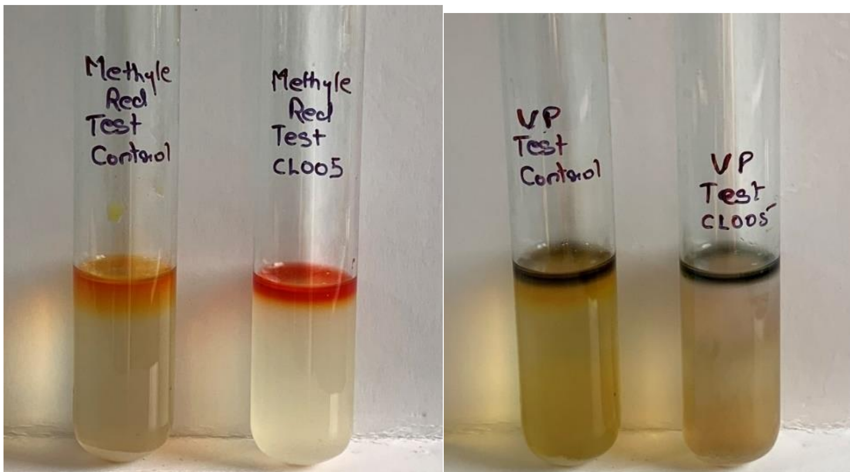


Catalase Utilization Test



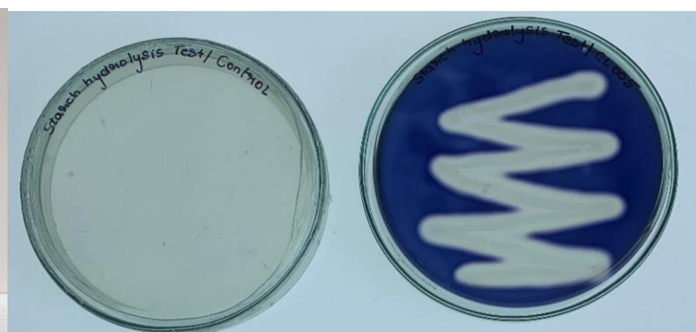
Catalase Test

Indole Test



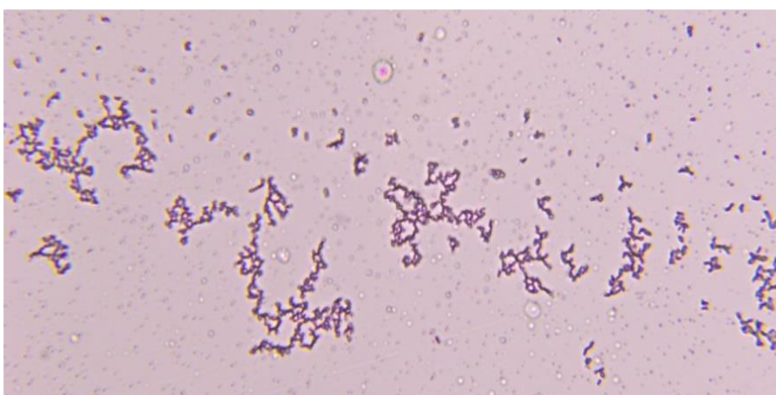
Methyle Red Test *VP Test*

Sugar Fermentation Test



Starch Hydrolysis Test

Gelatin Liquefaction Test



Gram's Stainin

TEST METHOD

Subculturing of isolate

Procedure:

- For the isolation of bacteria, initially Nutrient Agar Media (NAM) was prepared as per the standard composition and the media was autoclaved at 121C and 15psi for 15 minutes in autoclave.
- After the sterilization media was poured in sterile glass petri plates inside the Laminar air flow using the aseptic techniques, each plate was poured with 20ml of the culture media.
- The Plates were then allowed to solidify properly, then the media was inoculated with the respective bacterial isolate.
- After solidification, Flame sterilized the inoculating loop and allowed it to cool down.
- Touched the loop to a corner of culture plates and then streaked it into the plates.
- After streaking, inverted the plates and sealed them with cling tape and kept them in the incubator for 24-48 hours.
- Observed the plates after incubation period for the appearance of microbial colonies.

Citrate Utilization Test

Media Composition

S.No.	Composition	(g/lit)
1	Sodium Chloride	5
2	Sodium Citrate	2
3	Ammonium dihydrogen Phosphate	1
4	Magnesium phosphate	0.2
5	Bromothymol Blue	0.08
6	Agar	20
7	Distilled Water	1000ml

Procedure:

1. For the isolation of bacteria, initially Simmons citrate agar was prepared as per the standard composition and the media was autoclaved at 121°C and 15psi for 15 minutesin autoclave.
2. After the sterilization, media was poured in sterile glass test tubes inside the Laminar air flow using the aseptic techniques
3. The test tubes then were placed in tilt position to make a slant and allowed to solidify properly, then the media was inoculated withthe respective bacteria.
4. After solidification, Flame sterilized the inoculating loop and allowed it to cool down.
5. Touched the loop to a corner of culture test tube and then streak it into the test tube.
6. Streaked the slant from center of isolated colony and kept them in the incubator for 24-48 hours.
7. Next day, after incubation, green and blue colours were obtained.
8. A positive result indicated by the formation of blue colour and negative resultindicated by the absence of colour change.

Catalase test

Composition of Nutrient agar

S.No.	Composition	(g/lit)
1	Yeast extract	1
2	Peptone	5
3	Sodium chloride	5
4	Agar	15
5	Distilled Water	1000 ml

Procedure:

1. For this test, nutrient agar (NAM) was used the media was prepared as per the standard composition and autoclaved for sterilization.
2. After the test tubes cooled down and the agar was solidified completely, they were streak the tube aseptically by taking the growth from 24 hours culture and incubated at 37C for 24 hours
3. Then added 0.5ml H_2O_2 to the test tubes.
4. Place the tube against a white background and observe for immediate bubble formation.

Carbohydrate Fermentation Test

Phenol Red Carbohydrate Broth media composition

S.No.	Composition	Amount (g/lit)
1	Peptone	10
2	Sodium chloride	5
3	Yeast extract	1
4	Phenol red	0.018
5	Carbohydrate source	10
6	Distilled water	1000 ml

Procedure:

1. For this test, carbohydrate fermentation media broth media was used the media wasprepared as per the standard composition and autoclaved for sterilization.
2. After the media cooled down completely, they were inoculated the tube aseptically bytaking the growth from 24 hours culture and incubated at 37̊C for48 hours.
3. At the end of the incubation period, observe the observe the color change from red toyellow along the broth

Positive: Development of yellow color in the medium is indicative of a positivecarbohydrate fermentation reaction.
Negative: No color change is indicative of a negative carbohydrate fermentation reaction

Voges Proskauer Test

VP Broth media composition

S.No.	Composition	Amount (g/lit)
1	Buffered Peptone	7.0
2	Glucose	5.0
3	Dipotassium Phosphate	5.0
4	Distilled Water	1000 ml

Procedure:

1. For this test, carbohydrate MRVP broth was used. The broth was prepared as per the standard composition and autoclaved for sterilization.
2. After the broth cooled down completely, it was inoculated using organism taken from an 18-24 hours pure culture.
3. Incubate aerobically at 37 degrees C. for 24 hours.
4. Following 24 hours of incubation, aliquot 2 ml of the broth to a clean test tube.
5. Re-incubate the remaining broth for an additional 24 hours.
6. Add 6 drops of 5% alpha-naphthol, and mix well to aerate.
7. Add 2 drops of 40% potassium hydroxide, and mix well to aerate.
8. Observe for a pink-red color at the surface within 30 min. Shake the tube vigorously during the 30-min period.
9. At the end of the incubation period, observe the observe the color change from red toyellow along the broth
10. **Positive**: the development of yellow color in the medium is indicative of a positivecarbohydrate fermentation reaction.
11. **Negative**: No color change is indicative of a negative carbohydrate fermentation reaction

Methyl Red Test

MR Broth media composition

S.No.	Composition	Amount (g/lit)
1	Buffered Peptone	7.0
2	Glucose	5.0
3	Dipotassium Phosphate	5.0
4	Distilled Water	1000 ml

Procedure:
1. Prior to inoculation, allow medium to equilibrate to room temperature.
2. Using organisms taken from an 18–24-hour pure culture, lightly inoculate the medium.
3. Incubate aerobically at 37 degrees C. for 24 hours.
4. Following 24 hours of incubation, aliquot 1ml of the broth to a clean test tube.
5. Reincubate the remaining broth for an additional 24 hours.
6. Add 2 to 3 drops of methyl red indicator to aliquot.
7. Observe for red colour immediately.

Indole Test

Indole Broth media composition

S.No.	Composition	Amount (g/lit)
1	Peptone	10.0
2	Sodium Chloride	5.0
3	Tryptophan	1.0
4	Distilled Water	1000 ml

Procedure:
1. Take a sterilized test tubes containing 4 ml of tryptophan broth.
2. Inoculate the tube aseptically by taking the growth from 18 to 24 hrs culture.
3. Incubate the tube at 37°C for 24-28 hours.
4. Add 0.5 ml of Kovac's reagent to the broth culture.
5. Observe for the presence or absence of ring.

Starch Hydrolysis Test

Media composition

S.No.	Composition	(g/lit)
1	Peptic digest of animal tissue	5
2	Sodium Chloride	5
3	Yeast Extract	1.5
4	Beef Extract	1.5
5	Starch soluble	2.0
6	Agar	15
7	Distilled water	1000ml
	Final pH	7.4±0.2

Procedure
1. Using a sterile technique, make a single streak inoculation of organism to be tested into the center of labeled plate.
2. Incubate the bacterial inoculated plates for 48 hours at 37°C.
3. Following incubation, flood the surface of the plates with iodine solution with a dropper for 30 seconds.
4. Pour off the excess iodine.
5. Examine for the clear zone around the line of bacterial growth.
6. Positive result: A clear zone around the line of growth after addition of iodine solution indicates that the organism has hydrolyzed starch.

Gelatin Hydrolysis Method

Media composition

S.No.	Composition	(g/lit)
1	Enzymatic digest of gelatin	5
2	Beef extract	3
3	Gelatin	120
4	Distilled water	1000 ml
	pH	6.8

Procedure
1. Inoculate the gelatin deep with 4 to 5 drops of a 24-hour broth culture.
2. Incubate at 35°-37°C in ambient air for up to 14 days.

Note: Incubate the medium at 25°C if the organism grows better at 25°C than at 35°C.
3. Alternatively, inoculate the gelatin deep from a 24-hour-old colony by stabbing four or five times, 0.5 inch into the medium.
4. Remove the gelatin tube daily from the incubator and place at 4°C to check for liquefaction.

Note: Do not invert or tip the tube, because sometimes the only discernible liquefaction occurs at the top of the deep where inoculation occurred.
5. Refrigerate an un-inoculated control along with the inoculated tube. Liquefaction is determined only after the control has hardened (gelled).

Gram Staining
Reagents Used in Gram Staining
- Crystal Violet, the primary stain
- Iodine, the mordant
- A decolorizer made of acetone and alcohol (95%)
- Safranin, the counterstain

Procedure
1. Take a clean, grease free slide.
2. Prepare the smear of suspension on the clean slide with a loopful of sample.
3. Air dry and heat fix
4. Crystal Violet was poured and kept for about 30 seconds to 1 minutes and rinse with water.
5. Flood the gram's iodine for 1 minute and wash with water.
6. Then, wash with 95% alcohol or acetone for about 10-20 seconds and rinse with water.
7. Add safranin for about 1 minute and wash with water.
8. Air dry, blot dry and Observe under Microscope.

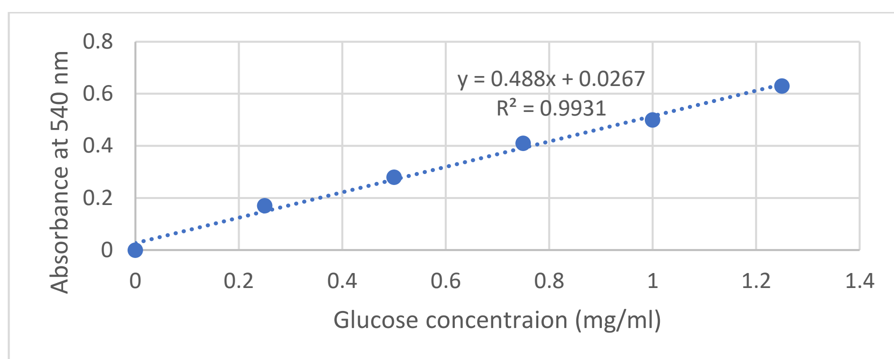
TEST REPORT: 2
Test
Enzyme production; Enzyme Assay; Optimization for Cellulose production (Ph, Temperature, Carbon source, Nitrogen source, Agro-based waste material); Production of enzyme using optimized condition; Ammonium sulphate precipitation; Lowry's assay; Application of Cellulase enzyme

Test Sample: CL002 and CL005

Test Methods: Spectrophotometric assay (PC Based UV-Vis Spectrophotometer Systronic 2202)

Result and Observation:
Table: Absorbance observed at 540 nm for glucose solution at different concentrations

S.No.	Concentration (mg/ml)	Absorbance at 540 nm
1	0	0
2	0.25	0.17
3	0.5	0.28
4	0.75	0.41
5	1	0.5
6	1.25	0.63



Graph: Calibration graph of Glucose for Enzyme activity Assay

Table: Result recorded for Optimization of pH- Glucose released and enzyme activity (U/mL) for isolates CL002 and Cl005

S.No.	Parameter	Sample	pH	Absorbance at 540 nm	Glucose released (mg/ml)	Enzyme activity (U/mL)
1	pH	CL002	7	0.11	0.171	0.126
2		CL002	8	0.38	0.724	0.536
3		CL002	9	1.21	2.425	1.795
4		CL002	10	1.28	2.568	1.901
5		CL002	11	0.27	0.499	0.369
1		CL005	7	0.09	0.130	0.096
2		CL005	8	0.17	0.294	0.217
3		CL005	9	1.35	2.712	2.007
4		CL005	10	1.33	2.671	1.977
5		CL005	11	0.93	1.851	1.370

Table: Result recorded for Optimization of Temperature- Glucose released and enzyme activity (U/mL) for isolates CL002 and Cl005

S.No.	Parameter	Sample	Temp (°C)	Absorbance at 540 nm	Glucose released (mg/ml)	Enzyme activity (U/mL)
1	Temperature	CL002	35	1.11	2.220	1.643
2		CL002	40	1.28	2.568	1.901
3		CL002	45	0.75	1.482	1.097
4		CL002	50	0.54	1.052	0.778
5		CL002	55	0.47	0.908	0.672
6		CL002	60	0.39	0.744	0.551
1		CL005	35	1.31	2.630	1.946
2		CL005	40	1.45	2.917	2.159
3		CL005	45	0.88	1.749	1.294
4		CL005	50	0.65	1.277	0.945
5		CL005	55	0.41	0.785	0.581
6		CL005	60	0.37	0.703	0.521

Table: Result recorded for Optimization of Carbon source- Glucose released and enzyme activity (U/mL) for isolates CL002 and Cl005

S.No.	Parameter	Sample	Carbon source	Absorbance at 540 nm	Glucose released (mg/ml)	Enzyme activity (U/mL)
1	Carbon source	CL002	Starch	1.15	2.302	1.704
2		CL002	Glucose	1.93	3.900	2.887
3		CL002	Maltose	1.04	2.076	1.537
4		CL002	Lactose	0.91	1.810	1.340
5		CL002	Fructose	0.83	1.646	1.218
1		CL005	Starch	1.85	3.736	2.765
2		CL005	Glucose	2.01	4.064	3.008
3		CL005	Maltose	1.54	3.101	2.295
4		CL005	Lactose	1.6	3.224	2.386
5		CL005	Fructose	1.22	2.445	1.810

Table: Result recorded for Optimization of Nitrogen source- Glucose released and enzyme activity (U/mL) for isolates CL002 and Cl005

S.No.	Parameter	Sample	Nitrogen source	Absorbance at 540 nm	Glucose released (mg/ml)	Enzyme activity (U/mL)
1	Nitrogen source	CL002	Yeast extract	1.86	3.757	2.780
2		CL002	Peptone	1.73	3.490	2.583
3		CL002	Urea	1.66	3.347	2.477
4		CL002	Ammonium Sulphate	1.41	2.835	2.098
1		CL005	Yeast extract	1.62	3.265	2.416
2		CL005	Peptone	1.84	3.716	2.750
3		CL005	Urea	1.33	2.671	1.977
4		CL005	Ammonium Sulphate	1.31	2.630	1.946

Table: Result recorded for Optimization of Agri-waste substrate- Glucose released and enzyme activity (U/mL) for isolates CL002 and Cl005

S.No.	Parameter	Sample	Agri-waste substrate	Absorbance at 540 nm	Glucose released (mg/ml)	Enzyme activity (U/mL)
1	Agri-waste substrate	CL002	Groundnut cake	0.64	1.257	0.930
2		CL002	Coconut cake	0.52	1.011	0.748
3		CL002	Soy cake	0.41	0.785	0.581
4		CL002	Wheat bran	0.78	1.544	1.142
1		CL005	Groundnut cake	0.34	0.642	0.475
2		CL005	Coconut cake	0.21	0.376	0.278
3		CL005	Soy cake	0.27	0.499	0.369
4		CL005	Wheat bran	0.73	1.441	1.067

Table: Result recorded for Optimization of Different concentration of Optimized C-source- Glucose released and enzyme activity (U/mL) for isolates CL002 and Cl005

S.No.	Parameter	Sample	% C-source (Glucose)	Absorbance at 540 nm	Glucose released (mg/ml)	Enzyme activity (U/mL)
1	Concentration of C-Source (Glucose)	CL002	1	0.15	0.253	0.187
2		CL002	2	0.18	0.314	0.232
3		CL002	3	1.63	3.285	2.432
4		CL002	4	1.72	3.470	2.568
5		CL002	5	1.82	3.675	2.720
1		CL005	1	0.09	0.130	0.096
2		CL005	2	0.07	0.089	0.066
3		CL005	3	1.15	2.302	1.704
4		CL005	4	1.47	2.958	2.189
5		CL005	5	1.59	3.203	2.371

Enzyme activity calculation at Optimized condition

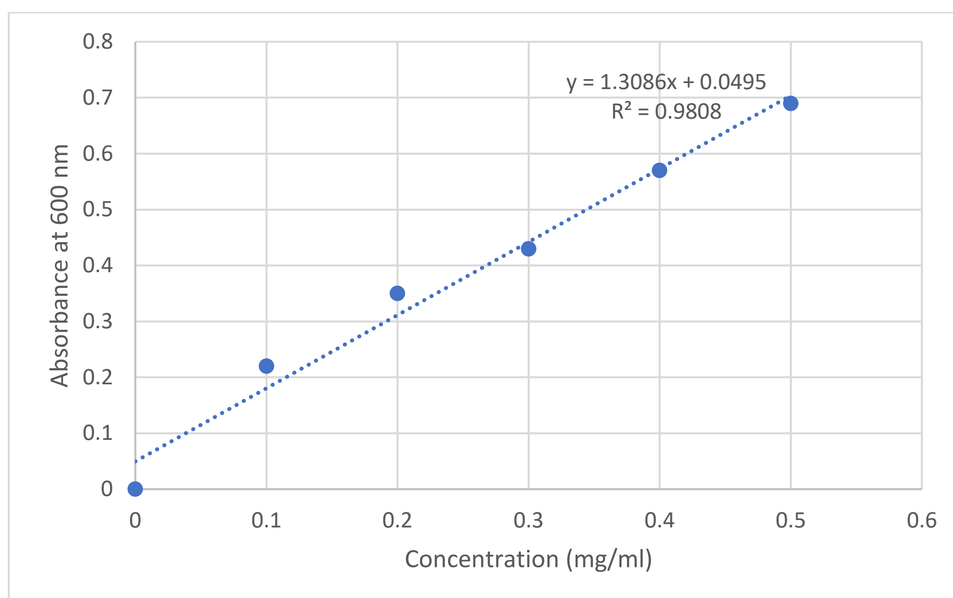
Table: Enzyme activity (U/mL) for isolates CL002 and Cl005 under Optimized conditions

S.No.	Sample code	Conditions	Absorbance at 540 nm	Glucose released (mg/ml)	Enzyme activity (U/mL)
1	CL002	pH-10; 40°C; N-source- yeast extract	2.24	4.699	3.478
2	CL005	pH-9; Temp-40°C; N-source- peptone	2.15	4.351	3.220

Protein Estimation using Lowry's Assay

Table: Absorbance observed at 600 nm for BSA solution at different concentrations

S.No.	Concentration (mg/ml)	Absorbance at 600 nm
1	0	0
2	0.1	0.22
3	0.2	0.35
4	0.3	0.43
5	0.4	0.57
6	0.5	0.69



Graph: Calibration graph of BSA for Protein estimation Assay

Table: Protein concentration observed in crude enzyme extracted from isolates

S.No.	Sample code	Absorbance at 600 nm	Protein concentration (mg/ml)	Enzyme activity (U/mL)	Specific activity (U/mg)
1	CL002	1.32	0.971	3.478	3.582
2	CL005	1.14	0.833	3.22	3.864

TEST METHOD

Enzyme Production Medium

In a conical flask with a capacity of 100 mL, ten mL of medium were withdrawn. After being sterilized in an autoclave at a temperature of 121°C for fifteen minutes, the flasks were allowed to cool before being inoculated with a bacterial culture that had grown overnight. After being inoculated, the medium was shaken for 24 hours at 37°C inside an incubator. After the fermentation process was complete, the culture medium was centrifuged at 5000 rpm for 15 min in order to obtain the crude extract, which was used as an enzyme source.

Production medium (g/L)

S.No.	Composition	Amount (g/L)
1	glucose	0.5 gm
2	peptone	0.75 gm
3	$FeSO_4$	0.01 gm
4	KH_2PO_4	0.5 gm
5	$MgSO_4$	0.5 gm

Ammonium Sulphate Precipitation

- Crude Enzyme was precipitated using Salting out method using Ammonium Sulphate. Desired saturation range of ammonium sulphate was 40-80%, therefore precipitation was conducted in three ranges of 0-20%,20-40% and then 40-80%.
- Precipitation was conducted by mixing calculated amount of ammonium sulphate salt in the extracted crude enzyme. The amount of ammonium sulphate and crude enzyme used were as follows:
- Initially, 20 ml of crude enzyme was taken and mixed with 2.30gm of ammonium sulphate crystals for 0-20% saturation. Stirred for 1 hour to fully equilibrate.
- Centrifugation was done at 10,000g for 15 minutes to pellet out protein.
- Added more saturated ammonium sulfate or solid ammonium sulfate to make next concentration, repeat stirring and centrifugation.
- Next, for 21.25ml of solution 2.61gms of ammonium sulphate crystals for 20-40% saturation. Stirred for 1 hour to fully equilibrate.
- Centrifugation was done at 10,000g for 15 minutes to pellet out protein.

- Then finally, for 22.67ml of solution 6.43gm of ammonium sulphate crystals for 40-80% saturation. Stirred for 1 hour to fully equilibrate.
- Centrifugation was done at 10,000g for 15 minutes to pellet out protein.
- Pooled and dissolved pellets obtained at three stages in PBS and moved to the next step to further dialyze out the Ammonium Sulfate.

Dialysis

- Dialysis tubing was carefully cut of 10 cm using sterile scissor. After cutting the proper length of the dialysis bag, it was activated by keeping in a beaker filled with distilled water at 80C.
- Now tie the membrane at one end with thread (make sure no leakage is there by tying it very tightly with the thread)
- Now add the precipitated enzyme in the tubing using sterile micropipette tip. Once added, press the free end of tubing using finger to prevent entrapment of any bubbles.
- Again turn the free end of tubing and tie with another piece of thread very tightly.
- The dialysis bag was then suspended in the dialysis buffer**. A stir bar was placed in the dialysis buffer and kept for stirring.
- Dialysis buffer was changed thrice during the process and stored at 4C overnight after third time changing the buffer.
- After completion of the incubation period, samples were collected from dialysis bag and centrifuged at 10000 rpm for 10 mins.
- After centrifuging, the supernatant was collected and store at -20°C

Enzyme Assay

The method developed by Miller **(Miller *et al*., 1959)** was used to determine the level of cellulase activity. In a nutshell, a reaction mixture that consisted of 0.2 mL of crude enzyme solution and 1.8 mL of 0.5% carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7) was incubated at 37 °C in a shaking water bath for 30 minutes. This was done in order to achieve the desired results. The reaction was stopped when 3 mL of DNS reagent was added to the mixture. After that, the colour was developed in the mixture by bringing it to a boil for five minutes. The optical density (OD) of the samples was measured at 575 nm in comparison to a blank that consisted of all the reagents except for the crude enzyme. The enzyme activity was calculated in terms of the micromoles of glucose units released in 1 minute using the standard graph of glucose.
U/ml = Released glucose concentration (mg/ml) * Reaction volume (ml) * Dilution factor * 1000/ Incubation time (min) * volume of enzyme (ml) * Mol. Wt. of glucose (mg/mol)
Where,
Reaction volume= 5.0 ml
Enzyme volume= 0.2ml
DF=160 (Enzyme pellet= 12.5 mg;Buffer used for dilution= 2 ml)
Mol wt= 180156 mg/mol

Process Optimization for Maximum Cellulase Production

pH

After taking flasks with broth that already contains the optimal concentration of substrate and carbon source, the pH of the broth is adjusted to 7.0, 8.0, 9.0, 10.0, and 11.0 in various flasks using 1 N HCl and 1 N NaOH, and then the broth is sterilised. The cultures are then inoculated before being placed in an incubator at 37C temperature. At the completion of the incubation period, the cell-free culture filtrate is extracted, and it is subsequently put to use as a source of enzyme for enzymatic activity determination.

Temperature

Production medium with a pH of 7 was inoculated with a selected bacterial strain that had been grown overnight. For a period of twenty-four hours, the broth was heated to 35, 40, 45, 50, 55, and 60 degrees Celsius at various intervals. At the ending of the incubation period, the cell-free culture filtrate is extracted, and it is subsequently put to use as a source of enzyme for enzymatic activity determination.

Carbon Sources

The effects of a variety of carbon sources, including starch, glucose, maltose, lactose, and fructose, were investigated in the production medium at concentrations ranging from 1% to 5%.

Nitrogen Sources

By substituting 0.5% of the peptone in the production medium with one of many other nitrogen sources, such as yeast extract, peptone, urea, or ammonium sulphate, the effects of these nitrogen sources on enzyme production were analysed.

Agro-Based Waste Material

In order to determine whether or not agro-based waste is suitable for use as a substrate in the production of enzymes, many different types of substrates, including groundnut cake, coconut cake, soy cake, and wheat bran, are placed in the growing medium while it is submerged. After twenty-four hours, the enzyme synthesis is evaluated by measuring the enzyme activity.

- **Production of enzyme using optimized condition**

Enzyme production was done using optimized condition as described above

- **Lowry's assay protein content determination**

Reagents:

F.C (Folin Ciocalteu) reagent
Bovine Serum Albumin (1mg/ml)
Reagent A – 2% Na_2Co_3 + 0.1N NaOH.
Reagent B - 2% $CuSO_4.5H_2O$.
Reagent C – 2% Potassium Sodium Tartarate
Reagent D – 99ml + 0.5 ml reagent B + 0.5 ml Reagent C.
Standard protein solution: Prepare BSA solution of concentration 1mg/ml.

Procedure:

Arrange the clean dry test tube on the stand and label them as per the table shows. Add the components to each labelled tube according to the information in the table below.

Table: Final components in the tube for Lowry estimation of protein

<table>
<tr><th>S. No.</th><th>Test tube name</th><th>Solution (µl)</th><th>Distilled water (µl)</th><th>Reagent D</th><th>Incubation time</th><th>FC reagent</th><th>Incubation time</th><th>OD at 630nm</th></tr>
<tr><td>1</td><td>Blank</td><td>0</td><td>1000</td><td>5 ml</td><td rowspan="7">10 minutes in Dark at RT</td><td>0.5 ml</td><td rowspan="7">30 Minutes in dark at RT</td><td></td></tr>
<tr><td>2</td><td>1 std.</td><td>100 BSA</td><td>900</td><td>5 ml</td><td>0.5 ml</td><td></td></tr>
<tr><td>3</td><td>2 std.</td><td>200 BSA</td><td>800</td><td>5 ml</td><td>0.5 ml</td><td></td></tr>
<tr><td>4</td><td>3 std.</td><td>300 BSA</td><td>700</td><td>5 ml</td><td>0.5 ml</td><td></td></tr>
<tr><td>5</td><td>4 std.</td><td>400 BSA</td><td>600</td><td>5 ml</td><td>0.5 ml</td><td></td></tr>
<tr><td>6</td><td>5 std.</td><td>500 BSA</td><td>500</td><td>5 ml</td><td>0.5 ml</td><td></td></tr>
<tr><td>7</td><td>Sample</td><td>100 sample</td><td>900</td><td>5 ml</td><td>0.5 ml</td><td></td></tr>
</table>

Add 5mL of reagent D to each tube including the blank. Mix well and allow it to stand for 10min. Then add 0.5mL of reagent D, mix well and incubate at room temp. in the dark for 30min. Blue color is developed. Take the reading at 660nm. Draw a standard graph and calculate the amount of protein in the sample.

Miller G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 1959;31(3):426–428.

TEST REPORT:3

Test: Application of Cellulase enzyme on General Biomedical waste

Test Sample: CL002 and CL005 ; Cotton swabs (CS), Dressings and bandages (DB), Plaster casts (PC), Discarded gloves (DG), Tissue and bits of papers (TP)

Test Methods: Spectrophotometric assay (PC Based UV-Vis Spectrophotometer Systronic 2202)

Result and Observation

1. % Degradation efficiency of Crude cellulase enzyme on general biomedical waste material

Table 1: % Degradation efficiency of Crude cellulase enzyme from isolates CL002 and CL005 on general biomedical waste material at variable temperature

Parameter	Sample	CL002			CL005		
Temperature		Initial wt. (mg)	final wt. (mg)	%Degradation	Initial wt. (mg)	final wt. (mg)	%Degradation
		30					
	CS	25	18.6	25.6	25	20.6	17.6
	DB	25	23.4	6.4	25	22.4	10.4
	PC	25	23.9	4.4	25	21.9	12.4
	DG	25	25	0	25	25	0
	TP	25	11	56	25	13.41	46.36
		35					
	CS	25	17.3	30.8	25	19.81	20.76
	DB	25	21.5	14	25	19.3	22.8
	PC	25	20.7	17.2	25	21.5	14
	DG	25	25	0	25	25	0
	TP	25	13.6	45.6	25	13.3	46.8
		40					
	CS	25	6.4	74.4	25	11.56	53.76
	DB	25	17.4	30.4	25	15.4	38.4
	PC	25	13.2	47.2	25	11.11	55.56
	DG	25	25	0	25	25	0
	TP	25	9.6	61.6	25	8.6	65.6
		45					
	CS	25	15.5	38	25	16.41	34.36
	DB	25	16.7	33.2	25	16	36
	PC	25	19	24	25	20.41	18.36
	DG	25	25	0	25	25	0
	TP	25	11.3	54.8	25	14.1	43.6
		50					
	CS	25	17.3	30.8	25	18.2	27.2
	DB	25	18.6	25.6	25	18.5	26
	PC	25	19.3	22.8	25	21.4	14.4
	DG	25	25	0	25	25	0
	TP	25	22.3	10.8	25	19.3	22.8

***** Cotton swabs -CS ; Dressings and bandages – DB ; Plaster casts – PC ; Discarded gloves – DG ; Tissue and bits of papers - TP

Table 2: % Degradation efficiency of Crude cellulase enzyme from isolates CL002 and CL005 on general biomedical waste material at variable pH

Parameter	Sample	CL002			CL005		
pH							
		Initial wt. (mg)	final wt. (mg)	%Degradation	Initial wt. (mg)	final wt. (mg)	%Degradation
		3					
	CS	25	17.8	28.8	25	14.3	42.8
	DB	25	23.5	6	25	21.4	14.4
	PC	25	14.7	41.2	25	17.64	29.44
	DG	25	25	0	25	25	0
	TP	25	14.9	40.4	25	14.9	40.4
		5					
	CS	25	17.3	30.8	25	18.6	25.6
	DB	25	16.3	34.8	25	14.3	42.8
	PC	25	20	20	25	18.33	26.68
	DG	25	25	0	25	25	0
	TP	25	16.7	33.2	25	15.8	36.8

		6					
	CS	25	24.7	1.2	25	12.9	48.4
	DB	25	23.3	6.8	25	17.1	31.6
	PC	25	20.9	16.4	25	11.7	53.2
	DG	25	25	0	25	25	0
	TP	25	17.9	28.4	25	10.3	58.8
		7					
	CS	25	20.6	17.6	25	21.1	15.6
	DB	25	18.1	27.6	25	24.6	1.6
	PC	25	21.09	15.64	25	24	4
	DG	25	25	0	25	25	0
	TP	25	17.7	29.2	25	20.5	18
		8					
	CS	25	19	24	25	19.33	22.68
	DB	25	19.7	21.2	25	18.5	26
	PC	25	18.5	26	25	18.5	26
	DG	25	25	0	25	25	0
	TP	25	18.7	25.2	25	17.2	31.2

***** Cotton swabs -CS ; Dressings and bandages – DB ; Plaster casts – PC ; Discarded gloves – DG ; Tissue and bits of papers - TP

Table 3: % Degradation efficiency of Crude cellulase enzyme from isolates CL002 and CL005 on general biomedical waste material at variable agitation speed

Parameters	**Sample**	**Initial wt. (mg)**	**final wt.** (mg)	**%Degradation**	**Initial wt.** (mg)	**final wt.** (mg)	**%Degradation**
Agitation speed	100						
	CS	25	23.3	6.8	25	20.13	19.48
	DB	25	21.56	13.76	25	20.41	18.36
	PC	25	22.5	10	25	20.02	19.92
	DG	25	25	0	25	25	0
	TP	25	23.8	4.8	25	19.8	20.8
	150						
	CS	25	12.4	50.4	25	13.5	46
	DB	25	13.7	45.2	25	12.7	49.2
	PC	25	16	36	25	15.21	39.16
	DG	25	25	0	25	25	0
	TP	25	9.07	63.72	25	12.08	51.68
	200						
	CS	25	17.9	28.4	25	19.45	22.2
	DB	25	17.9	28.4	25	21.4	14.4
	PC	25	15.4	38.4	25	16.33	34.68
	DG	25	25	0	25	25	0
	TP	25	16.3	34.8	25	18.4	26.4
	250						
	CS	25	21.05	15.8	25	22.5	10
	DB	25	23.8	4.8	25	22	12
	PC	25	23.1	7.6	25	23.5	6
	DG	25	25	0	25	25	0
	TP	25	24.5	2	25	24.8	0.8
	300						
	CS	25	21.3	14.8	25	20.4	18.4
	DB	25	23	8	25	21.4	14.4
	PC	25	23.4	6.4	25	22.5	10
	DG	25	25	0	25	25	0
	TP	25	23.7	5.2	25	21.6	13.6

***** Cotton swabs -CS ; Dressings and bandages – DB ; Plaster casts – PC ; Discarded gloves – DG ; Tissue and bits of papers – TP

Table 4: % Degradation efficiency of Crude cellulase enzyme from isolates CL002 and CL005 on general biomedical waste material at variable proportion of crude enzyme

Parameters	Sample	Initial wt. (mg)	final wt. (mg)	%Degradation	Initial wt. (mg)	final wt. (mg)	%Degradation
Concentration		**2**					
	CS	25	19.6	21.6	25	20.5	18
	DB	25	18.33	26.68	25	14.8	40.8
	PC	25	21.5	14	25	17.5	30
	DG	25	25	0	25	25	0
	TP	25	17	32	25	15.9	36.4
		4					
	CS	25	18.75	25	25	24.3	2.8
	DB	25	18.1	27.6	25	21.5	14
	PC	25	21.26	14.96	25	20.3	18.8
	DG	25	25	0	25	25	0
	TP	25	15.3	38.8	25	19.7	21.2
		6					
	CS	25	13.4	46.4	25	15.5	38
	DB	25	11.84	52.64	25	13.56	45.76
	PC	25	11.36	54.56	25	17.2	31.2
	DG	25	25	0	25	25	0
	TP	25	10.41	58.36	25	11.71	53.16
		8					
	CS	25	14.7	41.2	25	17.9	28.4
	DB	25	15.03	39.88	25	15.01	39.96
	PC	25	15.9	36.4	25	18.3	26.8
	DG	25	25	0	25	25	0
	TP	25	12.2	51.2	25	14.5	42
		10					
	CS	25	23.65	5.4	25	20.35	18.6
	DB	25	21.42	14.32	25	20.1	19.6
	PC	25	21.5	14	25	21.5	14
	DG	25	25	0	25	25	0
	TP	25	21.9	12.4	25	19.9	20.4

***** Cotton swabs -CS ; Dressings and bandages – DB ; Plaster casts – PC ; Discarded gloves – DG ; Tissue and bits of papers - TP

Table 5: Sugar estimation in degradation reaction from crude cellulase enzyme of isolates CL002 and CL005 on general biomedical waste material at variable temperature

Parameter	Sample	CL002		CL005	
		Absorbance	**Concentration (mg/ml)**	**Absorbance**	**Concentration (mg/ml)**
Temperature		**30°C**			
	CS	0.910	1.810	1.180	2.363
	DB	0.070	0.089	0.170	0.294
	PC	0.040	0.027	0.960	1.913
	DG	0.030	0.007	0.040	0.027
	TP	1.210	2.425	1.040	2.076
		35°C			
	CS	0.940	1.872	0.760	1.503
	DB	0.160	0.273	1.180	2.363
	PC	0.190	0.335	0.810	1.605
	DG	0.030	0.007	0.030	0.007
	TP	1.150	2.302	0.810	1.605
		40°C			
	CS	1.240	2.486	1.120	2.240
	DB	0.860	1.708	1.040	2.076
	PC	1.250	2.507	1.280	2.568
	DG	0.040	0.027	0.030	0.007
	TP	1.390	2.794	1.350	2.712

		45°C			
	CS	1.040	2.076	0.170	0.294
	DB	0.880	1.749	0.050	0.048
	PC	0.840	1.667	0.090	0.130
	DG	0.030	0.007	0.030	0.007
	TP	1.190	2.384	0.200	0.355
		50°C			
	CS	0.950	1.892	0.230	0.417
	DB	0.910	1.810	0.210	0.376
	PC	0.750	1.482	0.210	0.376
	DG	0.030	0.007	0.740	1.462
	TP	0.120	0.191	0.090	0.130

***Cotton swabs -CS ; Dressings and bandages – DB ; Plaster casts – PC ; Discarded gloves – DG ; Tissue and bits of papers – TP

Table 6: Sugar estimation in degradation reaction from crude cellulase enzyme of isolates CL002 and CL005 on general biomedical waste material at variable pH

Parameter	**Sample**	**CL002**		**CL005**	
		Absorbance	**Concentration (mg/ml)**	**Absorbance**	**Concentration (mg/ml)**
		3.000			
	CS	0.940	1.872	1.180	2.363
	DB	0.050	0.048	0.170	0.294
	PC	1.120	2.240	0.960	1.913
	DG	0.030	0.007	0.040	0.027
	TP	1.040	2.076	1.040	2.076
		5.000			
	CS	0.930	1.851	0.760	1.503
	DB	0.910	1.810	1.180	2.363
	PC	0.630	1.236	0.810	1.605
	DG	0.030	0.007	0.030	0.007
	TP	0.870	1.728	0.810	1.605
		6.000			
	CS	0.040	0.027	1.120	2.240
	DB	0.080	0.109	1.040	2.076
pH	**PC**	0.170	0.294	1.280	2.568
	DG	0.030	0.007	0.030	0.007
	TP	0.940	1.872	1.350	2.712
		7.000			
	CS	0.200	0.355	0.170	0.294
	DB	0.940	1.872	0.050	0.048
	PC	0.160	0.273	0.090	0.130
	DG	0.030	0.007	0.030	0.007
	TP	0.910	1.810	0.200	0.355
		8.000			
	CS	0.870	1.728	0.230	0.417
	DB	0.720	1.421	0.210	0.376
	PC	0.760	1.503	0.210	0.376
	DG	0.030	0.007	0.740	1.462
	TP	0.890	1.769	0.090	0.130

***** Cotton swabs -CS ; Dressings and bandages – DB ; Plaster casts – PC ; Discarded gloves – DG ; Tissue and bits of papers - TP

Table 7: Sugar estimation in degradation reaction from crude cellulase enzyme of isolates CL002 and CL005 on general biomedical waste material at variable agitation speed

Parameter	Sample	CL002		CL005	
		Absorbance	**Concentration (mg/ml)**	**Absorbance**	**Concentration (mg/ml)**
Agitation speed (rpm)		**100 rpm**			
	CS	0.08	0.109	0.63	1.236
	DB	0.14	0.232	0.57	1.113
	PC	0.11	0.171	0.75	1.482
	DG	0.03	0.007	0.04	0.027
	TP	0.05	0.048	0.78	1.543
		150 rpm			
	CS	1.290	2.589	1.24	2.486
	DB	1.130	2.261	1.28	2.568
	PC	0.940	1.872	0.98	1.953
	DG	0.030	0.007	0.03	0.0067
	TP	1.340	2.691	1.27	2.548
		200 rpm			
	CS	0.940	1.872	0.86	1.707
	DB	0.950	1.892	0.47	0.908
	PC	0.970	1.933	0.67	1.318
	DG	0.030	0.007	0.04	0.0272
	TP	0.910	1.810	0.92	1.830
		250 rpm			
	CS	0.150	0.253	0.11	0.170
	DB	0.060	0.068	0.14	0.232
	PC	0.120	0.191	0.06	0.068
	DG	0.030	0.007	0.04	0.027
	TP	0.080	0.109	0.04	0.027
		300 rpm			
	CS	0.130	0.212	0.77	1.523
	DB	0.140	0.232	0.84	1.666
	PC	0.110	0.171	0.89	1.769
	DG	0.030	0.007	0.89	1.769
	TP	0.120	0.191	0.85	1.687

***** Cotton swabs -CS ; Dressings and bandages – DB ; Plaster casts – PC ; Discarded gloves – DG ; Tissue and bits of papers – TP

Table 8: Sugar estimation in degradation reaction from crude cellulase enzyme of isolates CL002 and CL005 on general biomedical waste material at variable proportion of crude enzyme

Parameter	Sample	CL002		CL005	
		Absorbance	**Concentration (mg/ml)**	**Absorbance**	**Concentration (mg/ml)**
Concentration (%)		2 %			
	CS	0.790	1.564	0.58	1.133
	DB	0.810	1.605	0.93	1.851
	PC	0.110	0.171	0.66	1.298
	DG	0.030	0.007	0.04	0.027
	TP	0.870	1.728	0.69	1.359
		4.0 %			
	CS	0.740	1.462	0.09	0.129
	DB	0.930	1.851	0.17	0.293
	PC	0.140	0.232	0.6	1.175
	DG	0.030	0.007	0.03	0.0067
	TP	0.970	1.933	0.85	1.687
		6.0 %			
	CS	1.240	2.486	0.71	1.400
	DB	1.330	2.671	0.23	0.416
	PC	1.370	2.753	0.68	1.339

	DG	0.030	0.007	0.03	0.0067
	TP	1.340	2.691	1.29	2.588
		8.0 %			
	CS	1.170	2.343	0.95	1.892
	DB	1.150	2.302	0.97	1.933
	PC	0.960	1.913	0.87	1.728
	DG	0.030	0.007	0.03	0.0067
	TP	1.300	2.609	0.95	1.892
		10.0 %			
	CS	0.060	0.068	0.88	1.748
	DB	0.110	0.171	0.74	1.461
	PC	0.100	0.150	0.81	1.605
	DG	0.030	0.007	0.9	1.789
	TP	0.090	0.130	0.74	1.461

* Cotton swabs -CS ; Dressings and bandages – DB ; Plaster casts – PC ; Discarded gloves – DG ; Tissue and bits of papers - TP

TEST METHOD

- Cellulase from isolates CL002 and CL005 were used for the Degradation assessment in general biomedical waste.
- The samples included general wastes of health care [a] Cotton swabs (CS) [b] Dressings and bandages (DB) [c] Plaster casts (PC) (d) Discarded gloves (DG) [e] Tissue and bits of papers (TP). The samples were collected in polythene bags and cut into small pieces, and aliquoted for different experimental set up.
- Crude enzyme was dissolved in 50 mM sodium acetate/NaOH buffer (pH 4.5) to reach concentration of 3.0 U/ml for both isolates and then mixed with 25 mg (Initial weight-W1) of waste materials and incubated at 50°C for 2 h.
- After incubation the tubes were centrifuged for 15 min using and the supernatants were transferred into clean test tubes with the concentration of the produced sugars determined by the DNS method. All absorbance readings were taken using the Double beam UV Vis spectrophotometer. For the concentration determination, the glucose standards was used as in mentioned in earlier report.
- The precipitate of papers collected after centrifugation was rinsed with distilled water, oven dried and weighed (Final weight-W2).
- Degradation efficiency was calculated using formula

Degradation Efficiency (%) = (1-W2/W1)*100
where W2 and W1 are the weight of sample after reaction and the initial weight of the waste in experimental set up.

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