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Isolation, Screening and Production of Bacterial Cellulase from Cow Dung

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Abstract: ***Cellulases are the most important industrial enzyme due to their potential application in various industries including pulp and paper, textile, laundry, biofuel production, food and feed industry, brewing and agriculture. The present study was carried out to isolate and characterize cellulase producing microorganisms from different cow dung samples. Four different cow dung samples were collected from variety of cows namely Gir, Holstein, Jersey and Desi and isolation and screening was done to check cellulase producing microorganisms. The obtained 11 isolates were screened for their cellulase activity by using CMC (carboxymethyl cellulose) agar medium. In the current study, 10 cellulase producing isolates were obtained and were characterized morphologically from which 8 isolates were found Gram negative and 2 were found Gram positive. All the 10 cellulase producers were further confirmed for their cellulase producing ability by performing turbidity test. Out of these 10 isolates, GN4 and HN2 with optical density 0.35 and 0.28, respectively were found to be best cellulase producer and were selected for cellulase enzyme production and other further studies. Botssh the isolates were tested for their enzymatic activities by performing DNSA method and protein estimation by Lowry's method. Cellulase activities were found to be 7.33 µg/ml/min and 3.66 µg/ml/min and the total protein concentrations were found to be 40 µg/ml and 32 µg/ml for GN4 and HN2, respectively. Thus, dungs cow dung can be considered as the excellent source for cellulase producing bacteria.***
Keywords: Cellulase, Cellulase producing microorganisms, Cow dung, Isolation & screening, Enzyme production.

I. INTRODUCTION

Nowadays enormous amount of agricultural and industrial cellulosic waste has been accumulating in environment [10]. Cellulose is considered as the most important natural renewable resource for bioconversion, and is naturally present in the environment in various decomposable sources. This organic matter is decomposed by fungi, bacteria, and actinomycetes [16]. Among these microorganisms, bacteria are more capable of decomposing cellulose by the secretion of cellulase enzyme [5]. Cellulose, crystalline polymer of D-glucose residues connected by beta-1,4 glycosidic linkage, being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature [12]. Therefore, it has become considerable economic interest to develop processes for effective treatment and utilization of cellulosic waste as inexpensive carbon sources.

Complete enzymatic hydrolysis of enzyme requires synergistic action of 3 types of enzymes namely-Cellobiohydrolases, Endoglucanase or Carboxy methyl cellulase (CMCase) and alpha -glucosidases [3]. Cellulose is commonly degraded by an enzyme called cellulase. Cellulase refers to a class of enzymes produced chiefly by Fungi, bacteria, and protozoans that catalyze the cellulolysis (or hydrolysis) of cellulose [2].

Cellulolytic enzymes are the third most important industrial enzyme due to its versatile application in various industries such as paper and pulp, textile and detergent industry [15]. Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juices, and in baking. A potential challenging area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals [15].

Due to increased concern about the greenhouse effect, depleting oil reserves and rising global oil prices, as well as the focus on utilizing renewable fuels such as bioethanol cellulase enzyme have become quite important to keep the environment active and interactive [16].

II. METHODOLOGY

A. Sample Collection

4 different cow dung samples namely Gir, Holstein, Jersey, Desi, were collected. Samples were transferred to labelled plastic bags and processed on the same day of collection. For isolation of bacteria, suspensions were prepared by mixing one gram of each of the fresh cow dung sample to 9 ml of sterile distilled water and was vigorously shaken for proper mixing of sample [14].

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B. Isolation and Purification of Bacteria from Cow Dung

The cow dung suspensions were streaked on sterile Nutrient agar plates by streak plate method. All the plates were incubated at 37˚C for 24 hours. After the incubation, isolated colonies were further purified using streak plate technique. The purified bacterial cultures were maintained over sterile Nutrient agar slant. The purity of culture was cross checked by Gram's staining procedure [9].

C. Screening of Purified Cultures for Cellulase Activity

The purified cultures were screened for cellulase production by streaking them as line on sterile CMC (carboxy methyl cellulose) agar plates centrally and incubating them for 72 hours at 37˚C. After incubation period, the plates were flooded with Gram's iodine to visualize clear zones formed by cellulase positive strains. Positive and better zone producing strain was chosen and continued for further studies [4].

D. Characterization of Cellulase Producing Isolates

All the positive isolates were studied for different colony characteristics such as shape, size, surface, edge, elevation, opacity and pigmentation. Morphological characteristics of colonies of each isolate were examined on CMC plate [14].

1) *Morphological Characterization:* Morphological characterization was done by performing Gram's staining.
2) *Turbidity Measurement for Cellulase Activity:* The isolated colonies were inoculated in different sterile CMC broths and were incubated at 37˚C for 48 hours. After incubation period, the broths were observed for their turbidity. Absorbance reading at 650 nm was taken to determine the optical density.

E. Production of Cellulase

For the cellulase production, two best cellulase producing isolates were chosen for cellulase production. On the basis of staining and turbidity, two best cellulase producer isolates were grown in 100 ml different fermentation medium at pH 7.0. To the fermentation medium, 1 ml of selected bacterial cultures was added. The inoculated flask was incubated for 96 hours on shaker incubator (120 rpm) at 37˚C. After incubation, for crude extracellular cellulases, the fermentation broth was centrifuged at 5000 rpm for 5 minutes at 4˚C. The supernatant obtained was used as crude enzyme for enzyme activity [1].

F. Enzyme Activity Assay

1) *Cellulases Activity (DNSA Method):* Cellulase activity was assayed by a modification of Dinitrosalicyclic acid (DNS) method using Carboxy methyl cellulose (CMC) as a substrate. The reaction mixture contained 0.5 ml of 1.0% (w/v) CMC in 0.5 M solution of sodium acetate buffer, pH 5.0 & 0.5 ml of the cell free culture supernatant and incubated at 55˚C for 15 min. After incubation, reaction was stopped by addition of 2 ml of DNS reagent and boiled at 100˚C in the water bath for 10 min. Sugar liberated were determined by measuring absorbance at 540 nm. A concentration glucose calibration curve was used to convert color to reducing sugar equivalent.one unit of enzyme activity was defined as the amount of enzyme required to liberate 1µg of glucose from the appropriate substrate per ml per min under the assay condition. Cellulases assay was performed according to the method of S. Sadashivam & A. Manikam [17].

Calculation of Enzyme Assay (EA)-

The cellulases production was estimated by using glucose standard curve. The activity of cellulases was determined by using the following formula.

$$EA = \frac{\text{Absorbance of enzyme solution} \times (\mu g/ml)}{\text{Time of incubation (min)}}$$

2) *Protein Determination:* Protein concentration in crude extracellular extract was determined by Lowry's method. Bovine serum albumin (BSA) was used as protein standard. 1 ml of supernatant of filtrates were mixed with 5 ml of alkaline copper solution, All the tubes were incubated at room temperature for 15 minutes, then 0.5 ml of approximately diluted Folin-Ciocalteu reagent was added. All the tubes were incubated at room temperature (dark) for 30 minutes. After incubation, color was changed, the optical density was measured at 750 nm by using spectrophotometer. The optical density was compared with the BSA standard curve to calculate the amount of protein µg/ml [11].

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III. RESULTS AND DISCUSSION

A. Sample Collection

In the present study, 4 different cow dung samples were collected from variety of cow namely Jersey, Desi, Holstein, and Gir (Fig.1).



Fig. 1: Different cow dung samples

Since most of the natural wastes were degraded by the native microbes present in it, the present study deals with the analyzing the microbes present in the cow dung for their ability of producing cellulase [2]. Degradation of cellulosic materials is a complex process requiring participation of microbial enzymes. Habitat that contains these substrates are best source in which these organisms are present [7]. Cellulosic material if stand as such in the environment can be very problematic that's why in this study, we are searching for the cellulase producers, so that we can convert the wastage celluloses into useful products [6].

For isolation of bacteria, all cow dung were prepared by mixing one gram of the fresh cow dung sample was mixed to 9 ml of sterile distilled water and vigorously shaked for proper mixing of sample [14].



Fig. 2: Different cow dung suspension

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B. Results of Isolates Obtained from Cow Dung

Around 13 different bacterial colonies were obtained on nutrient agar plates.

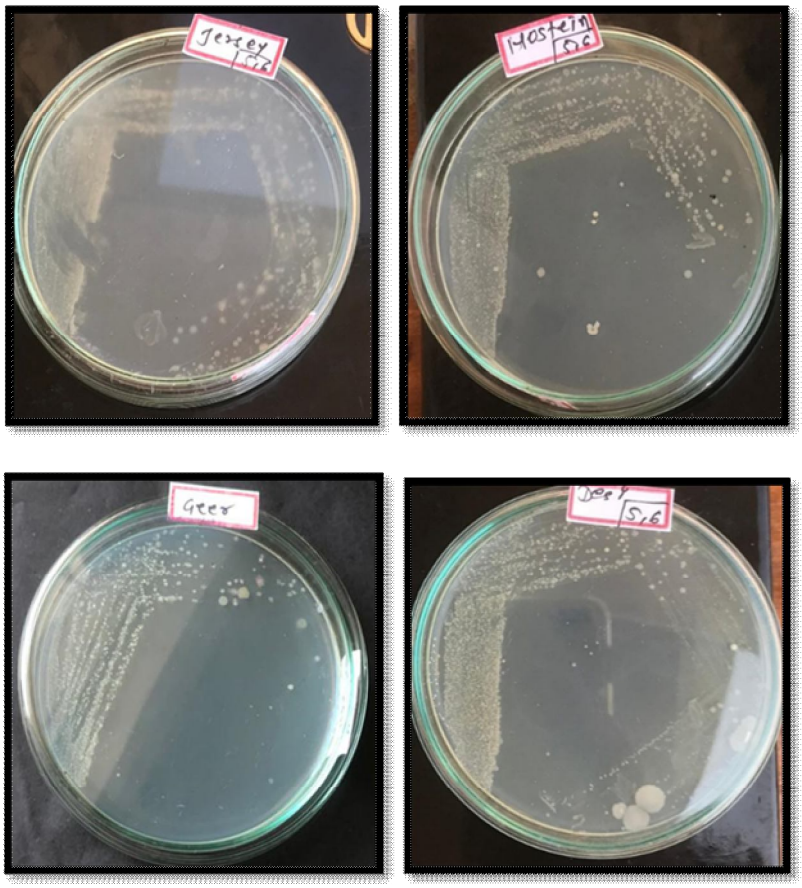


Fig. 3: Isolation and Purification of bacteria obtained from cow dung

Cow dung was selected as a source for obtaining desirable cellulase producing microorganisms, because it is rich source of diverse group of cellulolytic microorganisms. Owning to diet of the ruminants which consist of higher amount of cellulosic matter. Around 13 different bacterial colonies were obtained on nutrient agar plate from different cow dung sample. Out of 13 different bacterial colonies, 11 colonies were purified. All the purified isolates were studied for their Gram's staining and morphology. Further screened for their cellulase activity by the Gram's iodine or Congo red test.

TABLE 1
Gram's reaction of the purified bacterial colonies

Sample 1	Gram positive long rods
Sample 2	Gram negative rods
Sample 3	Gram negative short rods
Sample 4	Gram negative short rods
Sample 5	Gram negative rods
Sample 6	Gram positive cocci
Sample 7	Gram negative cocci
Sample 8	Gram positive cocci
Sample 9	Gram negative short rods
Sample 10	Gram negative rods
Sample 11	Gram negative short rods

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C. Results of Screening of Purified Isolates for Cellulase Activity

Among the total of 11 isolates were screened on CMC agar plate, of which 10 isolates showed clear zone around colonies after flooded the plate with Gram's iodine (Fig.4).

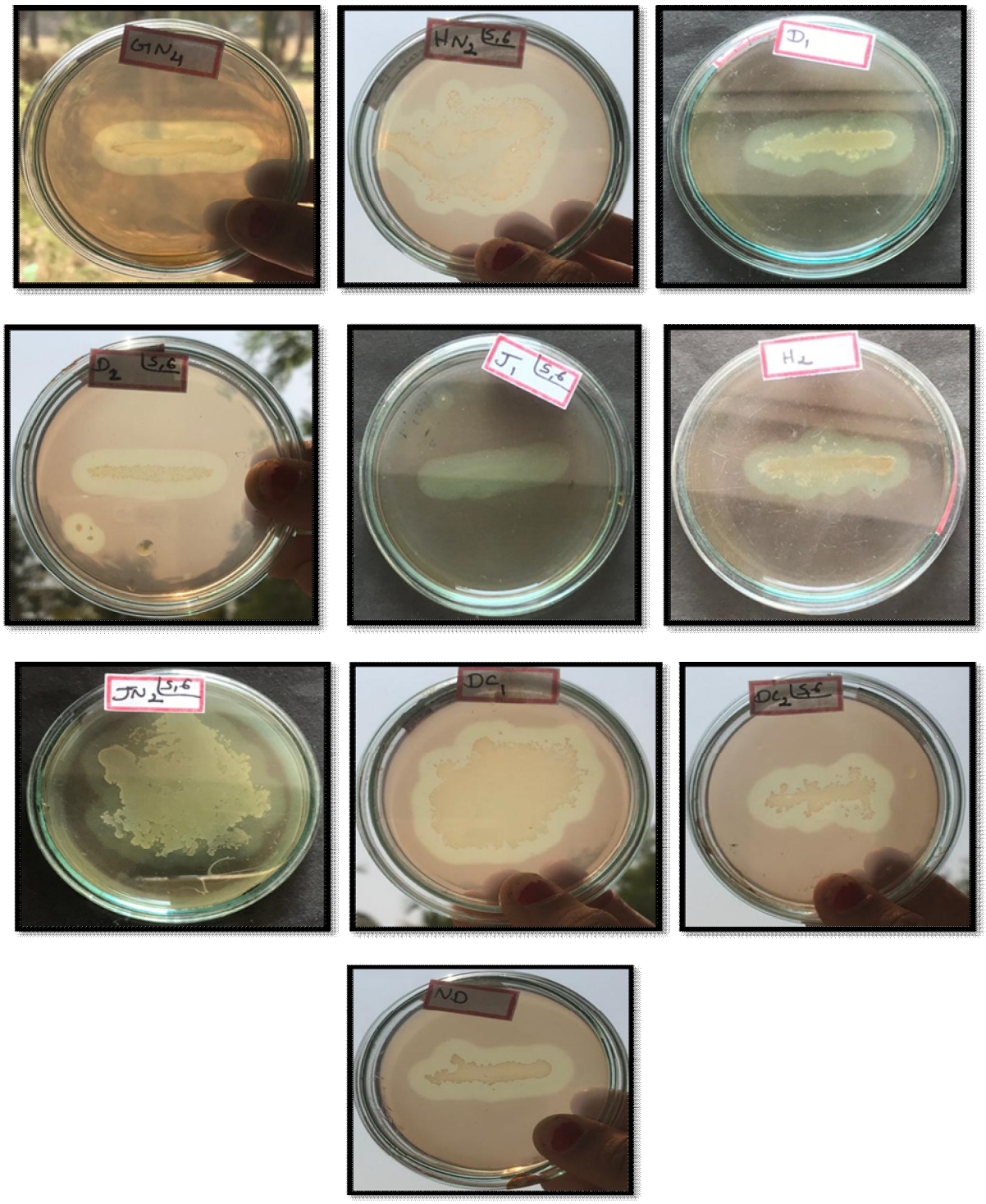


Fig. 4: Screening of cellulase producing bacteria

10 isolates were screened on CMC agar plate were found to be cellulase producers. All the cellulase producing isolates was coded as GN4, HN2. D1, D2, J1, H2, DC1, DC2, JN2, ND. Screening for the isolates with cellulolytic activity revealed that the spore formers were more prolific producers of the enzyme [13]. All the cellulase producing isolates were characterized morphologically.

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D. Characterization of Cellulase Producers

All the cellulase producers screened on CMC agar plates, were studied for their colony characteristics such as shape, size, surface, edge, elevation, opacity and pigmentation, moreover, all the cellulase producers were morphologically characterized by performing Gram's staining which are depicted in TABLE.2

The below table shows the result of staining and colony characteristics of the cellulase positive culture.

Table 2
Colony characterization of positive isolate

Code of isolates	Colony characteristics
GN4	Rhizoidal, small, convex, rough, opaque, brick-red pigmentation.
HN2	Rhizoidal, large, convex, rough, opaque, brick-red pigmentation.
D1	Rhizoidal, intermediate, convex, rough, translucent, brick-red pigmentation.
D2	Rhizoidal, small, convex, rough, translucent, brick-red pigmentation.
J1	Round, small, convex, smooth, translucent, no pigmentation.
H2	Round, intermediate, raised, smooth, opaque, white pigmentation.
JN2	Round, intermediate, convex, smooth, opaque, white pigmentation.
DC1	Round, intermediate, convex, smooth, opaque, white pigmentation.
DC2	Round, large, flat, smooth, opaque, no pigmentation.
ND	Irregular, small, slightly raised, rough, opaque, white pigmentation.

All the isolates were different in their size, shape, opacity, surface and many with each other. From all isolates some isolates were large or in small in size. Most of the isolates were having round and some have irregular and rhizoidal shape. 5 isolates having smooth surface and another 5 isolates having rough. All isolates were opaque accept 3 (D1, D2, J1) which was translucent. Most of the isolates was having brick-red pigmentation, white pigmentation and only two were non- pigmentation.

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1) *Morphological Characterization*: In the present study, all the isolates were characterized morphologically by performing Gram's staining.

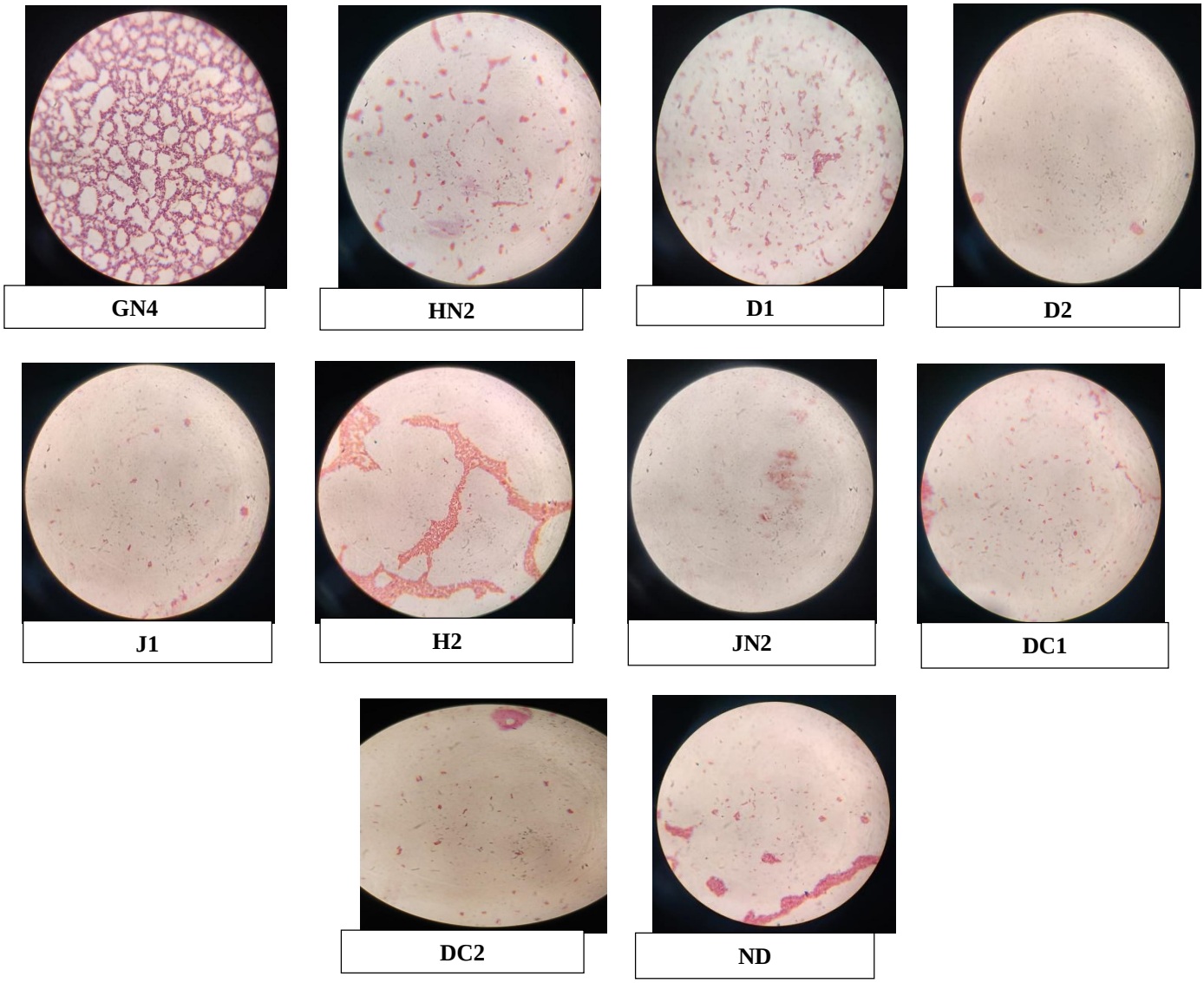


Fig. 5: Microscopic observation of Gram's staining

In the above Fig.5, microscopic observation of Gram's staining is shown. In total 10 cellulase producing isolates, 2 were Gram positive (coded as GN4, JN2) and 8 were Gram negative (coded as HN2, D1, D2, J1, H2, DC1, DC2, ND) (TABLE 3). Similar type of work also carried out by Bharti Sharma *et al.*, 2015, in their study they also reported, out of four strains, three strains B1, B3 and B5 were gram positive, cocci form and rest of the strain B4 is gram negative.

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TABLE 3
Characterization of Cellulase Producer Isolates

Isolates	Gram staining
GN4	Gram positive long rods occurring in chain.
HN2	Gram negative short rods occurring singly.
D1	Gram negative short rods occurring singly or in chain.
D2	Gram negative short rods occurring singly.
J1	Gram negative shorts rods occurring singly.
H2	Gram negative cocci occurring singly and in bunch.
JN2	Gram positive cocci occurring singly and in bunch.
DC1	Gram negative short rods occurring singly.
DC2	Gram negative short rods occurring singly or in chain.
ND	Gram negative short rods occurring singly.

2) *Result of Turbidity Test for Cellulase Activity:* In the present study, all the 10 cellulase producers were further tested for their cellulase activity by performing Turbidity test. (Fig. 6).



Fig. 6: Turbidity of Bacterial Isolates in CMC Broth

Turbidity test was performed in CMC broth that contained cellulose as a sole source of nutrient. By performing the test, all the samples gave turbidity which confirmed their cellulase production ability (Fig. 7).

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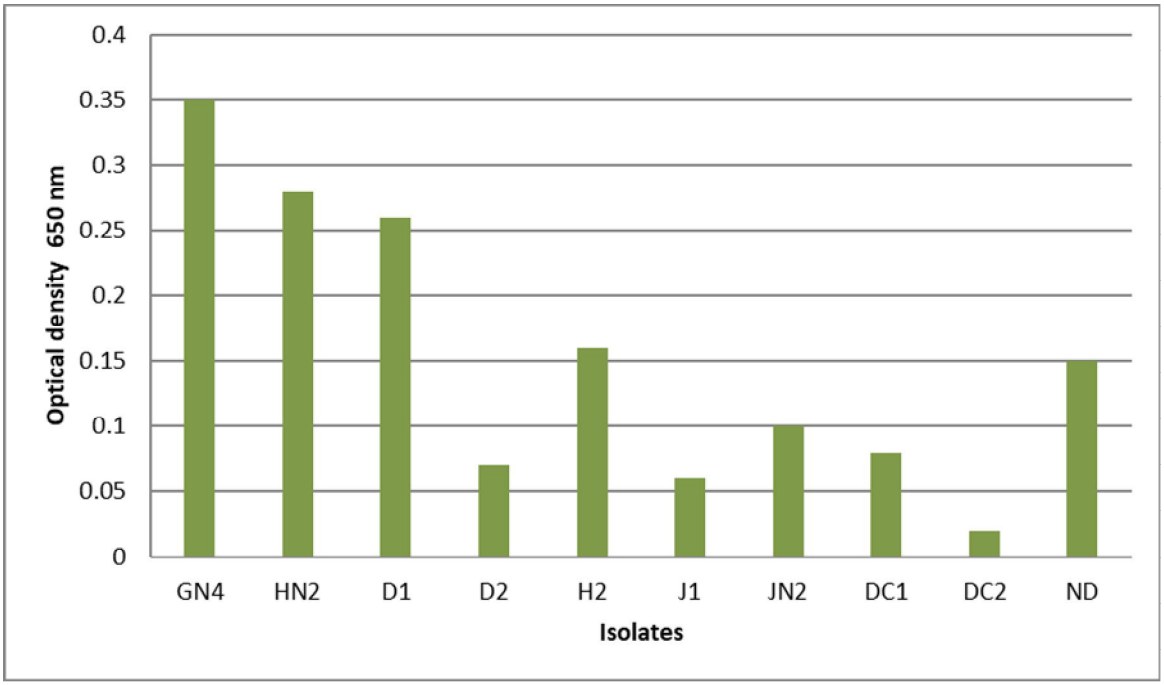


Fig. 7: Graphical Representation of Turbidity Measurement of Different Isolates.

Out of these 10 isolates, GN4 and HN2 with optical density 0.35 and 0.28 respectively were found to be best cellulase producer and were selected for cellulase production (Fig. 7).

E. Results of Cellulase Enzyme Production

Two bacterial isolates, GN4 and HN2 were inoculated to the fermentation medium, after incubation period, both the fermentation broths were centrifuged at 5000rpm for 5 minutes at 5˚C to extract crude cellular cellulase. Supernatant was used as crude enzyme for enzyme activity.



Fig. 7: Production of cellulase enzyme (GN4 and HN2 isolates)

On the basis of colony characteristics, morphology, turbidity measurement, two bacterial isolates GN4 and HN2 found to be best cellulase producers. Cellulase is an inducible enzyme and it is affected by the nature of substrate used for production.

In 2004, Haung and monk from their studies, have reported that when CMC is used as substrate, the enzyme activity was observed to increased, they also reported as incubation period increases the produced value of cellulase also increases.

In the present study, cellulase activity was checked by using CMC as substrate. Similar study was also carried out by Wood and Bhat in 1988, Wood and Bhat have also reported that CMC is a substrate gave the highest yield of enzyme.

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F. Results of Enzyme Activity Assay

1) Cellulase Activity: Cellulase activity was performed by DNSA method for selected 2 isolates (GN4 and HN2). Among them GN4 give higher cellulase activity (Fig. 8). Standard graph of glucose (1000µg/ml) for cellulase activity is shown in Fig. 9.

Table 4
Bacterial Cellulase Activity

Sr no.	Isolates	Enzyme activity (µg/ml/min)
1.	GN4	7.33
2.	HN2	3.66

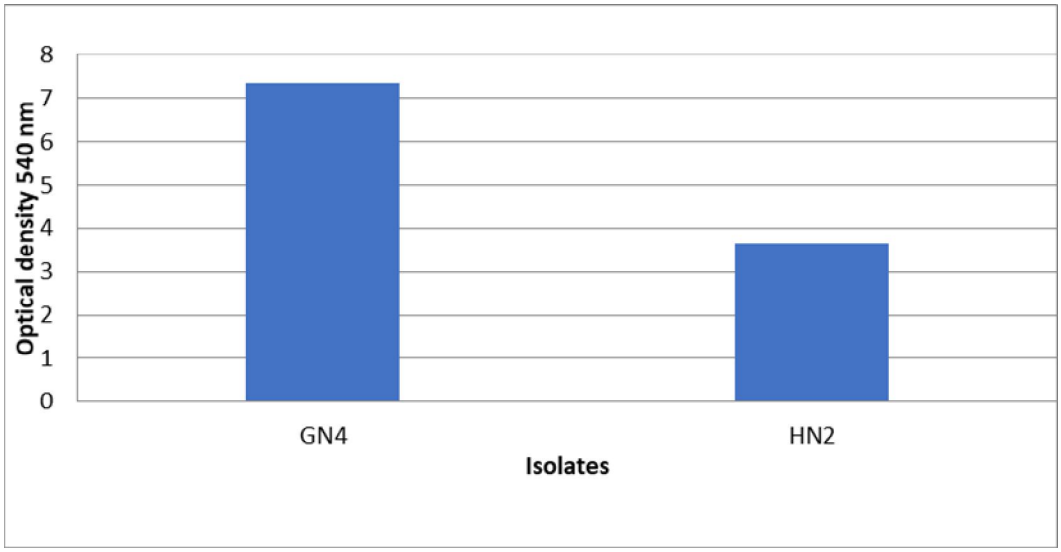


Fig. 8: Graphical representation of cellulase activity (GN4, HN2 isolates)

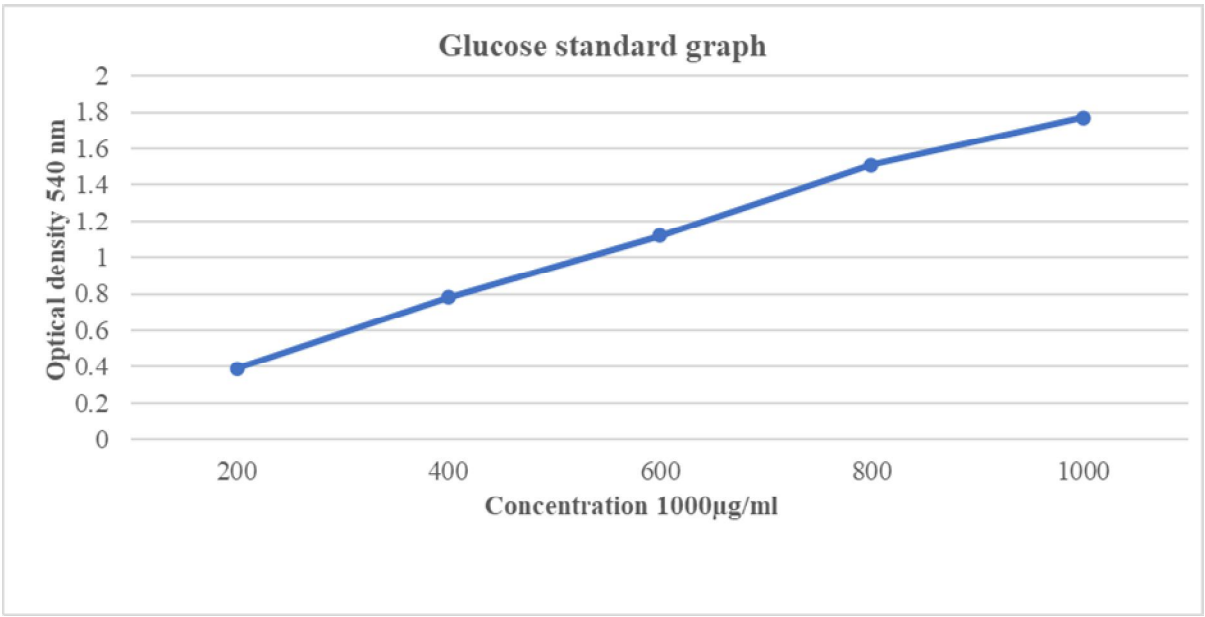


Fig. 9: Standard graph of glucose (1000µg/ml)

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2) *Protein Determination:* Protein concentration for GN4 and HN2 isolates was determined by Lowry's method using crude extract. Among them GN4 gave higher protein concentration (Fig. 10). Standard graph of protein (BSA) (200µg/ml) is as shown in Fig. 11.

TABLE 5
Bacterial cellulase protein assay

Sr no.	Isolates	Protein concentration (µg/ml)
1.	GN4	40
2.	HN2	32

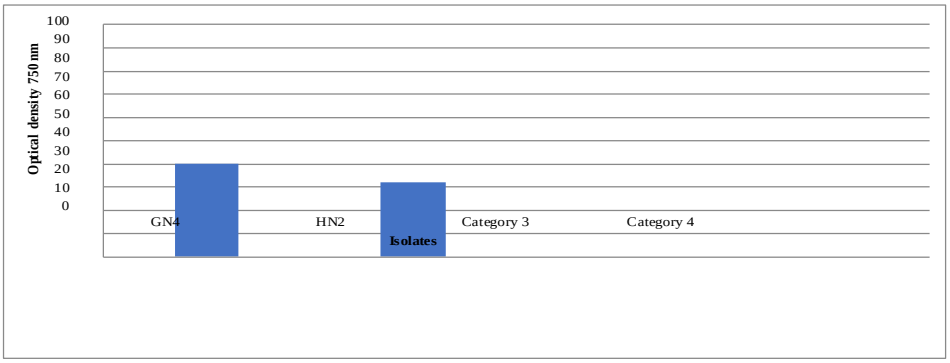


Fig. 10: Total protein concentration of GN4 and HN2 isolates

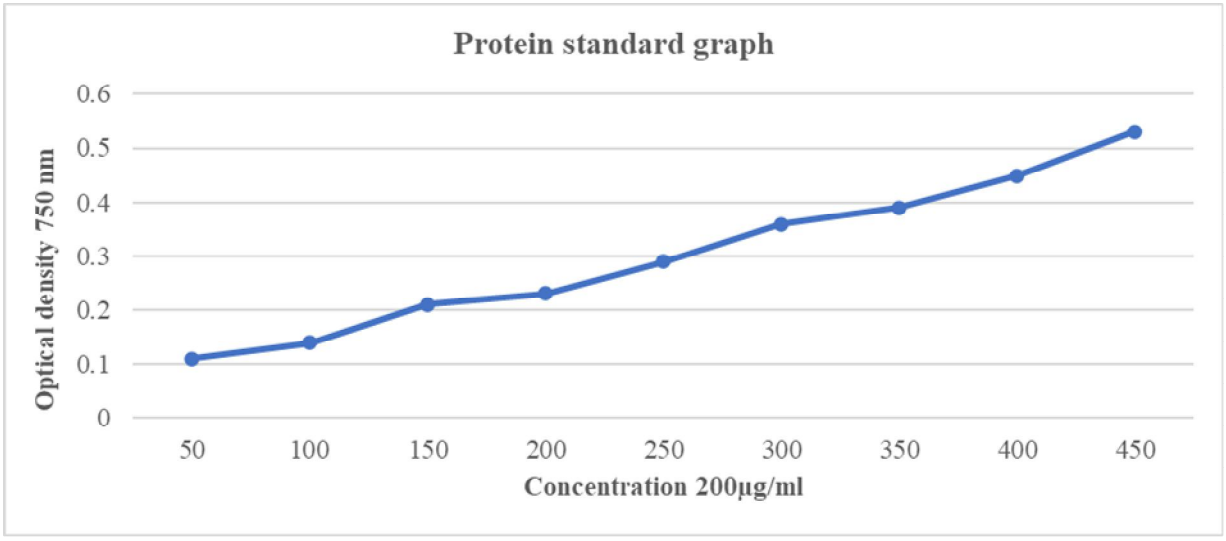


Fig. 11: Standard graph of protein (BSA) (200µg/ml)

The cellulase activity for GN4 and HN2 were found to be 7.33 µg/ml/min and 3.66µg/ml/min respectively. Wherein, protein concentration for GN4 and HN2 were found to be 40µg /ml and 32 µg/ml respectively.
Here, GN4 was found to be the best cellulase producer with cellulase activity 7.33 µg/ml/min and protein concentration 40 µg/ml.

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IV. CONCLUSIONS

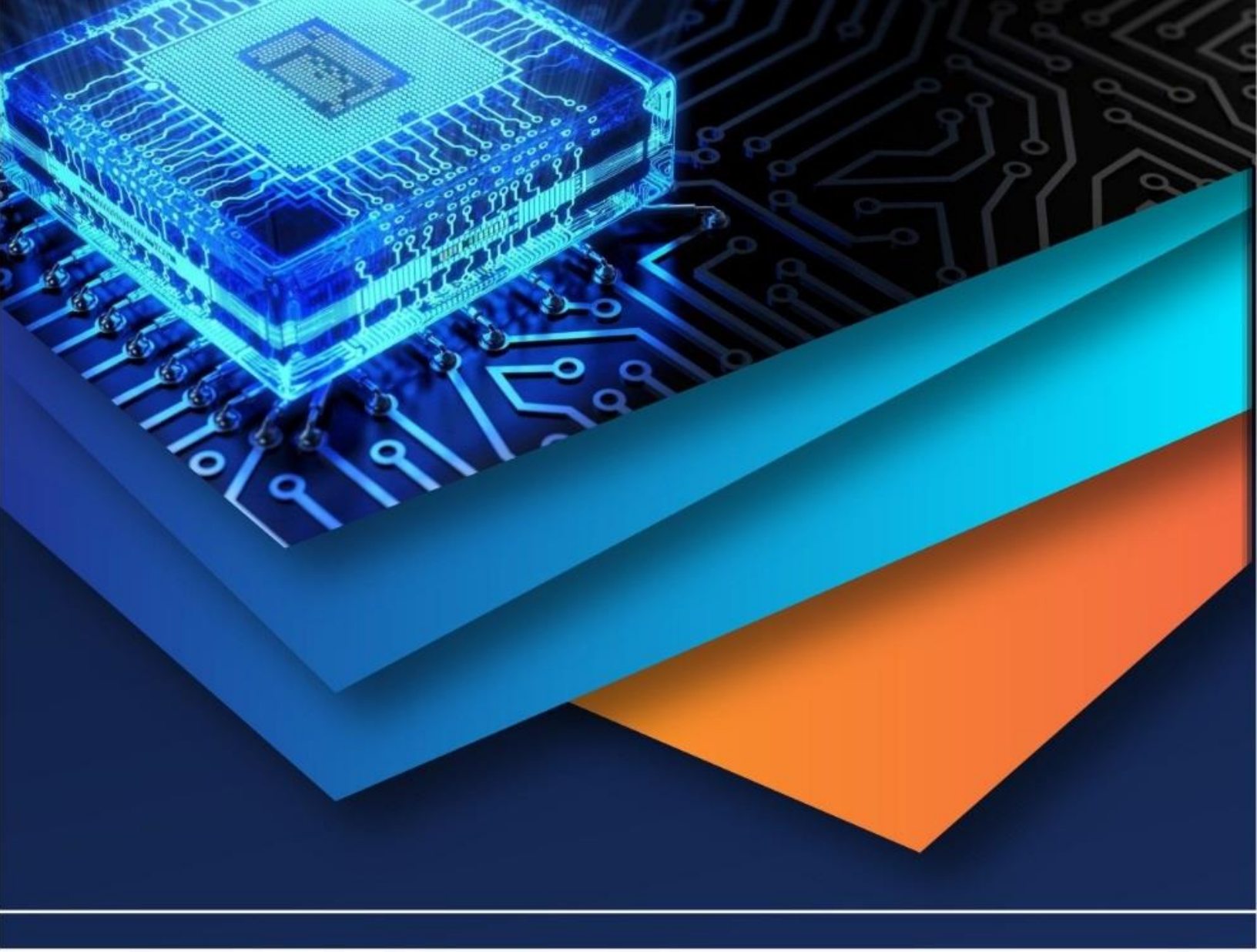
In the present study, total 13 bacteria were isolated from the cow dung samples collected from different variety of cows namely, Holstein, Jersey, Desi and Gir. From 13 isolates, 10 isolates were found to be cellulase producers. All the 10 isolates were characterized morphologically from which 8 isolates were found Gram negative and 2 were found Gram positive. All the 10 cellulase producers were further confirmed for their cellulase producing ability by performing turbidity test. Out of these 10 isolates, GN4 and HN2 with optical density 0.35 and 0.28 respectively were found to be best cellulase producer and were selected for cellulase production.

GN4 and HN2 were selected for cellulase enzyme production and further studies. Both the isolates were tested for their enzymatic activities and protein estimation. Cellulase activities were found to be 7.33 µg/ml/min and 3.66 µg/ml/min and the total protein concentrations were found to be 40 µg/ml and 32 µg/ml for GN4 and HN2, respectively. Finally, from the present study, it can be concluded that cow dungs are the excellent source for cellulase producing bacteria.

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