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Isolation, identification, characterization, and production of cellulolytic bacterium from soil and cow dung

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***Abstract**: The aim of the present study was to isolate, identification, screening, and characterization of cellulolytic bacterium from soil and cow dung. The observations entirely based on morphological, physiological, and biochemical characteristics as well as 16S rRNA analysis, a cellulolytic bacterium was isolated from soil and cow dung and identified as Bacillus spp. Its increase curve entered the logarithmic portion after 8-12 hours, according to an analysis of its increase qualities, and the strong increase section was between 20-40 hours. Corn flour was used as the 1% fine carbon source for fermentation, while peptone and yeast powder compound were used as the 2% fine nitrogen reasserts. With a 4% inoculum, the initial pH during fermentation increased to 5.5, producing a large and potent amount of enzyme in 24-48 hours. Bacillus sp. was identified as the cellulase-producing bacteria that was removed from the soil pattern. Cellulase enzyme production parameters were obtained and should be used in further benefits.*

***Keywords**: Isolation, screening identification, fermentation, cellulolytic bacterium, soil and cow dung.*

1. INTRODUCTION:

Cellulose is the major plentiful biological compound of terrestrial and aquatic ecosystem. It is foremost element of plant biomass [1]. Plant cellulose, is a chief element of plant cell walls [2]. It is the dominant waste fabric from agricultural enterprise in the shape of stalks, stems and husk. There was remarkable hobby in utilizing cellulose as a useful resource and feed. Cellulose is a natural compound with the formula ($C_6H_{10}O_5$), a polysaccharide consisting a linear chain of numerous hundred to over 10000 β -1, four related D-glucose unit. Plant cellulose is used especially for fuel, animal feed and manure, and in the paper enterprise. However, whilst the usage of plant cellulose is low, the corresponding environmental pollutants is considerable. Although acid, alkali, and steam heating remedy strategies produce fairly desirable results, their packages were significantly limited as they require complex gadget and feature hazards which include secondary pollutants [3,4].

Cellulose is normally degraded via way of means of Cellulolytic enzyme gadget includes fundamental additives including endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and glycosidase (EC 3.2.1.21). These enzymes act as synergistic gadget and convert cellulose right into a utilizable strength supply [5]. The cost of cellulose as a renewable supply of strength has made cellulose hydrolysis the concern of severe studies and business interest [6]. There has been much studies aimed toward acquiring new microorganisms generating cellulase enzymes with better unique sports and extra efficiency [7]. Cellulolytic enzymes play a crucial role in herbal biodegradation procedure wherein plant lignocellulosic substances are correctly degraded via way of means of cellulolytic fungi, micro-organism, actinomycetes and protozoa. Cellulolytic enzymes are synthesized via way of means of some of microorganisms. Fungi and micro-organism are the primary herbal retailers of cellulose degradation [4]. The cellulose making use of populations encompass cardio and anaerobic mesophilic micro-organism, filamentous fungi, thermophilic and alkaliphilic micro-organism, actinomycetes and positive protozoa. However, fungi are widely recognized retailers of decompositions of natural matter, in general, and of cellulosic substrate in particular [8]. Insect like termites (Isopteran), are determined to have syntrophic symbiotic microflora of their guts answerable for cellulosic feed digestion. *Chaetomium, Fusarium, Myrothecium, Trichoderma, Penicillium, Aspergillus* are a number of the pronounced fungal species answerable for cellulosic biomass degradation.

Cellulolytic bacterial species encompass *Clostridium thermocellum, C. cellulolyticum, C. cellulovorans, B. subtilis, Pseudomonas spp, Cellulomonas spp. Micrococcus spp,* etc. First, there may be degradation of wastes and discount of pollution of the surroundings for the betterment of the nice of lifestyles of human-beings and established order of a green surroundings for the generations to come. Secondly, the technique of cellulose degradation outcomes with inside the manufacturing of glucose that may be applied as a supply of food, feed and fuel. Cellulases is used bio-sharpening of fabrics, as softness and brightness, in processing of fruit juices, in paper and pulp industry, and de-inking of paper [9]. However, microorganisms are characterized through a totally fast growth, therefore, the technique of microbial degradation of cellulose may be taken into consideration as financially possible and appears to be the sensible choice [5]. Hence, the existing venture became assigned to isolation, identification, fermentation, cellulolytic bacterium, soil and cow dung.

2. MATERIALS AND METHOD:

Sample Collection

For isolation of cellulase producing microorganism, two samples were collected, first was soil sample and second was cow dung sample. Soil sample is collected from saw mill, Jayshree Timber Mart, Ahmednagar and Cow dung sample from farmers farm Ahmednagar.

Screening and isolation of cellulase producing bacteria

Cellulolytic bacteria were isolated from soil sample and cow dung sample by using serial dilution method. 1gm of soil sample and cow dung sample dissolved in 100 ml sterile distilled water and prepared serial dilution suspensions, which were ranging from 10^{-1} to 10^{-7}. Then 0.1ml diluted samples from each tube was spread on carboxymethyl cellulose agar plates, which were incubated for 72 hrs at 37°C [5].

After incubation, the plates were flooded with 1% Congo red solution and allowed to stand for 15min at room temperature for counter staining. 1M NaCl was used, to distain the Congo red dye. Colonies showing zone of hydrolysis further isolated for repeated screening. Isolate showing largest clear zone was selected for further studies [5].

Characterization and Identification of isolate

Isolated bacteria characterized morphologically and biochemically as per Bergey's Manual of Determinative Bacteriology. Fresh culture was used for all the tests.

Endospore staining- Endospore staining was performed by Schaeffer-Fulton Method.

Tests for different enzymes- The ability of organism to produce different enzymes like catalase, oxidase was checked.

a. **Catalase-** Hydrogen peroxide (3%) solution was taken in the tubes and single well isolated colony was immersed in to the tube and observed for effervescence.
b. **Oxidase-** Filter paper strip dipped in N, N, N, N tetra-methyl para-phenylene di-amine dihydrochloride reagent was smeared with single colony and observed for colour change.

Sugar Fermentation test

Fermentation of different sugars by the isolates was carried out by inoculating the test organism in the sterile peptone water base supplemented with, 1% of the required sugars containing phenol red as indicator and an inverted Durham's tube. Then incubated for 24 hrs at 37°C, change in the colour of the medium from red to yellow was recorded as positive for acid production and formation of gas bubble in Durham's tube indicated the formation of gas.

Starch hydrolysis test

Starch agar media was prepared and spot inoculated with isolate. Then starch agar plates were incubated at 37^0C for 24 hrs. After incubation iodine solution was flooded on the media for 1 minute and observed for the zone of hydrolysis around the colony of the isolate.

Gelatin liquefaction

A heavy inoculum of a 24 hrs old test isolate was stab-inoculated into tubes containing nutrient gelatin. The inoculated tubes and uninoculated control tubes were kept at 37^0C for 24 hrs for incubation. After 24 hrs of incubation, observed for gelatine liquefaction.

Nitrate reduction test

Sterile Nitrate broth was prepared and inoculated with test organism. Inoculated broth was incubated at 37^0C for 24 hrs. After incubation 1drop of Sulfanilic acid reagent and 1 drop of α–naphthylamine reagent was added. Mix well and observed for red colour formation. If the test is negative then add a very small pinch of zinc dust which will convert nitrate to nitrite.

Inoculum preparation

For preparation of inoculum, isolate which showed a maximum zone of hydrolysis were inoculated in 50 ml sterile CMC broth and incubated for 24 hrs at 37°C [10].

Production media preparation

Production media was prepared by transferring 1ml of inoculum in 250 ml Erlenmeyer flasks, which contain 100ml sterile CMC broth. After inoculation flask was incubated at 37°C for 72 hrs [5].

Preparation of crude enzyme

After incubation of production medium, 1ml of culture was centrifuged at 5000 rpm for 15 minutes. Supernatant which was obtained after centrifugation serves as crude enzyme and used for analysis of enzyme activities [5, 11].

Estimation of cellulase activity

Cellulase activity was assayed by using Dinitrosalisilic acid (DNSA) reagent by estimation of reducing sugars released from CMC. 1ml of crude enzyme was added to 1ml of 1% CMC in 0.05 M phosphate buffer of pH 7 and incubated at 50°C for 30 min. After incubation, reaction was stopped by addition of 1ml of DNSA reagent and boiled at 100°C 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. One unit of enzyme activity is expresses as the amount of an enzyme, which is required to release 1µmol of glucose per minute under standard assay conditions [5, 8, 11].

Optimization of different parameters for cellulase production

Effect of Carbon sources

Effects of different carbon sources like galactose, starch, fructose, mannitol, sucrose on cellulase production were checked by inoculating isolate in CMC broth containing different carbon source (1%) and then incubating at 37^0C for 72 hrs [5].

Effect of nitrogen sources

Effect of different nitrogen sources like peptone, Gelatin, Ammonium chloride, Ammonium Nitrate on cellulase production was studied by inoculating isolate in CMC broth containing different nitrogen source (replaced original nitrogen source) and then incubating at 37^0C for 72 hrs [5].

Effect of incubation time

Effect of incubation time on cellulase production was carried out at different time interval, such as 24 hrs, 48 hrs, 72 hrs, 96 hrs and 120 hrs [5].

Effect of different pH on cellulase production

Effect of pH on cellulase production was determined by adjusting different pH (4, 5, 6, 7, 8, 9 and 10) of CMC broth by 1N NaOH and 1N HCl and then inoculated with isolate, incubated at 37^0 C for 72 hrs [11].

3. RESULTS:

Screening and isolation of cellulase producing bacteria

In the present study, a total of 11 isolates were recovered from the two samples (soil and cow manure), labeled CB1, CB2, CB3, CB4, CB5, CB6, CB7, CB8, CB9, CB10 and CB11. All isolates were screened for additional cellulase production and only 4 isolates CB4, CB6, CB8 and CB9 were observed to show a zone of hydrolysis (Table 1). Of 4 isolates, CB4 shows the highest area of hydrolysis and is therefore selected for further investigation.

Previous work reported using CMC-Na as the sole carbon source in selective media followed by a Congo red staining method for the preliminary isolation of cellulolytic bacteria is widely considered the best method for the preliminary screening of cellulolytic bacteria because it is quick and easy [11]. However, plaque screening methods using dyes are not quantitative or sufficiently sensitive, and due to the poor correlation between enzyme activity and the size of the compensation zone, selective media cannot accurately reflect l cellulolytic activity of bacteria [13], which requires repeated screening. Many cellulolytic bacteria have been selected from different environments, such as bovine rumen [11], soil [14], organic waste [15] and ruminant animal waste [3,17]. Most have been identified as Bacillus, Clostridium, rumen bacteria or Bacteroides [18,19].

In the present study, the isolated species was grown at 37 ° C on solid CMC-Na medium for 24 hours and the subsequent Congo red stain showed a noticeable zone of compensation. Furthermore, by examining the enzymatic activity, we selected the strain showing the highest enzyme activity for the subsequent research. According to previous research, the enzymatic activity of this strain (1.52 U / mL) is higher than that of other strains grown for 24 hours [17,20], indicating that this strain shows stable inheritance and has a strong CMCase activity (Fig. 1).

Table 1: Zone of hydrolysis of different isolates

Sr. No.	Isolate No.	Colony Diameter (mm)	Zone diameter (mm)
1	CB4	8.2	11
2	CB6	5.6	8
3	CB8	4.2	6.3
4	CB9	3.1	5.4

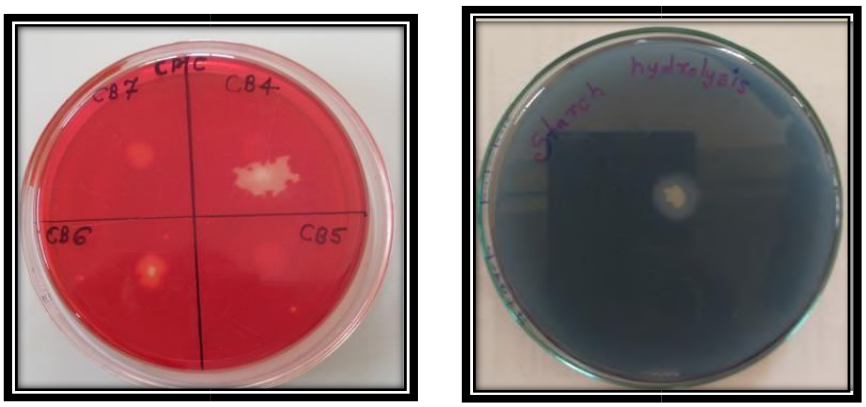

Fig.1. Zone of hydrolysis after addition of 1% Congo-red and Starch hydrolysis

Identification & characterization of isolate CB4 -

Morphological characteristics: - Character of colony observed on the CMC plates after 72 hours of incubation at 37°C. Morphological examination revealed that the surface of the colonies was irregular in shape, large, white in colour, filiform at the edge, opaque, dry in consistency and mobile, and found to be a bacillus mobile gram positive.

Biochemical character of CB4

Culture and biochemical tests were performed according to Bergey's Determinative Bacteriology Manual, the CB4 isolate was identified as Bacillus sp. Catalase, oxidase, starch hydrolysis, nitrate reduction gave positive results and all sugar fermentations tested were reported as acid and gas (Table 2).

Table 2: Biochemical characteristic of CB4

Sr. No.	Biochemical test	Results
1	Catalase	+
2	Oxidase	+
3	Starch hydrolysis	+
4	Gelatinase	+
5	Nitrate reduction	+
6	**Sugar fermentation**	
i)	Xylose	A+G
	Glucose	A+G
ii)	Lactose	A+G
iii)	Sucrose	A+G
iv)	Maltose	A+G
v)	Mannitol	A+G

A=Acid G=Gas

Optimization of different parameters-

Effect of different carbon sources on the production of cellulase

Carbon is considered the main nutrient necessary for the growth of bacteria, which is why the effect of various carbon sources on cellulase production has been verified. Carbon sources such as galactose, starch, fructose, lactose, mannitol, sucrose have been analyzed for cellulase production. The maximum activity of cellulase (28.42 U / ML) was observed when lactose served as a carbon source, while less (10.9 U / ML) was observed when the handle was used as a source of carbon (Fig. 2).

Since cellulas are inducible enzymes, the medium of fermentation cellulase production generally contains substrates rich in cellulose as a source of carbon [20]. Different carbon sources at different concentrations were examined to study their effects on BY-2 cellulase production under identical conditions. The results showed that the BY-2 strain could utilize different carbon sources and the maximal activity of CMCase (2.91 U / mL) was observed when a mixture of 1% corn powder and 1% CMC % was used as the sole carbon source. However, when MCC was

used as the sole carbon source, negligible CMCase activity was observed. Therefore, cheap and readily available corn dust proved to be optimal and was used as a carbon source in the following experiments [20].

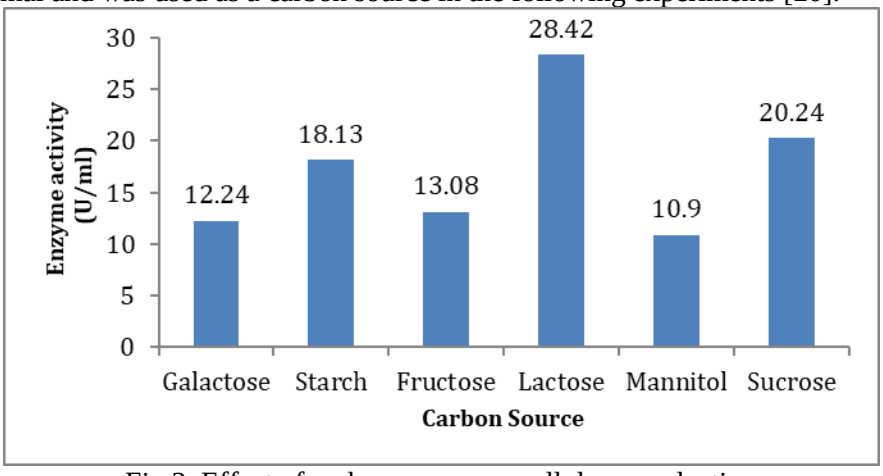

Fig.2. Effect of carbon source on cellulase production

Effect of Nitrogen sources on the production of cellulase

The effect of different nitrogen sources was realized in the CMC medium where ammonium sulfate was replaced by peptone, gelatin, ammonium, chloride and ammonium nitrate. Among the different nitrogen sources tested, Peptone was found to be the best nitrogen source for cellulase production (Fig.2), with an activity of 42.36 U/ml.

Various nitrogen sources (1% peptone, 1% yeast extract, 2:1 mixture of 1.5% peptone and yeast extract, 1:1 mixture of 2% peptone and extract, 1% urea and 1% NH4NO3) were examined with other identical conditions. The Bacillus species isolated by Rajoka [21] and Ray et al. [22] showed a similar ability to utilize nitrogen sources. The cellulolytic B. subtilis developed by Sadhu et al. [23] were able to use both inorganic and organic nitrogen sources. Additionally, the strain of B. subtilis isolated by Acharya and Chaudhary [24] could not utilize inorganic nitrogen sources when CMC was used as the carbon source, but could when wheat straw and rice hulls were used as a carbon source. Overall, the results show that the original environment and the environmental conditions are closely related to the enzymatic properties and the productivities of the different strains of Bacillus.

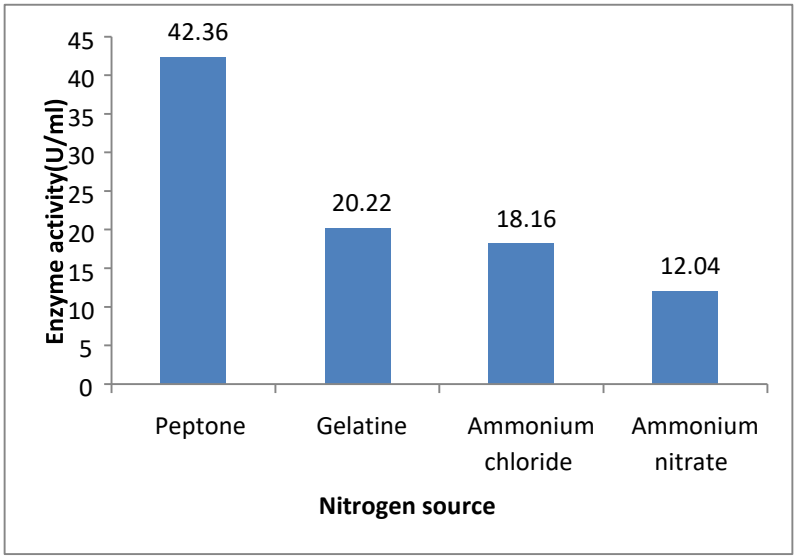

Fig.3. Effect of nitrogen source on cellulase production

Effect of incubation time on the production of cellulase

The effect of incubation time on cellulase production was determined by monitoring enzymatic activity at 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. A gradual increase in production occurred starting at 24 hours (Fig. 4) and the maximum production was observed after 72 hours with the enzymatic activity of 54.36 U / ml. The incubation time depends on the nutrients present in the medium and the cultural conditions of the organism.

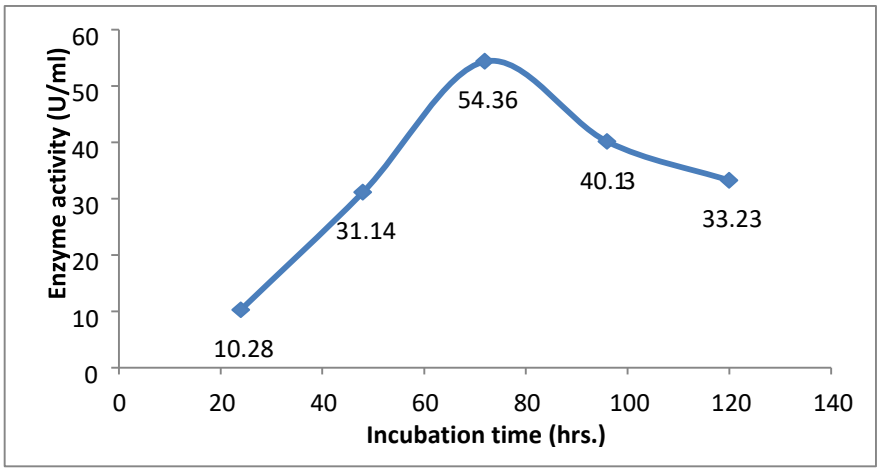

Fig.4: Effect of incubation time on cellulase production

Effect of different pH on the cellulase production

The effect of different pHs on cellulase enzyme production was performed in CMC medium, where the pH was adjusted from 4-10 and incubated at 37 ° C for 72 hours. The highest activity (54.21 U / mL) was observed at an initial pH of the medium 7 and the lowest activity (2.1 U / mL) at pH 10 (Fig. 5).

Furthermore, the activity of CMCase was found to be stable over a wide range of pH values (5.5-8.0), comparable to the pH of the faecal content of the Tibetan pig. These results indicated the potential use of the obtained cellulose to improve the nutritional quality and digestibility of animal feed. The optimal starting pH of strains from different sources varies: for example, the optimal starting pH for cellulase production from Bacillus isolated from fertilizer [22] and B. subtilis isolated from a hot spring [24] were 6.0 and 9.0, respectively. Therefore, appropriate pH conditions can promote the growth of the strains and increase the yield of cellulase; this may be related to the negative feedback mechanism of the enzymes.

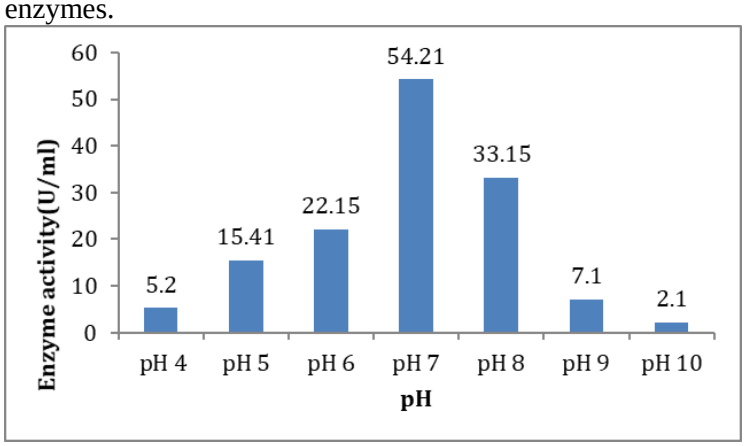

Fig.5. Effect of different pH on the cellulase production

4. DISCUSSION:

In the prevailing paintings, it become located that 11 isolates which have been remoted from soil and cow dung pattern simplest CB4 isolate confirmed maximum region of hydrolysis, which become then taken for in addition take a look at, the parameters like pH, incubation time, impact of carbon supply, impact of nitrogen supply, have been optimized for cellulase manufacturing and pastime.

Nutrient resets have been based to be important aspect for cellulase manufacturing. Since carbon is taken into consideration because the primary nutrient for bacteria, specific carbon reassets like Fructose, Lactose, Mannitol, Sucrose, Galactose and starch have been analyzed for the most cellulase manufacturing. Maximum pastime (28.42 U/ml) of cellulase located while lactose become served as carbon supply. Similar end result become acquired within side the paintings via way of means of Saraswati *et al*. [25] In their take a look at it become located that cellulase manufacturing become better with 23.96U/ml enzyme pastime, while lactose become served because the carbon supply. While Yang *et.al*. [26] located that their isolate (BY-2) may want to make use of numerous carbons reasserts and the most CMCase pastime become located while a combination of 1% corn powder and 1% CMC become used as the only carbon supply. Muhammad Irfan *et al*., [8] located that the glucose considerably multiplied the cellulase pastime.

The impact of numerous nitrogen reassets at the manufacturing of cellulase have been accomplished and it became located that Peptone become served as first-rate nitrogen supply for max cellulase manufacturing which display maximum enzymatic pastime i.e. 42.36 U/ml. Similar end result have been acquired with inside the take a look at Mukesh et al., In their take a look at they located that peptone confirmed maximum enzymatic pastime 29.63 U/ml. While withinside the paintings via way of means of Payen [27] stated that natural nitrogen supply has been determined to be greater appropriate for optimizing cellulase manufacturing via way of means of *Bacillus sp*. Shankar [28] proved that *Bacillus subtilis* remoted from earthworm intestine indicates higher manufacturing of cellulases while Malt extract used as nitrogen supply.

Effect of various pH at the manufacturing of cellulase have been accomplished and it became determined that on the pH 7 cellulase manufacturing become better with enzyme pastime 54.21 U/ml. Similar end result acquired via way of means of Shaikh *et al*. [9] it become located that sluggish multiplied withinside the enzyme pastime because the pH will increase from pH 6-7. While withinside the paintings of Bakare *et al*. [28] had been stated that most desirable pH values for cellulase have been ranging 4.5-8.00.

5. CONCLUSION:

A cellulase producing micro-organism remoted from soil pattern turned into diagnosed as *Bacillus sp.* parameters for manufacturing of cellulase enzyme turned into optimized. It turned into discovered that, the enzymatic hobby turned into maximum (54.21 U/ml) at pH 7. Lactose is maximum appropriate carbon supply for cellulase manufacturing with most hobby (28.42 U/ml). While amongst all nitrogen reassets tested maximum enzymatic hobby (42.36 U/ml) received with peptone. Celluase hobby step by step will increase as time of incubation will increase and most hobby (54.36 U/ml) discovered after 72 hrs. of incubation in a while it decreases. The present studies indicated that remoted *Bacillus spp.* successfully produced cellulases and can be applied for business manufacturing of cellulase. Hence, this isolate may also have the capacity to be used at a business level.

CONFLICT OF INTEREST: The author(s) claim no war of interest.

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