Isolation and Identification of Cellulose Degrading Bacteria From Grave Yard Soil And Termite Tubes and Its Application In Ethanol Production

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ABSTRACT

Cellulose holds the reputation of the most abundant renewable biomass on the earth. Ethanol produced from cellulosic biomass is one of the most promising renewable fuel source replacing fossil fuels.

With the advantage of zero net contribution of carbon dioxide to the atmosphere. Hence, the bioconversion of cellulose into ethanol plays an important role for sustainable development .micro-organism having the great ability to degrade cellulosic compounds is of great importance from different ecological point of view. The present study focuses on the isolation and identification of cellulose degraders from sources like Grave Yard Soil And Termite Tubes aims at isolation and characterization of cellulose degrading bacterial fruit and vegetable decaying waste such a sugarcane bagasse, rice hay, tadgola fruit, grass, cellulose degraders were isolated and identified and simultaneously the effect of different environmental parameters such as pH (5-8), Temperature (25-65^{o}c), Substrate concentration (0.2%-1.5%), and incubation period (48hours-120 hours) were studied for optimization of growth. Enzyme production was carried out from the isolated degraders. The enzyme was partially purified by Ammonium sulphate precipitation and dialysis. Enzyme assay was carried by dinitrosalycylic acid (DNSA) method to confirm production of cellulose.

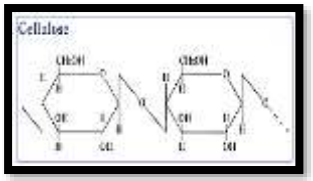
There for current study mainly focuses on isolation, optimization and identification of cellulose-degraders from grave yard soil and termite tubes selected bacterial isolates were co-cultured with *Saccharomyces cerevisiae* for simultaneous saccharification and fermentation. Ethanol production was positively tested after five days of incubation with acidified potassium dichromate. Our results suggested that enzyme secreted by this bacteria strain could be a potential source of cellulase enzyme for the development of hydrolysis cellulase for cellulosic biomass and for a further aid of bioconversion of ethanol.

Keywords: Cellulose–degraders.Cellulose, optimization, bio-ethanol production, renewable fuel source.

INTRODUCTION

1.0 INTRODUCTION

Cellulose is the most common organic compound on Earth. It is well known that plants are the most common source of renewable carbon and energy on the earth. Cellulose has no taste, is odourless & is hydrophilic The cellulose content of cotton fiber is 90%, that of wood is 40–50%, and that of dried hemp is approximately 57%Cellulose is the structural component of the primary cell wall of green plants, Cellulose is an organic compound with the formula$(C_6H_{10}O_5)$n, many forms of algae and the oomycetes. Some species of bacteria secrete it to form biofilms. Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units, this is a hydrolysis reaction. Because cellulose molecules bind strongly to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides. Consisting of a linear chain of several hundred to many thousands of β(1→4) D-glucose units. The total amount of cellulose on earth has been estimated at $7*10^{11}$ tons.



Cellulose – abundance and a renewable source of energy
The combustion of petroleum-based fossil fuels has become concern with respect to global climate change due to accelerated carbon emissions Burning of fossil fuels has also created a concern forum stable and uncertain petroleum sources, as well as, the rising cost of fuels. These concerns haves shifted global efforts to utilize renewable resources for the production of a 'greener' energy replacement which can also meet the high energy demand of the world. The Canadian renewable fuel standard has been raised so that fuel will contain 5% ethanol by2010 the US Environmental Protection Agency raised their renewable fuel standard

to 10.21% ethanol mixed fuels by 2009 while, the current mandate for mixing ethanol in fuel for Brazil is 25% The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest .It is the primary product of photo synthesis in terrestrial environments, and the most abundant renewable Bio resource produced in the biosphere(100 billion dry tons/year) Approximately 70% of plant biomass is locked up in 5- and 6-carbon sugars (D- xylose, D-arabinose, D- glucose, D- galactose, D mannose) which are found in lingo cellulosic biomass comprised of mainly cellulose, lesser hemicelluloses and least of all lignin Cellulose is perhaps the most abundant renewable organic material on the Earth with the annual biomass production estimated over 1.5×10^{11} tons, and is considered as almost inexhaustible source of raw material for different products. Cellulose is synthesized in variable living organisms, from wide distribution in higher plants to less range in algae, fungi, bacteria, invertebrates, and even several marine animals like tunicates. Cellulases are a group of enzymes catalyzing the hydrolysis of cellulose. They are produced by Fungi, Bacteria, and Protozoa, also by other organisms like plants and animals.

With decades of studies on cellulose bioconversion, cellulases have been playing an important role in producing fermentable sugars from lignocellulosic biomass. Usually, cellulases are mainly composed of three types of synergistic enzymes:
1Endoglucanasn that hydrolyze the exposed cellulose chains of the cellulose polymer.
2 Exoglucanases (cellobio hydrolases,) that act to release cellobiose from the reducing and nonreducing ends.
3β-glucosidases that help to cleave the cellobiose and short-chain cello-oligosaccharide into glucose.

Cellulose – system and its type
Endoglucanases- it act by cleaving internal β-glycosidic bonds in the cellulose chain, thereby making chain ends accessible to cellobio hydrolase. The end product cellobiose is further broken down to glucose units by β-glucosidase. Endoglucanases have an enzyme classification and belong to the broader enzyme group called glycosyl hydrolases, which also includes other cellulases such as exoglucanase and β-glucosidase. According to the CAZy database endoglucanases are part of 13 distinct glycosyl hydrolase families, distributed in several archeal, bacterial, fungal, and eukaryotic organisms.

Exoglucanase- act in a processive manner on the reducing or non reducing ends of cellulose polysaccharide chains, liberating either cellobiose or glucose as major products. Exoglucanases can effectively work on micro-crystalline cellulose, presumably peeling cellulose chains from the microcrystalline structure CBH is the most-studied exoglucanase. Different CBHs are produced by many bacteria and fungi.

β-Glucosidase- β-glucosidase (β-D-glucoside glucohydrolase; cellulase 1,4-β-glucosidase) that hydrolyzes cellobiose to glucose . β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose . Thus, β-glucosidase not only produces glucose from cellobiose, but also reduces cellobiose inhibition, allowing endo glucanase and exo glucanase enzymes to function more efficiently.
Uses of cellulases
Pulp and Paper Industry, Textile Industry, Bio-ethanol Industry, Wine and Brewery Industry, Food Processing Industry, Animal Feed Industry, Agricultural Industries, Olive Oil Extraction, Detergent Industry, Waste Management .

MATERIALS AND METHODS

2.0 MATERIALS AND METHODS

2.1 Collection of sample

Cellulose degrading bacterial strain was isolated from degraded wood sample. Samples was collected from moist place and grave yard place.

2.2 Enrichment & Isolation of cellulose degrading bacteria

One gram of sample from both grave yard and termite tube was enriched in 100 ml of carboxy methyl cellulose broth (CMC) COMPONENT in g/l Carboxy Methyl Cellulose -10g,Trypton-2g, KH_2PO_4-4g, Na2HPO_4-4g, $MgSO_4$ -0.2g,$CaCl_2$-0.001g,F_2SO_4 -0.004g,AGAR-20g for 5 days at rotary shaker at 100 Rpm.

2.3 Screening of cellulose degrading bacteria

After incubation, quantitative assay using Congo red dilution assay was performed where zone of clearance was observed visually by staining the plate media (CMC) with 0.1% Congo red and distaining with 1M NaCl / Grams iodine. Colonies showing discoloration of grams iodine was taken as positive cellulose-degrading bacterial colonies. Indicating cellulose hydrolysis, Bacterial colonies was purified by repeated streaking only these were taken for further study

The bacterial colonies having the largest clear zone were selected for identification and cellulose production in submerged system.

2.4 Maintenance of pure culture

The bacterial colonies showing significant clear zone was plated on the minimal agar medium (CMC) (devoid of CMC and tryptone) and analyzed for colony characteristics and sub-cultured on the minimal medium containing 1% CMC and incubated at 37 for 24hrs and then stored at 4ºC.

2.5 Morphology and biochemical characterization

The bacterial isolates from both the sample was subject to gram staining and various biochemical test suggested as per Bergey's manual.

2.6 Optimization of cellulase production

The optimum parameters will determined for cellulase production from the efficient bacterial culture. The cellulase fermentation was carried out at different ranges of parameters included temperature, pH, incubation period, substrate concentration. After fermentation at different parameters the crude enzyme sample was collected from each to check the enzyme activity.

2.6.1 Optimization of temperature:

1 ml of the bacterial culture was dispensed in the tubes containing 5 ml of (CMC) broth each with 1% CMC and was adjusted at pH-7.0. All were incubated at various temperatures in the range of 25ºC, 37ºC, 45ºC, 55ºC and 65ºC for 48hrs. Following incubation, growth of the cultures was measured by observation of the optical density at 560 nm.

2.6.2 Optimization of pH:

1 ml of the bacterial culture was dispensed in the tubes containing 5 ml of (CMC) broth each with 1% CMC and was adjusted from various pH range 5-8 incubated at 37ºC for 48 hrs. Following incubation, growth of the cultures was measured by observation of the optical density at 560 nm.

2.6.3 Optimization of incubation period:

1 ml of the bacterial culture was dispensed in the tubes containing 5 ml of (CMC) broth each with 1% CMC and was adjusted at pH-7 incubated at 37ºC at various time intervals 24,48,72 and 96 hrs. Following incubation, growth of the cultures was measured by observation of the optical density at 560 nm.

2.6.4 Optimization of substrate concentration:

1 ml of the bacterial culture was dispensed in the tubes containing 5 ml of (CMC) broth each with variable concentration of substrate, 0.2%, 0.5%, 1%, and 1.5% CMC and was adjusted at pH-7. Incubated at 37ºC for 48hrs.Following incubation, growth of the cultures was measured by observation of the optical density at 560 nm.

2.7 Submerge Fermentation process

Standard inoculums.

For preparation of standard inoculum, those isolates showed a maximum zone of hydrolysis were cultured in 20 ml inoculums medium Carboxymethyl cellulose (CMC) 0.5g individually and incubated at 37 ºC for 24 h where an average viable count of 2-3.5x106 cells /ml culture was obtained. By keeping it on 100 rpm on rotary shaker this was used as inoculums for the production medium.

Fermentation was carried out in 250 ml Erlenmeyer flasks, each containing 100 ml sterile production medium and inoculated with 5ml of media (CMC) of standard inoculums (containing 2-3.5x10^6cells /ml). The flasks were incubated at 37^0C on a rotary shaker at 150 rpm for 72hrs.

2.7.1 Preparation of crude enzyme

After incubation of the production medium cultures were centrifuged at 5000 rpm for 20 min at 4°C and supernatant was used as a source of crude enzyme. The crude enzyme solution was utilized for determination of enzyme activities.

2.7.2 Partial purification of cellulase enzyme

2.7.2.1 Ammonium sulfate precipitation

About 20 ml of the crude enzyme solution was brought to 80% saturated (10.32 g of solid ammonium sulfate in 20 ml crude enzyme). By solid ammonium sulfate and the mixture was left overnight at 4ºC for precipitation. The precipitates were collected by centrifugation and dissolved in 10 ml of 50mM sodium acetate buffer (pH- 5.5).

2.7.2.2 Dialysis

For partial purification, enzyme collected after ammonium sulphate precipitation was dialyzed against 30mM sodium acetate buffer (pH-5.5) at 4ºC with three changes of buffer at 2 hours interval.

2.8 Estimation of cellulose enzyme activity using DNSA method.

A standard DNSA assay was run using glucose (1000ug/ml) as a standard,
Diluents – distilled water, Range -200-1000ug/ml, Total volume -3ml
For unknown sample – 0.5ml of purified cellulose enzymes sample will be taken and mixed with 1ml of 0.05m citrate buffer ph 4.8 solution in two test 1% of cellulose substrate (CMC) and the resulting reaction mixture will be incubated at 50^0c for 60 minutes after reaction time , 1ml of DNSA reagent will be added to the reaction mixture and would be boiled exactly for 5minutes to terminate the reaction in vigorously Boiling water bath After cooling the tubes record the absorbance which was measured by calorimetrically at 540 nm against blank without enzyme the activity would be measured using a calibration curve for glucose. One unit of activity will be defined as the amount of enzyme that released one micro gram per ml of glucose.

2.9. To check the Amylolytic and Proteolytic activity of the isolated culture.

2.9.1 Screening for Amylase Activity. (Starch Iodine Test)

Isolated culture was spot inoculated by adding loop full of culture on the starch agar plates components g/100ml Nutrient broth 100ml, Starch 2g, Agar 2g with starch as the only carbon source. After incubation at 37ºC for 24-48 hrs individual plates were flooded with Gram's iodine to produce a deep blue colored starch-iodine complex. In the zone of degradation no blue colour forms, which is the basis of the detection and screening of an amylolytic strain.

2.9.2 Screening for protease Activity.

Isolated culture was spot inoculated by adding loop full of culture on Frazier's gelatin agar plates components g/100ml Nutrient broth 100ml, Gelatine 1g, Agar 2g. After incubation at 37ºC for 24-48 hrs individual plates were flooded with 10% tannic acid. A development of a transparent zone of proteolysis was detected on the casein agar plates.

2.10.1 Application of most efficient cellulase producer in biodegradation.

Most efficient isolate will be selected and used for filter paper and cotton degradation. For this a sterile mineral buffered solution with pH-7.5 would be individually supplemented with filter paper strips and cotton as a sole source of carbon and the (CMC media) devoid of CMC and tryptone will be supplemented with two drops of 10 mM glucose to possibly induce cellulase production . Then, the log phase culture of selected most efficient isolate will be separately inoculated into this medium. The culture will be incubated for maximum 6 days at 50ºC in shaking condition at 120 rpm and observed daily for visual evidence of degradation.

2.10.2 Estimation of Bio-ethanol production by dichromate method.

A stock : 1mg/ml ethyl alcohol solution ,Diluents : distilled water, Sensitivity rang : 1-5 mg /system,End point : light blue to colourless For unknown sample- 5ml of the sample was taken after the fermentation process

A 5 ml mixed culture of cellulose degrading bacteria was added in (CMC media) devoid of CMC and tryptone in two different sets, 50 ml each one containing filter paper and the other containing cellulose powder 1g as substrate and then was incubated at 37°C at 100 rpm on rotary shaker for 3 days.

2.10.3 Bio-ethanol production from cellulose degrading waste material.

In this case biodegradable waste material such as sugarcane bagasse, rice hay, tadgola fruit, grass were taken and cut in small pieces to increase the surface area for degradation 2g each from the above sample was taken and then subjected to heat sterilized and added to 50 ml of (CMC media) devoid of CMC and tryptone. 5 ml mixed culture of cellulose degrading bacteria was added in the media for production of cellulolytic enzyme and to initiate saccharification process. Culture was incubated at 37°C at 100 rpm on rotary shaker for 3 days.

After completion of three days of incubation, the above culture broth was conditioned for co-culturing by adding (5ml) *Saccharomyces cerevisae* culture. The simultaneous saccharification and fermentation was carried out at 27°C for 5 days in stationary condition. At the end of incubation, the culture broth was qualitatively tested for alcohol production using the $K_2Cr_2O_7$ reagent test.

RESULTS

3.1 Isolation of cellulose degraders

Cellulose degraders were isolated from grave yard soil and termite tubes sample isolated colonies were shown in the fig 1:

Fig-1 Isolation of colonies on Congo red agar plate

3.2 To compare between Congo red and Gram's iodine stain.

Formation of a cleared halo zone indicates enzymatic activity that is visible around the colony by the counter stain of 1M NaCl solution that elutes the dye in the clearing zone where the cellulose has been degraded into simple sugar by the enzymatic activity. Grams iodine gave more effective results as compared to Congo red and 1M NaCl by showing clearer zone of hydrolysis.

The Results Are Depicted In Fig- 2&3.

Fig-2: To compare between congo red and gram's iodine stain.

Fig -3 Repeated streaking was carried out to obtain pure culture for identification and further studies.

3.3. Morphological and biochemical characterization

Gram staining and various biochemical tests were performed as per Bergey's manual for the identification of both the cellulose degraders obtained from grave yard soil and termite tubes and the results observed were as follows:

Grave Yard Soil.

Observation table no - 1

TESTS	RESULTS
Gram character	Gram positive
Morphology	Cocci
Catalase	+
Oxidase	+
Glucose	-
Mannitol	-
Maltose	-
Xylose	-
Lactose	+
TSI	Yellow/pink Slant butt
Indole	-
Proteolytic	+
Amylolytic	-

Key: (+) = Positive test (-) = Negative test

Biochemical test performed as per Bergey's manual for identification of Cellulose-degrader from grave yard soil sample,For confirmation of the isolate it was given to infexn laboratories and identified as *Staphylococcus lugdunensis.*

For -Termite tube sample.

Observation table no - 2

TESTS	RESULTS
Gram character	Gram positive
Morphology	Rods
Catalase	+
Oxidase	+
Glucose	+
Mannitol	+
Maltose	-
Xylose	-
Lactose	-
TSI	Yellow/pink Slant butt
Indole	-
Proteolytic	+

Amylolytic	+

Key: (+) = Positive test (-) = Negative

Biochemical test performed as per Bergey's manual for identification of Cellulose-degrader from termite tube sample. For confirmation of the isolate it was given to infexn laboratories and identified as *Bacillus cereus*.

3.4 optimization of parameters

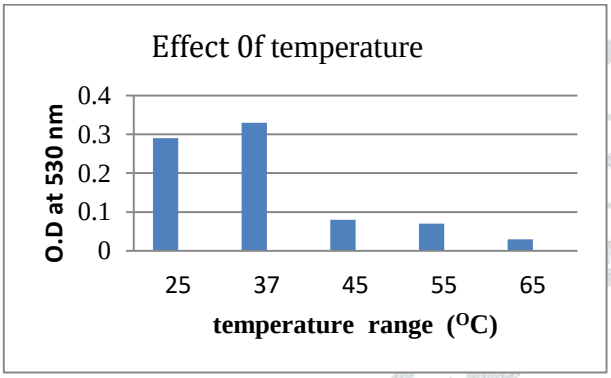
optimization of various physical and nutrition growth parameters affected growth of cellulose degraders and production of enzyme which is evident by the following results obtained.

3.4.1 optimization of temperature

All the tubes were maintained at pH 7.0 at different temperatures from 25ºC, 37ºC, 45ºC, 55ºC and 65ºC containing 1.0% of substrate (CMC)

Optimization of growth of cellulose –degraders under different temperatures is depicted in graph-1 &fig-6

Optimum temperature was found to be 37ºC.



Graph 1 -Effect of Temperature

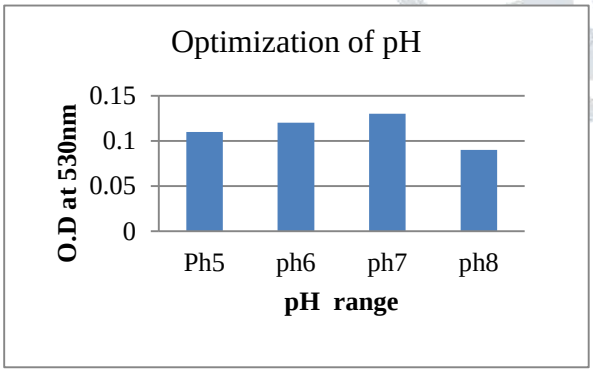
Fig-6- Effect of temperature

3.4.2 optimization of pH

All the tubes were maintained at different pH of 5, 6, 7, and 8 at 37ºC for 48 hours containing 1.0% of substrate (CMC)

Optimization of growth of cellulose–degraders under different pH is depicted in graph-2&fig-7.

Optimum pH was found to be pH-7.



Graph2 -Effect of pH

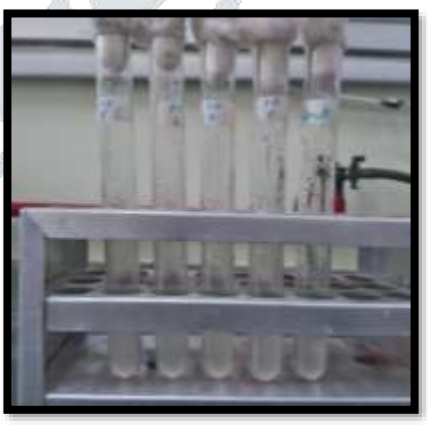


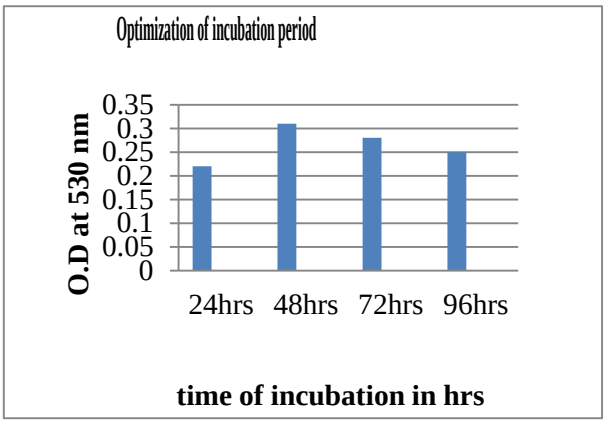
Fig-7- Effect of pH

3.4.3 optimization of incubation period.

All tubes were maintained at pH 7.0, containing 1.0% of substrate (CMC), incubated at 37ºC for different time intervals that is 24, 48,72 and 96hours respectively.

Optimization of growth of cellulose –degraders under different incubation time is depicted in graph-3&fig-8.

Optimum time was found to be 48hrs.



Graph 3 -Effect of incubation period

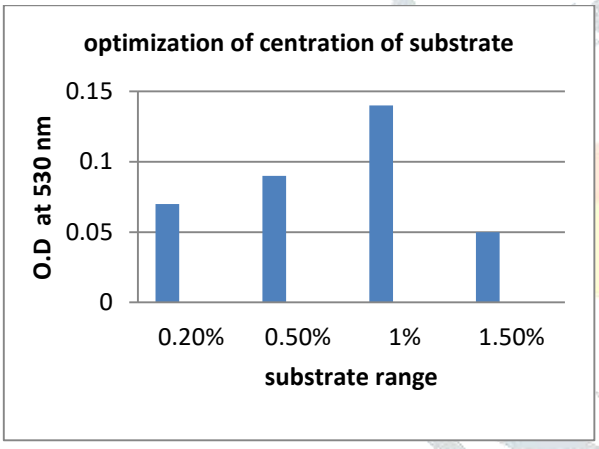
Fig-8 Effect of incubation period

3.4.4 optimization of concentration of substrate

All the tubes were maintained at pH 7.0 at 37ºC for 96 hours containing amount of cellulose substrate (CMC) that is 0.2%, 0.5%, 1.0% and 1.5%.

Optimization of growth of cellulose –degraders under different concentrations of substrate is depicted in graph-4&fig-9

Optimum substrate concentration was found to be 1%.



Graph 4 -Effect of substrate concentration

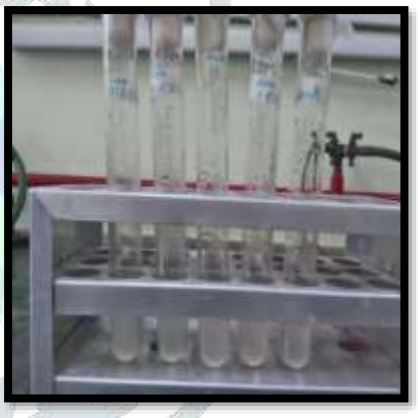


Fig-9 Effect of substrate concentration

3.5 PARTIAL PURIFICATION OF ENZYME CELLULAS

3.5.1 Ammonium sulphate precipitation

20 ml of crude enzyme was brought to 80% saturation by adding 10.32g of solid ammonium sulphate in 20 ml crude enzyme left at 4ºc for overnight and then centrifuge the mixture dissolved in 10 ml of 50 mM sodium acetate buffer pH (5.5).

Fig-10 Ammonium sulphate precipitation

3.5.2 Dialysis

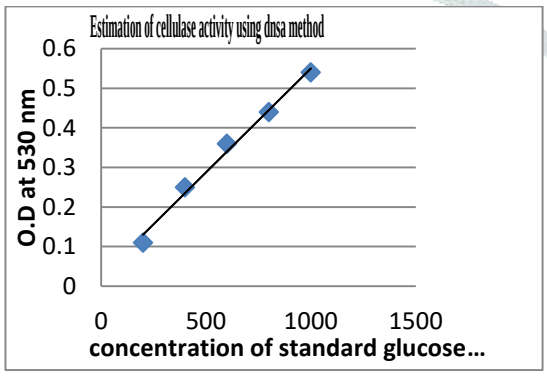
For partial purification, enzyme collected after ammonium sulphate precipitation was dialyzed against 30mM sodium acetate buffer (pH-5.5) at 4ºC with three changes of buffer with 2 hr interval.

Fig – 11 Dialysis three changes of buffer

The swelling of the dialysis bag conformed that the partial purification of enzyme cellulase is carried out

3.6 Estimation of Cellulase Enzyme Activity Using DNSA Method

Activity of enzyme cellulase was estimated for the extracted sample using DNSA method colorimetrically at 540 nm and the activity was calculated to be 300 mcg/ml which is illustrated in graph -5.



Graph- 5 Estimation of cellulase activity using DNSA method

Fig 12 Estimation of cellulase activity by DNSA method (standard and unknown)

3.7. Amylolytic and Proteolytic Activity of the Isolated Culture

3.7.1 AMYLOLYTIC ACTIVITY ON DAY -2 (48hrs)

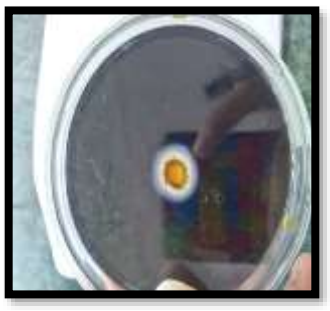


Fig -13 (a) *Bacillus cereus*

Fig-13(b) *Staphylococcus lugdunensis*

3.7.2 PROTEOLYTIC ACTIVITY ON DAY-2(48hrs)

Fig-14(a) *Bacillus cereus*

Fig-14(b) *Staphylococcus lugdunensis*

3.7.3 Zone Of Inhibition After Carrying out the Amylolytic and Proteolytic Activity of Both Cultures.

Observation Table No - 3

After 48 hrs of incubation	Proteolytic activity on Frazier's Gelatin agar plate	Amylolytic activity on Starch agar plate
Bacillus cerieas	Large zone 55mm	Medium zone of clearance 20 mm
Staphalococcus lugdunensis	Small zone of clearance 17mm	No zone of clearance --

APPLICATION

3.8.1 USE OF CELLULASE ENZYME IN BIODEGRADATION

3.8.1.1 COTTON

Cotton was selected to check the biodegradation and not much degradation was seen in fig 15(b)

Fig-15 (a) Cotton before Degradation

Fig-15(b) Cotton after Degradation

3.8.1.2 FILTER PAPER

Filter paper was selected to check the biodegradation and effective degradation was seen in fig 16(b)

Fig-16(a) Filter Paper before Degradation

Fig-16(b) Filter Paper After Degradation

3.8.2.0 Estimation of ethanol production by dichromate method

Observation Table No - 4

Con (mg/ml)	Std (m)	D/w (ml)	$K_2Cr_2O_7$ (ml)	Treatment	Burette reading (ml)	B-T
1	1	4	10	Incubate at RT for 30 min. add 100 ml of KI and mix well Titrate with	4	1.5
2	2	3	10		3.5	2.0
3	3	2	10		3.1	2.4
4	4	1	10		2.7	2.8
5	5	0	10		2.0	3.5
BLANK			10		5.5	0
UK 1-1			10		3.1	2.4

gm (CMC) (1:2diluted) (5ml)				$Na_2S_2O_3$ using 1 % starch indicator end point blue to colorless		
UK 2-Filter paper(5ml)			10		4.6	1.2

7.8.2.1 Estimation of bio-ethanol production from cellulosic biodegradable waste material.

Observation Table No – 5

waste material	volume (ml)	$K_2Cr_20_7$ (ml)	Treatment	burette reading (ml)	B-T
Sugarcane bagasse	5ml	10ml	Incubate at RT for 30 min add 100 ml of KI and mix well Titrate with $Na_2S_2O_3$ using 1 % starch indicator End point blue to Colorless	3.8	3.7
Rice hay	5ml	10ml		4.5	3
Grass	5ml	10ml		5.5	2
Tadgola fruit	5ml	10ml		6.2	1.3
Blank D/w	5ml	10ml		7.5	0

Graph is estimated after plotting the standard concentration of bio-ethanol and on the graph and then plotting bio-ethanol production from cellulatic biodegradable waste material. Where sugar cane should the maximum at 5.4mg/ml

Graph 6: Estimation of Bio ethanol Production

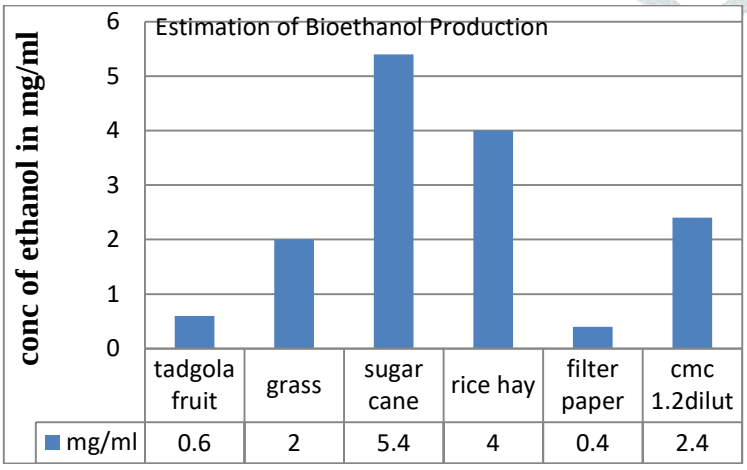


Fig-17(a) Biodegradable waste after heat sterilization.

Fig-17(b) Biodegradable waste after 3 days of shaker incubation.

Fig-17(c) Biodegradable waste after 5 days of static incubation.

Fig-17(d) Estimation of bio-ethanol production by dichromate method.

DISCUSSION

Cellulose is the main building block of plants and have major fraction of organic carbon in soil. Microorganisms, which live in soil, are accountable for recycling of this organic carbon to the environment. Degradation of cellulosic materials is a complex process and requires participation of microbial cellulolytic enzymes. Habitats where these substrates are present are the best sources for isolation of cellulolytic microorganisms. About one fifth of fresh water and soil samples yield cellulose degrading bacteria after enrichment but some samples did not bear such kind of bacteria. This is due to existence of microenvironments where different growth conditions for cellulose degrading bacteria are present. These bacteria are generally found in well manure soils. Several microorganisms have been discovered for decades which have capacity to convert cellulose into simple sugars but the need for newly isolated cellulose degrading microorganism still continues.

Habitats that contain these substrates are the best sources in which to find these microorganisms. So the sites for sample collections were selected as those were rich in cellulosic biomass such as grave yard soil and termite tubes, hence there were maximum possibilities to get potential cellulase producing bacterial strain.

A rapid primary screening of isolates was carried out for their cellulase activity by using media containing 1 % CMC as a sole source of carbon and after incubation the plates were flooded with 1% Congo red dye, after 15mins the dye was decanted and the plates were again flooded with 1M NaCl for 15 mins. Technique1. In Technique2 Grams iodine was selected where it gave more effective results then using technique 1 Colonies showing discoloration of grams iodine was taken as positive cellulose-degrading bacterial colonies. Indicating cellulose hydrolysis, Bacterial colonies was purified by repeated streaking only these were taken for further study

The bacterial colonies having the largest clear zone were selected for identification and cellulose production in submerged system.

The intra-and intermolecular bonds that hold enzymes in their secondary and tertiary structures are disrupted by changes in temperature and pH. This affects shapes and so the catalytic activity of an enzyme is pH and temperature sensitive. This also optimizes the working conditions for bacterial growth affecting glucose biosynthesis by preselected isolate. Therefore, environmental factors also play an important role in growth of (cellulose degrading bacteria) CBD and production of cellulase. Hence primarily the condition in the growth medium was optimized by varing pH from 5, 6, 7 and 8. optimization of pH was important because it is one of the most critical environmental parameter affecting growth, enzyme production and the transport of various components across the cell membrane in the current study, a pH of 7 with an O.D of 0.13 proved to be the best parameter for maximum enzyme production .whereas ph 6 and higher pH showed a marked decrease in supporting growth. Further, the growth was carried out at variant temperatures of 25ºC 35º C, 45ºC,55ºC and 65ºC as a result, the growth increased with the initial increase in temperature and maximal peak growth was at 37ºC with 0.33 O.D,while further increased in temperature showed a decreasing trend optimization of substrate would increased the growth as more the carbon source would be available in the medium more would be the enzyme activity .with this intention, the medium was supplemented with 0.2%,0.5%,1.0%and 1.5% of CMC . An increased trend was seen in growth as the amount of carbon source was increased from 0.2% to 1.0% beyond which a decrease in growth was observed it may be because a high substrate concentration can cause substrate inhibition which sub-stially caused reduction in growth. Therefore, O.D of 0.14 at concentration of 1.0% proved to be the best parameter to be used for growth of CBD. Similarly other parameter such as incubation period also plays an important role affecting the growth of CBD and ultimately affecting production of enzyme cellulase thus growth of isolates was carried out at variant time of incubation of 24hrs, 48hrs, 72 hrs, 96 hrs and 120 hrs as a result the growth increased with the increased in incubation period and maximal peak was observed to occur at 48hrs giving O.D of 0.31 while further increase in time showed a decreasing trend. This must have been because the incubation time depends on the nutrients present in the medium and the cultural condition of the organisms thus, after optimizing each parameter at a time, the optimized physical and nutritional parameters that are 1.0% of cellulose substrate in the medium at 37ºC and pH of 7 were amalgamated and incubated for 72hrs in order to produce maximum growth and cellulase.

The both organism should significant growth when changing the carbon source from (CMC) to gelatin and starch this showed that the organism can be used in production of gelatinase and amylase.
These, isolated were identified on the basis of standard biochemical test given in Bergey's manual for conformation it was sent to infexn laboratories for identification. Gram staining showed that the bacterial isolates were gram positive cocci for grave yard soil and identified as *Staphalococcus lugdunensis* and gram positive bacillus was identified as *Bacillus cereus* for termite tube.

From which *Staphalococcus lugdunensis* gave more zone of hydrolysis then the other one thus was used for further studies

Later the extracellular cellulases produced by *Staphalococcus lugdunensis* was partially purified by ammonium sulphate precipitation and dialysis which is used to purify proteins by altering their solubilities and a 80% saturation was achieved.

This CDB *S. lugdunensis* was further applied for the biodegradation of filter paper and cotton and after the 6 day incubation it was found that the CDB *S.lugdunensis* was able to degrade the filter paper completely but not the cotton, its ability to degrade filter paper represent the production of more than one type of enzyme and ability to degrade crystalline cellulose but it might require incubation for cotton treatment. The efficient isolate *S.lugdunensis* showed a potential to convert cellulose into reducing sugars which could be readily used in many applications such as animal foods and a feedstock for production of valuable organic compounds.

The other application is to obtain cheap bio-ethanol from the cellulitic biodegradable waste material where sugarcane bagasse, rice hay, tadgola fruit waste, grass. Was used after fermentation and incubation at static condition with *S.cerevisae* from which sugarcane bagasse gave bio-ethanol production upto 5.4mg/ml. thus becoming a part of renewable source of energy and helping farmers. To save on energy cost and preventing pollution by using green fuel.

CONCLUSION

5.0 CONCLUSION
The present work was carried out for isolation and identification of potential Cellulase producing bacterial strain and for the two abundantly available sources such as grave yard soil and termite tubes were utilized physical and nutritional parameters like pH, temperature, substrate concentration and incubation time period were optimized for enhanced growth and cellulase production this resulted in increased enzyme activity.
Two isolates were obtained from grave yard soil and termite tubes each these two were characterized for their morphological cultural and biochemical analysis, to conform the results outsourcing of the culture was done for identification of the organism and were identified as *Staphylococcus lugdunensis* and *Bacillus cereus* where *Staphylococcus lugdunensis* gave more zone of clearance on using grams iodine. Partial purification of cellulase was done and the enzyme activity was determined.
Thus the results reveal that the grave yard soil and termite tubes served as the good isolation source for cellulase producing microorganisms as they are rich in cellulose.
Besides the utilization of CMC organism should significant growth on other carbon source such as gelatin and starch.
Consequently, the cellulase enzyme produced is used for biodegradation of celluloseic material such as filter paper, and cotton which broke down large organic molecules into smaller molecules that can be used by the biotic community. This in turn will

provide some medium to utilize renewable sources of energy with the help of microorganism and processes they undergo during degradation of complex polymer to simpler sugars.
Further, it was also found that cellulases not only served as the cellulosic enzyme but also having applications on various industries. It also helps in bioethanol production from cellulatic biodegradable waste material such as sugarcane bagasse, rice hay, tadgola fruit waste, and grass after fermentation.

Therefore it can be concluded that cellulose-degraders have a multi-utility product which can be exploited in many fields from our day to day life.

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