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Isolation and Production of Cellulase from Bacteria Using Agro Waste

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The development of appropriate procedures for the efficient treatment and utilization of wastes containing cellulose as an inexpensive carbon source has grown to be of substantial economic relevance. Cellulase enzyme, which is known to be produced by bacteria is responsible for degrading cellulose. Thus, isolation of Bacteria producing cellulase was performed using soil sample that were identified as Bacillus subtilis, Pseudomonas aeruginosa, Enterobacter cloacae using CMC medium. The medium for fermentation was optimized for maximum cellulase to be produced by the potential isolate. Various parameters like the time of Incubation, temperature, pH, nitrogen sources and carbon sources, were considered for optimization. The culture condition was optimized and found to be 40°C at pH 7 with maximum activity in the presence of ammonium sulphate and lactose as nitrogen and carbon sources respectively. Amongst these isolates the maximum cellulase activity was shown by Enterobacter cloacae followed by Pseudomonas aeruginosa and Bacillus subtilis by comparative study. The supplement for the medium was various agricultural waste added as an alternate source of carbon to produce cellulase. The medium with the presence of rice husk (1.76 IU/ml), followed by wheat husk(1.51 IU/ml) and castor seed waste (0.65 IU/ml), had the highest cellulase activity. Thus, this work aimed to compare the potential of all the above-mentioned isolates to use agro-waste for production of cellulase at optimized parameters.

Keywords: Agro waste; Bacteria; Cellulase; CMC; optimization; production.

The planet is abundant with cellulose.[1]. It is one of the plentiful renewable resources in nature and is primarily found during the process of photosynthesis in plants of the terrestrial environment.[2,3]. Cellulase is the enzyme that degrades cellulose. Several bacteria and fungi are found to produce this enzyme [4–7].

Cellulose is mostly found in the cell walls of terrestrial plants. The microfibrils of plants present in cellulose are the source present in plants. These microfilaments are responsible for providing strength to the plants. Utilization of cellulosic biomass is done enormously worldwide but still there are some raw materials or waste materials which need to be exploited and used efficiently. [1]

It is important to develop the technique for the economic use of this cellulose that is profitable. The Synergistic approach of three enzymes helps in the complete hydrolysis of cellulose, namely, carboxymethylcellulose (CMCase) or endoglucanase, cellobiohydrolase, and beta-glucosidases.[8]

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Various Bacteria with higher growth rates possess good potential to produce cellulase as compared to fungi. However, this approach of using bacteria for the production of cellulase is not used widely. Indeed, the use of fungi is a more common way for producing cellulases. It was reported that there are several bacterial genera like *Bacillus, Micrococcus,*[7], *Pseudomonas*,[9]. *Cellulomonas, Cellvibrio sp.* has cellulolytic properties. It is necessary to control various parameters for maximum cellulase production during optimization. These parameters work in the relationship and are majorly responsible for an overall yield of cellulase which includes pH, inoculum size, temperature, carbon and nitrogen sources, inducers, etc. [7].

There are various sectors releasing waste materials containing cellulose like industries, agriculture and municipal. Due to high processing costs, this trash keeps building up and is used inefficiently, which hinders solid waste management. [10]. One of the many types of carbohydrates that can be found in nature, cellulose is the primary structural element of the plant cell wall. It is made up of glucose residues made up of beta 1, 4 linkages. [11]. Cellulose is an inexpensive carbon source and it is extremely essential to develop a process that helps in the proper utilization of cellulose at an economic level. Cellulase enzyme helps to hydrolyze this polymer and release glucose [12].

Cellulose is released from various wastes released from industries, agricultural and urban sewage and this source of carbon helps in the production of methane by the process of anaerobic digestion. Agricultural waste includes residues of crops, excreta of animals, wastes from crop processing industries and activities of forestry including products of wood. It is now a requisite to effectively use these wastes as carbon sources and not consider them valueless. [13]

The utilization of cellulase can be done in various sectors like the preparation of washing powders, extracting juice from fruits and vegetables and processing of starch[14]. Microorganisms are responsible for the production of cellulase and this enzyme can be intracellular or extracellular. Several microorganisms are responsible for cellulase production but very few are capable of degrading the crystalline structure of cellulose. [15].

The process of finishing denim and softening cotton in textile industries is done by cellulases , this enzyme can be used for cleaning in the laundry, for various food processing, in the pulp and paper business, to improve and modify fibre, and in the pharmaceutical industry.[16] This indicates that cellulases have applications in various industries and near future there will be a high demand of the enzymes that are stable , active and produced from renewable low-cost raw materials. Thus research to achieve cellulase production using agro waste will prove to be cost-effective and emphasis should be given to the specificity of substrate and enzyme-specific activity. This work was done to optimize various parameters for maximum production of cellulase using potential microbes. It also aimed to study comparatively the isolates showing maximum cellulase-producing ability and to analyze the utilization of agricultural waste as a carbon source by the most potential isolate in the current world production of cellulaes.

MATERIALS AND METHODS

Isolation of Bacteria

The isolation of Bacteria was done using soil as a sample from the Carpentry shop area located in Mehsana District, Gujarat (C1) and Krishi Vigyan Kendra, Ganpat University, Kherva, Mehsana, Gujarat (C2). The sample was diluted and spread plate method was performed on the CMC agar plate. Incubation of plates was done for 24 hours at 37 °C and 55°C. To observe the zone of hydrolysis, the plate was flooded with congo red (0.1 percent) for 15 minutes and then washed using NaCl (1 M). [17] The diameter of the clear zone created by the colonies on the plate was measured in order to assess the activity of cellulase. The Clear zone diameter of colonies was measured to find the enzyme activity quantitatively. Out of 31 isolates,5 isolates were selected for further process. Additionally, the DNS approach was used to calculate the amount of released reducing sugars.[18] The isolate with the highest level of enzyme activity was chosen for improvement.

Identification of Bacteria

For Identification of Bacteria various morphological and Biochemical characterizations were done presumptively. These parameters include the morphology of the colony, Catalase test, Indole test, Citrate Utilization test, Urea

hydrolysis, Methyl red Test (M-R) test, Voges-Proskauer (V-P) test, Starch hydrolysis, Gelatin hydrolysis, Carbohydrate fermentation test for Glucose, Lactose, Sucrose, Mannitol, Maltose. Bergey's Manual of Determinative Bacteria was used to compare the results obtained. [19]

Medium for Producing Enzyme

The following ingredients were used to create the production medium: peptone 0.75 g, FeSO4 0.01 g, MgSO4 0.5 g, KH2PO4 0.5 g, and glucose 0.5 g (all per litre). In a 100 ml conical flask, 10 ml of medium were added. These flasks underwent a 20-minute autoclave at 121°C. The flasks were further allowed to cool before being inoculated with a bacterial culture that had been cultivated overnight. After that, it was shaken in an incubator for 24 hours at 37°C. To produce a crude extract, the cultured medium was centrifuged at 6000 rpm for 10 minutes.

Enzyme Assay

Cellulase activity was assessed using the Miller technique.[18] After that, the reaction mixture was incubated at 37°C for 30 minutes using crude enzyme (0.2 mL) and carboxymethyl cellulose (1.8 mL) produced in sodium phosphate buffer (50 mM-pH 7). To stop the reaction, 3 mL of DNS reagent

Table 1. Sampling sites, location and their cellulosic sources

No	Sample Code	Site	Location	Cellulose source
1	HC	Himmatnagar, Gujarat, India	23.6°N 72.95°E	Soil from Police housing Corporation construction site
2	VC	Mansa, Gujarat, India	23.43°N72.67°E	Vermi Compost soil from farm
3	C1	Mehsana, Gujarat, India	23.6°N 72.4°E	Soil from Carpentry shop area
4	C2	Kherva, Mehsana, Gujarat,India	23.5443°N 72.443161°E	Soil from Krishi Vigyan Kendra, Ganpat University

Table 2. Zone of Diameter

Isolate no.	Code of isolates	Mean Clear zone diameter (ZD mm)	Mean Colony diameter (CD)mm
2	C1-A	3±0.10	1±0.10
4	C1-B	5±0.15	1±0.11
5	C1-D	7±0.05	1±0.20
11	C1-E	8±0.10	2±0.1
12	C2-A	12±0.15	1±0.15

Table 3. Cultural and morphological characteristics

Isolates	C1-A	C1-B	C1-D	C1-E	C2-A
Size	Small	Big	Small	Small	Small
Shape	Round	Round	rod	Rod	Rod
Margin	Regular	Irregular	Irregular	Regular	Regular
Texture	Smooth	Rough	Rough	Smooth	Smooth
Consistency	Moist	Dry	Moist	Moist	Moist
Elevation	Low convex	Flat	Convex	Low convex	Low convex
Opacity	Translucent	Opaque	Translucent	Translucent	Translucent
Pigmentation	nil	nil	nil	Pale yellow	nil
Gram Reaction	Positive	Positive	Positive	negative	Negative

was added to the mixture. After 5 minutes of boiling the reaction mixture, colour emerged. The optical density (OD) of the samples was measured at 575 nm for additional analysis.

Optimization of process for Maximum Cellulase Production

Incubation Time

The production medium was inoculated with selected colonies, and enzyme activity was monitored every 24 hours.

pH

The pH was adjusted with 1 N HCl and 1 N NaOH to 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 in several sterile flasks containing broth with the ideal concentration of carbon source and substrate. At 37 °C, the flasks underwent inoculation and incubation. Following the incubation period, filtrate containing cell-free culture was taken for use as an enzyme source in subsequent processes.

Temperature

A chosen bacterial strain that had been cultivated overnight was injected into a production medium at pH 7. The broth was incubated for 24 hours at various temperatures ranging from 35, 40, 45, 50, 55, and 60°C. The cell-free culture filtrate is obtained at the end of the incubation period and used as a source of enzymes.

Carbon Sources

To test their effects, several carbon sources including glucose, lactose, sucrose, fructose, maltose, and CMC were added to the production medium at concentrations ranging from 1 to 5%.

Nitrogen Sources

By substituting urea, yeast extract, and ammonium sulphate for the peptone (0.5%) in the production medium, the effects of various nitrogen sources were studied.

Table 4. Biochemical characterization

Biochemical Test	C1-A	C1-B	C1-D	C1-E	C2-A
Catalase test	Positive	Negative	Positive	Positive	Positive
Indole test	Negative	Negative	Negative	Negative	Negative
Citrate Utilization test	Negative	Positive	Positive	Positive	Positive
Urea hydrolysis	Negative	Negative	Negative	Negative	Negative
Methyl red Test (M-R)test	Negative	Negative	Negative	Negative	Negative
Voges-Proskauer (V-P)test	Positive	Negative	Positive	Negative	Positive
Starch hydrolysis	Positive	Positive	Positive	Positive	Negative
Gelatin hydrolysis	Positive	Positive	Positive	Negative	Positive
Glucose	Positive	Positive	Positive	Negative	Positive
Lactose	Negative	Positive	Positive	Negative	Negative
Sucrose	Positive	Positive	Positive	Negative	Positive
Mannitol	Positive	Positive	Positive	Positive	Positive
Maltose	Positive	Positive	Positive	Negative	Positive

Table 5. Enzyme activity by CMC Assay

Cultural filtrate (Days)	Isolate C1-D(IU)	Isolate C1-E(IU)	Isolate C2-A(IU)
1	61.05±0.12	127.66±0.05	199.47±0.05
2	77.71±0.32	160.97±0.27	233.13±0.46
3	116.56±0.24	172.07±0.13	244.23±0.22
4	105.46±0.15	199.82±0.81	294.18±0.17
5	66.60±0.31	116.56±0.22	344.33±0.11
6	122.11±0.02	94.36±0.36	199.82±0.09
7	49.95±0.10	83.26±0.14	116.56±0.12

Agro-Based Waste as substrate

Various agricultural waste products, including castor seed waste, wheat bran, and rice husk, were used as the carbon source in the production medium. They were introduced to the growth media while submerged, and after 24 hours, the enzyme activity was assessed.

RESULT AND DISCUSSION

So,a soil sample was used to isolate bacteria that produce cellulase. They were recognised based on their biochemical and physical traits. The isolates showing maximum cellulase activity were found to be *Enterobacter cloacae* (ISOLATE-C2-A) followed by *Pseudomonas aeruginosa* (ISOLATE-C1-E) and *Bacillus subtilis* (ISOLATE-C1-D).

Incubation Time

Enzyme activity was checked for all the three isolates by incubating by CMC assay for 7 days. Maximum activity shown by Isolate C1-D, C1-E and C2-A was on days 6th ,3rd and 5th respectively. Amongst these isolates, maximum activity was exhibited by isolate C3 (344.33 IU).

Effect of pH

All three isolates were grown on media with pH of different ranges from 5.0 to 11.0. Cellulase activity was maximum observed in a medium with pH 7.0–8.0 for *Enterobacter*

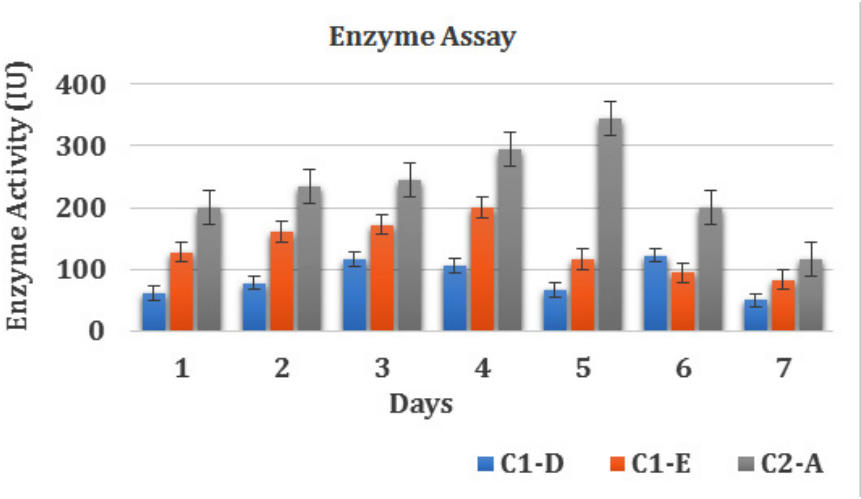

Fig. 1(a). Enzyme Essay

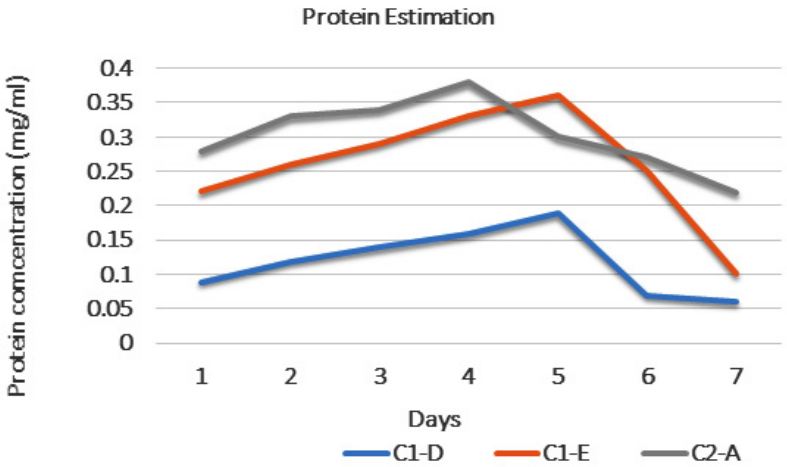

Fig. 1(b). Protein Estimation

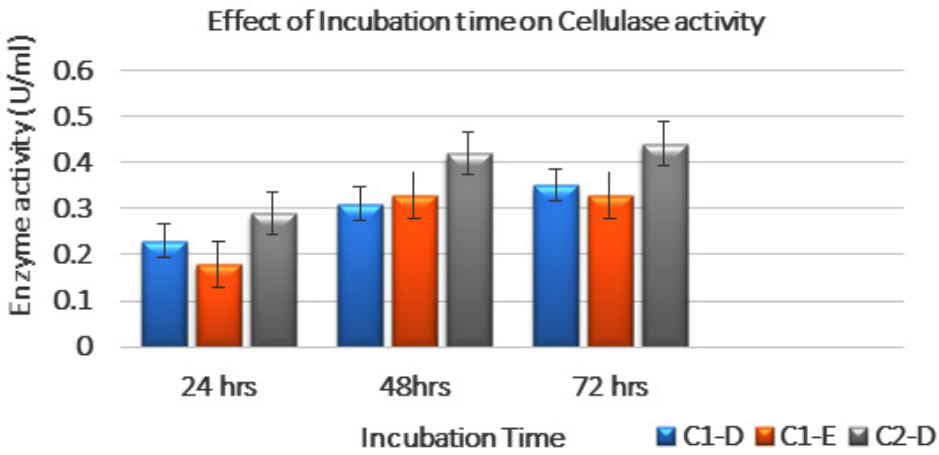

Fig. 1(c). Effect of Incubation time on Enzyme Activity

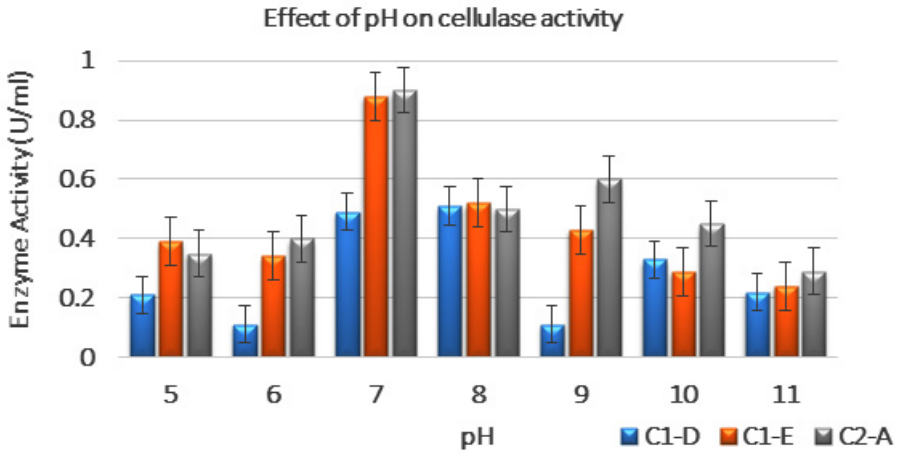

Fig. 2. Effect of pH on Enzyme Activity

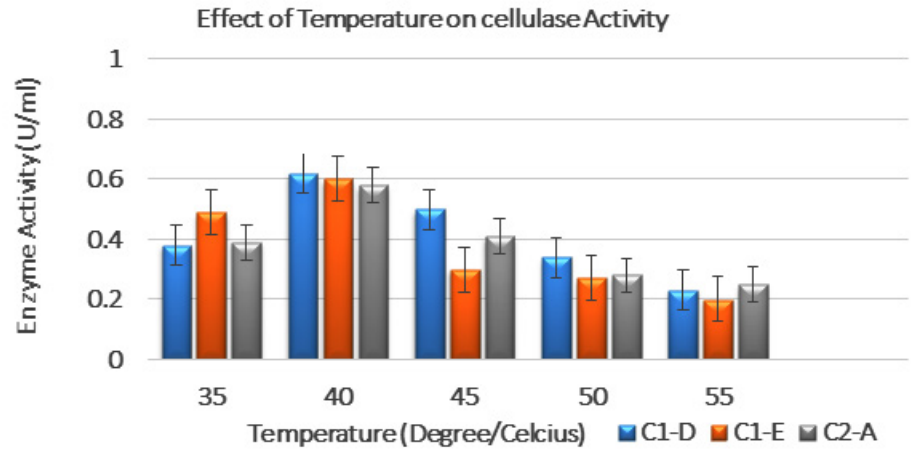

Fig. 3. Effect of pH on Enzyme Activity

cloacae, Pseudomonas aeruginosa and *Bacillus subtilis* (Figure 2). The result was also related to the findings of some other researchers for a strain of *Bacillus subtilis.* [20–22][Figure-2]

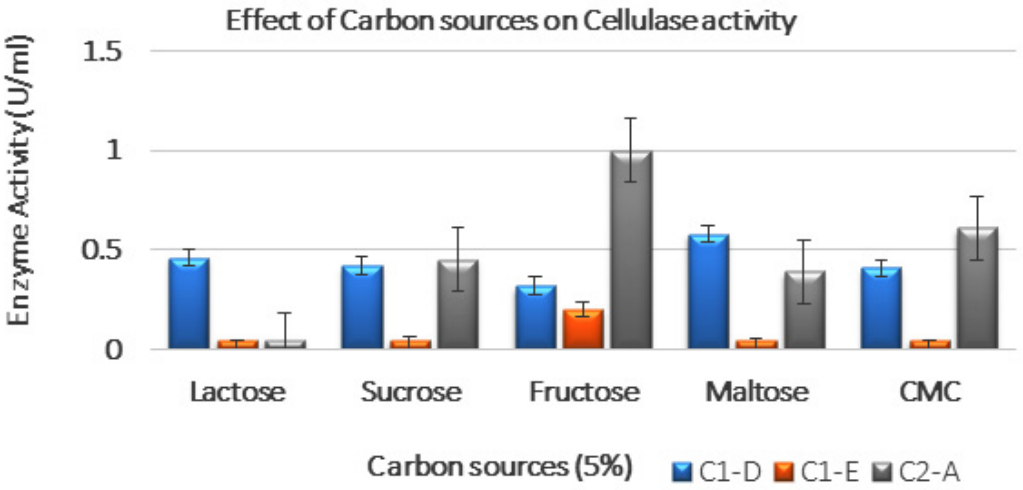

Fig. 4. Effect of Carbon sources on Enzyme Activity

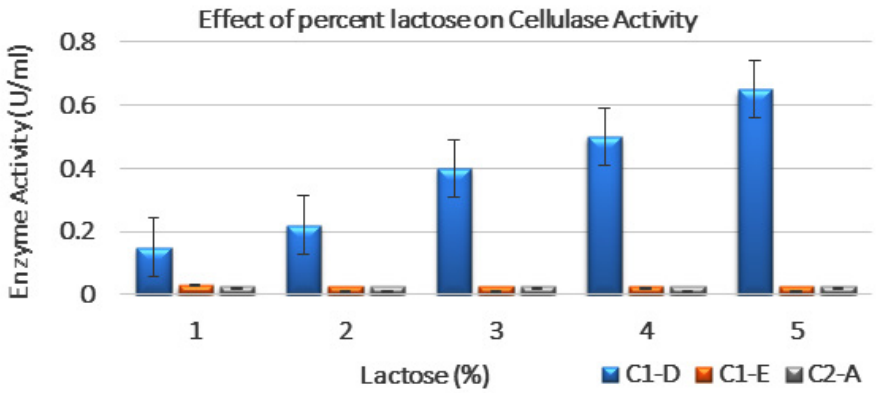

Fig. 5-a. Effect of percent lactose on Enzyme Activity

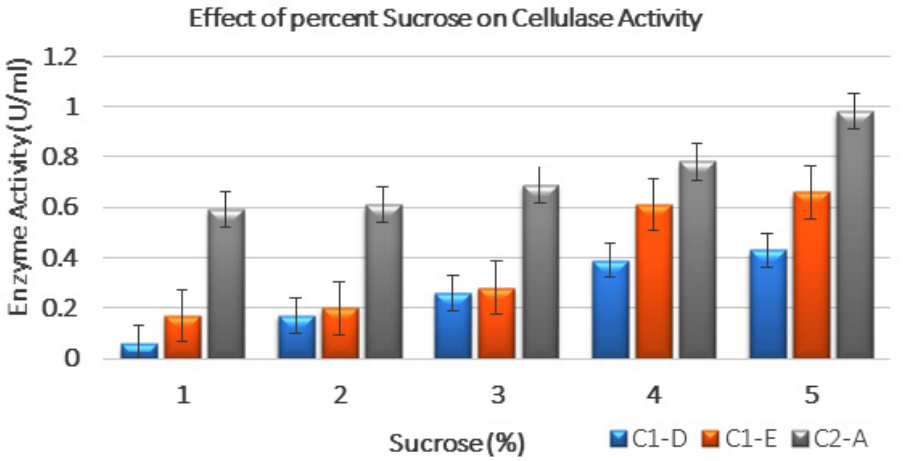

Fig. 5-b. Effect of percent Sucrose on Enzyme Activity

Effect of Incubation Temperature

The production of cellulase peaked at a temperature of 40°C for all three isolates when enzyme activity was measured at various temperatures. [Figure .3]. The secretion of extracellular enzymes was found to be influenced by temperature by causing the cell membrane to change its physical properties. At a temperature of 40°C, these isolates were determined to have the highest enzyme activity[23]. The results thus

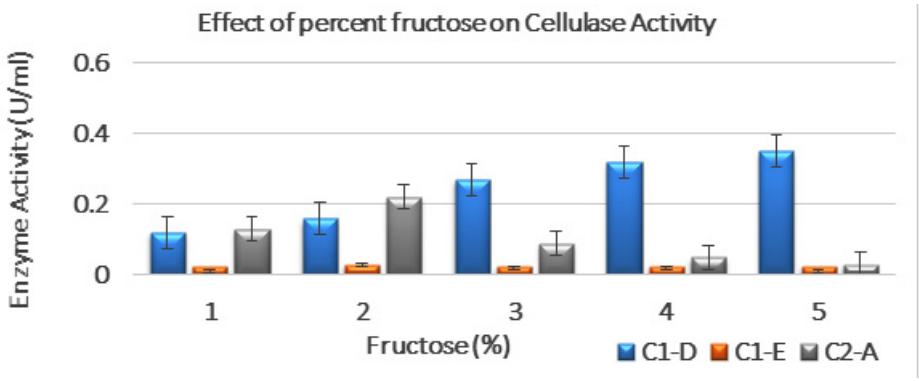

Fig. 5-c. Effect of percent fructose on Enzyme Activity

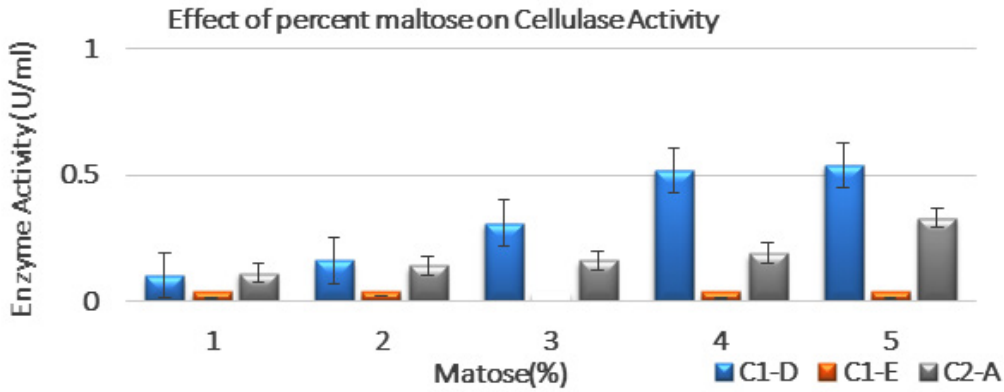

Fig.5-d. Effect of percent Maltose on Enzyme Activity

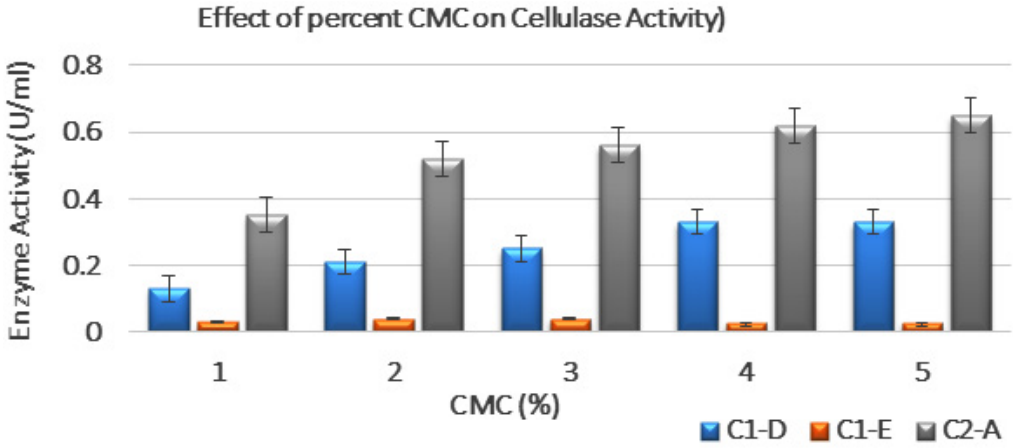

Fig. 5-e. Effect of percent CMC on Enzyme Activity

obtained had similarity to those of Bakare *et al*. (2005)[24] which suggests that optimum activity for cellulase enzyme obtained from *Pseudomonas* was at temperatures ranging from 35 to 40°C. Ray *et al*. [25] observed that the yield of cellulase obtained in a fermentation medium at 45°C was reported to be minimal, while at 40°C, the yield of cellulase was found to be maximum in *Bacillus subtilis*. Immanuel *et al*. [7]reported the activity of endoglucanase to be maximum in *Bacillus, Micrococcus* sp. and *Cellulomonas* , at 40°C and 7 pH [Figure-3]

Effect of Carbon Source

The source of carbon in growth media was replaced by various other carbon sources like lactose, sucrose, fructose, maltose and CMC. Maximum activity was shown by fructose and lactose as substrate after 24 hours of incubation. The best substrate for the formation of cellulase is glycerol, with an efficiency of 28.7% on the weight of additional substrate. [26] When D-xylose is employed as a carbon source, Ishihara *et al*. showed that purportedly creates cellulase membrane.[27]Also, it does not show good production of enzyme by selected isolates. Cellulase production was observed at optimum level in presence of the sucrose, maltose and CMC [28.] [Figure 4]

Effect of Different Concentrations of Carbon Sources

The original concentration of sugar in the growth medium was maintained at 1% which was then replaced by other carbon substrates having

Table 6. Maximum enzyme activity of three isolates considering optimization parameters

Maximum Enzyme activity considering optimization parameters	Isolate C1-D	Isolate C1-E	Isolate C2-A
Incubation time	72 hrs(0.29±0.13 U/mL)	72 hrs(0.42±0.31U/mL)	72 hrs(0.44±0.14 U/mL)
Optimum pH	8 (0.51± 0.33U/mL)	7 (0.88±0.30 U/mL)	7 (0.90± 0.11U/mL)
Optimum Temperature	40 (0.62± 0.04U/mL)	40 (0.60±0.21 U/mL)	40 (0.58±0.02 U/mL)
Carbon source	Maltose (0.58± 0.03U/mL)	CMC(0.012±0.13 U/mL)	Fructose(1.11±0.21 U/mL)
Lactose(%)	5%(0.65±0.23 U/mL)	5%(0.03±0.02 U/mL)	5%(0.02±0.01 U/mL)
Sucrose(%)	5%(0.43± 0.33U/mL)	5%(0.66± 0.16U/mL)	5%(0.98± 0.01U/mL)
Fructose(%)	5%(0.35± 0.12U/mL)	5%(0.03±0.04 U/mL)	3%(0.22±0.03 U/mL)
Maltose(%)	5%(0.54±0.08 U/mL)	5%(0.02±0.06 U/mL)	5%(0.33±0.12 U/mL)
CMC(%)	5%(0.33± 0.04U/mL)	5%(0.04±0.01 U/mL)	5%(0.65±0.11 U/mL)
Nitrogen source	Ammonium sulphate (0.30±0.11 U/mL)	Ammonium sulphate (0.76±0.05 U/mL)	Ammonium sulphate (0.17±0.07 U/mL)

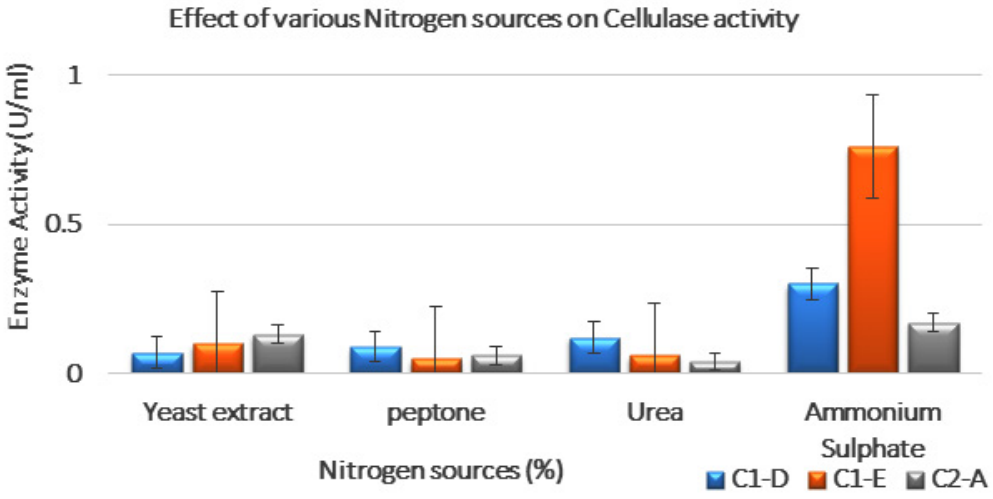

Fig. 6. Effect of Nitrogen sources on Enzyme Activity

5 percent concentration. Lactose and fructose showed the highest production of cellulase when the medium was supplemented with a 5% carbon source after incubation of 24 h. [Figure 5 a-e]

Effect of Nitrogen Source

The extracellular production of cellulase depends on the different sources of carbon and nitrogen added to the medium. The role of

Table 7. Specific activity of three isolates considering optimization parameters

Specific activity considering optimization parameters	Isolate C1-D	Isolate C1-E	Isolate C2-A
Incubation time	72 hrs(1.52±0.02 U/mg)	72 hrs(1.16±0.11U/mg)	72 hrs(1.15±0.13 U/mg)
Optimum pH	8 (2.63±0.01 U/mg)	7 (2.44±0.23 U/mg)	7 (2.36±0.12 U/mg)
Optimum Temperature	40 (3.26±0.02 U/mg)	40 (1.66±0.12 U/mg)	40 (1.52± 0.05U/mg)
Carbon source	Maltose(3.05± 0.23U/mg)	CMC(0.03±0.02 U/mg)	Fructose(2.92±0.03 U/mg)
Lactose(%)	5%(3.42±0.22 U/mg)	5%(0.08± 0.23U/mg)	5%(0.05±0.02 U/mg)
Sucrose(%)	5%(2.26± 0.21U/mg)	5%(1.83±0.01 U/mg)	5%(2.57±0.21U/mg)
Fructose(%)	5%(1.84±0.13 U/mg)	5%(0.08±0.02 U/mg)	3%(0.57±0.20 U/mg)
Maltose(%)	5%(2.84±0.11 U/mg)	5%(0.05±0.02 U/mg)	5%(0.86±0.13 U/mg)
CMC(%)	5%(1.73± 0.02U/mg)	5%(0.11±0.01 U/mg)	5%(1.71± 0.01U/mg)
Nitrogen source	Ammonium sulphate (1.57±0.12 U/mg)	Ammonium sulphate (2.11±0.23 U/mg)	Ammonium sulphate (0.44±0.12U/mg)

Table 8. Specific activity of three isolates considering optimization parameters

Isolate	Incubation time	Optimum pH	Optimum Temperature	Carbon source	Nitrogen source	Maximum Enzyme Activity	Protein content	Maximum specific activity
Isolate C1-D	72 hrs	8	40	Lactose (5%)	Ammonium Sulphate	0.65±0.23 U/ml	0.19 mg/ml	3.42±0.22 U/mg
Isolate C1-E	72 hrs	7	40	Sucrose (5%)	Ammonium Sulphate	0.88±0.16 U/ml	0.36 mg/ml	2.44±0.02 U/mg
Isolate C2-A	72 hrs	7	40	Fructose (5%)	Ammonium Sulphate	1.11±0.98 U/ml	0.38 mg/ml	2.92± 0.21 U/mg

Table 9. The potential isolate C3's potential to hydrolyze cellulose from the culture medium and release glucose was used to measure the cellulase activity. The amount of glucose released was measured every 24 hours

Period of Fermentation mL	Rice husk IU/mL (hours)	Wheat husk IU/mL	Castor seed waste IU/mL	CMC I U /
24	0.3	0.12	0.33	0.35
48	0.61	0.2	0.21	0.53
72	0.67	0.23	0.45	0.56
96	1.4	0.34	0.44	0.61
120	1.48	1.45	0.65	1.92
144	1.76	1.51	0.13	1.32
168	0.86	1.23	0.32	0.73
192	0.52	0.33	0.12	0.65
216	0.44	0.42	0.09	0.27
240	0.43	0.23	0.4	0.23

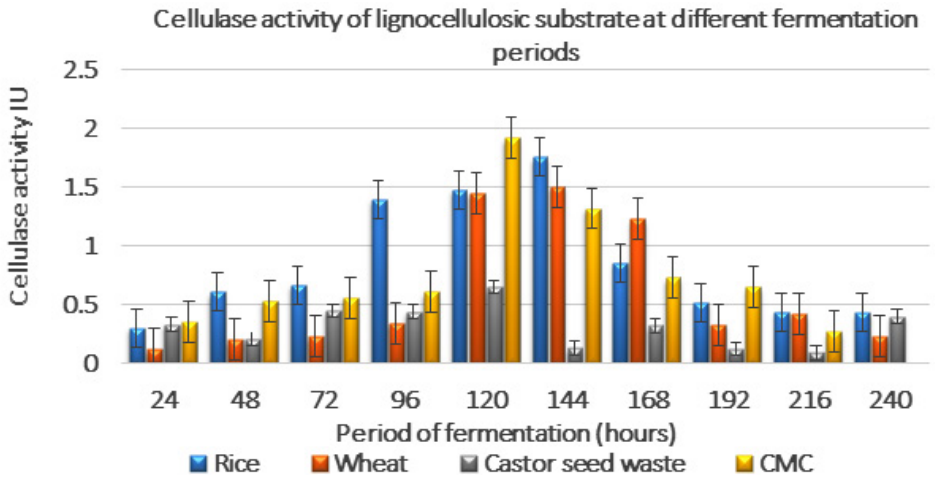

Fig.7. Cellulase activity of lignocellulosic substrate at different fermentation periods by potential isolate C2-A.

different nitrogen sources was checked on the growth of isolates by replacing the peptone that was used in the original medium. The various other nitrogen sources used were yeast extract, urea and ammonium sulphate. Amongst these sources, Ammonium sulphate was found to produce maximum cellulase as compared to others. The major element found in the cell proteins contains nitrogen and the uptake of ammonium sulphate salt indicates its direct assimilation in the synthesis of cell proteins.[29] [Figure 6, Table-6, Table-7, Table-8]

Effect of Agro-Based Waste Material

Agro waste has been used as a substitute source of carbon in the fermentation medium for the manufacture of cellulase, according to several reports. In contrast to wheat husk and castor seed waste, selected isolate 3 produced the most enzymes, according to the current investigation, when rice husk served as an inducer. [Table-9, Figure 7]

CONCLUSION

In this study, potential cellulase-producing bacteria from soil samples were isolated and identified. Maximum production of cellulase was shown by *Enterobacter cloacae* followed by *Pseudomonas aeruginosa* and *Bacillus subtilis.*

The optimum temperature and pH during cellulase production were found to be 40°C and 7–8. Various carbon and nitrogen sources were used and amongst them the best result was given by lactose and ammonium sulphate as compared to others. As a result, this research has assisted in identifying numerous optimized criteria for the microorganism's maximal cellulase production. The production of cellulase was carried out after optimization of various parameters at 40°C, pH 7 for 48 hours in shaking incubator at 120 rpm. It is found that in comparison to fungi, bacteria show a higher growth rate with good potential to produce cellulase. However, using bacteria to produce cellulase is not a common practice.

Reports suggested the presence of cellulolytic activity in some bacterial genera like *Cellovibrio, Sporocytophaga, Cellulomonas*, *Pseudomonas*, spp. [9], *Bacillus,* and *Micrococcus* [7]. This research suggested that several parameters are responsible for the production of the enzyme in microorganisms and control the overall process of optimization. These parameters include pH, inoculum time, temperature, carbon and nitrogen sources, etc. which play a vital role in the total yield of cellulase. Enzyme activity and specific enzyme activity for each isolate were calculated and thus the potential of these isolates was compared. The ability of these bacteria to produce cellulase in the presence of different carbon sources can be beneficial in optimizing the production process for each isolate.

Additional research on the purification and use of cellulase in several commercial domains is ongoing. The detergent, food, and pharmaceutical industries can all benefit from the utilisation of the refined cellulase. Cellulase enzymes' high activity and stability at high temperatures and neutral to

alkaline pH will be useful in a variety of industrial and biotechnological applications.

Conflict of Interest

There is no conflict of interest.

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