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# PRODUCTION OF EXTRACELLULAR *CELLULASE* USING *BACILLUS SPECIES* ISOLATED FROM RED SOIL AND OPTIMIZATION OF *CELLULASE* ACTIVITY

V. Bharathi * and M. Kanaka

PG and Research Department of Biochemistry, S.T.E.T .Women's College, Mannargudi, Tamilnadu-614001, India.

**Keywords:**

*Bacillus species*, Incubation time, pH, substrate concentration and temperature

**Correspondence to Author:**
**V. Bharathi**

Assistant Professor,
Department of Biochemistry,
Sengamala Thayaar Educational Trust Women's College,Mannargudi, Tamilnadu - 614001. India.

**E-mail**: bharathiviswanathanbio@gmail.com

**ABSTRACT:** Enzymes plays a major role in industries like textile, leather, sugar, paper, rubber, tea etc., Microbes plays important role in enzyme production due to their fast growth rate, easy to manipulate for getting highly stable enzymes through genetic engineering and requires shorter time for production and purification steps. In our present study, we concentrated on the enzymes used in textile industries. Recently, many novel enzymes that prove to be the efficient in the process of desizing, peroxide removal, bio polishing have been developed for textile industries. In commercial point of view, finding of new enzymes with high activity and desired properties that can be potentially used for those processes is a continuous process. Hence, we tried to isolate the cellulolytic bacteria from red soil and produced the enzyme *cellulase* using specific media. The isolated bacterial species was identified as *Bacillus species* by morphological and biochemical analysis. Further the *cellulase* activity was analyzed and the parameters like pH, temperature, substrate concentration, and incubation time were also optimized.

**INTRODUCTION:** Enzymes were discovered in the second half of the nineteenth century, and since then it has been extensively used in several industrial processes. Enzymes are extremely efficient and highly specific biocatalysts. Commercial sources of enzymes are obtained from three primary sources, i.e., animal tissue, plants and microbes. These naturally occurring enzymes are quite often not readily available in sufficient quantities for food applications or in industrial use.

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However, by isolating microbial strains that produce the desired enzyme and optimizing the conditions for growth, commercial quantities can be obtained. Most of the industrial enzymes are produced by relatively few microbial hosts like *Aspergillus* and *Trichoderma* fungi, *Streptomyces*, and *Bacillus* bacteria. Yeasts are not good producers of extracellular enzymes and are rarely used for this purpose. There are a large number of microorganisms which produce a variety of enzymes [1, 2]. Enzymes are used in many environmental-friendly industrial purposes, as they are efficient, selective, accelerate and speed up reactions by forming transition state complexes with their substrate which reduces the activation energy of the reaction.

Enzymes are useful in various areas of industrial applications like manufacturing of food and

foodstuffs, cosmetics, medicinal products as a tool for research and development. Technical enzymes are applied in for fuel production and for the production of pharmaceuticals and as chiral substances in the chemical industry. Enzymes can be used in chemical analysis and as a research tool in the life sciences.

**Enzyme applications in textile preparatory process:**

Especially in textile manufacturing, the use of enzymes has a long tradition. Enzymes used in textile and their effects are shown in **Table 2**. The current applications in the textile industry involve mainly hydrolases and now to some extent is oxidoreductase.

The **Table 3** and **Table 4** respectively which exemplify such textile applications. The enzymatic desizing of cotton with α-amylases is state- of-the-art since many decades [3]. Moreover, Cellulases, Pectinases, Hemicellulases, Lipases and Catalases are used in different cotton pre-treatment and finishing processes.

**TABLE 3: APPLICATION OF HYDROLASE ENZYME IN FABRIC PRECIPITATION**

| Enzymes | Role of action in industries |
|---|---|
| Amylase | Desizing |
| Cellulases and Hemicellulases | Biostoning of Jeans , Desizing of CMC ,Stylish effect on cellulose fibres |
| Pectinase | Scouring of vegetables as well as best fibres e.g. Cotton, Jute |
| Proteases | Scouring of animal fibres, degumming of silk and modification of wool properties |
| Lipases | Elimination of fat and waxes |

**TABLE 4: APPLICATION OF OXIDOREDUCTASE IN FABRIC PREPARATION**

| Enzyme | Substrate | Textile Application |
|---|---|---|
| *Amylases* | Starch | Starch desizing |
| *Cellulase* | Cellulose | Stone wash –Bio-Polishing<br>Bio finishing for handle modification<br>Carbonization of wool |
| *Pectinase* | Pectin | Bio scour replacing caustic soda |
| *Catalase* | Peroxides | In situ peroxide decomposition without any rinse in bleach bath |
| *Lipase* | Fats and oils | Improve hydrophilicity of PET in place of alkaline hydrolysis |

**Enzymatic Desizing:**

In the textile industry, amylases are used to remove starch-based size for improved and uniform wet processing. Amylase is a hydrolytic enzyme which catalyses the breakdown of dietary starch to short chain sugars, dextrin and maltose. The advantage of these enzymes is that they are specific for starch, removing it without damaging to the support fabric. An amylase enzyme can be used for desizing processes at low-temperature (30-60ºC) and optimum pH is 5.0- 5.5 [4].

**Enzymatic Scouring (Bioscouring):**

Scouring is removal of non-cellulosic material present on the surface of the cotton. In general cellulase and pectinase are combined and used for Bioscouring. In this process, pectinase destroy the cotton cuticle structure by digesting the pectin and removing the connection between the cuticle and the body of cotton fibred whereas cellulase can destroy cuticle structure by digesting the primary wall cellulose immediately under the cuticle of cotton. Biological Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) of enzymatic scouring process are 20-45 % as compared to alkaline scouring (100%). Total Dissolved Solid (TDS) of enzymatic scouring process is 20-50% as compared to alkaline scouring (100%).

**Enzymatic Bleaching:**

The purpose of cotton bleaching is to decolourise natural pigments and to confer a pure white appearance to the fibres. Mainly flavanoids are responsible for the colour of the cotton [5]. The most common industrial bleaching agent is hydrogen peroxide. Conventional preparation of cotton requires high amounts of alkaline chemicals and consequently, huge quantities of rinse water are generated. However, radical reactions of bleaching agents with the fibre can lead to a decrease in the degree of polymerisation and thus, to severe damage. Therefore, replacement of hydrogen peroxide by an enzymatic bleaching system would tend to make the fabric world as well as industrial operations in a safe mode. It not only lead to better product quality due to less fibre damage but also to substantial savings on washing water needed for the removal of hydrogen peroxide.

**Bio polishing:**

Bio polishing is a finishing process that improves fabric quality by mainly reducing fuzziness and pilling property of cellulosic fibre. The objective of

the process is elimination of micro fibrils of cotton through the action of cellulase [6, 7, 8]. The main characteristics imparted to the fabric during bio polishing treatment are as follows;

- Cleaner surface is obtained conferring a cooler feel
- Lustre is obtained as a side effect
- Fabric obtains softer feel.
- Tendency of the fabric to pill ends.

**Enzymatic treatment to denim Denim:**
Denim is heavy grade cotton. In this, dye is mainly adsorbed on the surface of the fibre. That is why fading can be achieved without considerable loss of strength. Disadvantage of using pumice stones for bio polishing method are as follows;

- Pumice stones cause large amount of back-staining.
- Pumice stones are required in very large amount.
- They cause considerable wear and tear of machine.

**Cellulases:**
*Cellulases* are hydrolytic enzymes that catalyse the breakdown of cellulose to smaller oligosaccharides and finally glucose. Cellulase activity refers to a multicomponent enzyme system combining at least three types of cellulase working synergistically. Cellobiohydrolases or exo-cellulases start their action from the crystal- line ends of cellulose chains, producing primarily cellobiose. Cellobiohydrolases act synergistically with each other and with endoglucanases, thus mixtures of all these types of enzymes have greater activity than the sum of activities of each individual enzyme alone. Cellobiose and soluble oligosaccharides, produced by exo-cellulases, are finally converted to glucose by β-4-glucosidase.These enzymes are commonly produced by soil- dwelling fungi and bacteria, the most important being *Trichoderma, Penicillium and Fusarium* [9, 10, 11].

Many of the fungal cellulases are modular proteins consisting of a catalytic domain, a carbohydrate-binding domain (CBD) and a connecting linker. The role of CBD is to mediate the binding of the enzyme to the insoluble cellulose substrate [12]. Based on their sensitivity to pH, they are classified as acid stable (pH 4.5-5.5), neutral (pH 6.6-7) or alkali stable (pH 9-10). The application of *cellulases* in textile processing started in the late 1980s with finishing. Currently, in addition to biostoning, *cellulases* are also used to process cotton and other cellulose-base.

## MATERIALS AND METHODS:

**Sample Collection:**
The soil sample was collected from the sugar cane field near Lalgudi, Trichy. The sample was collected in a sterile container and stored at 4°C until used. It was further used for isolation of cellulolytic bacteria mainly *Bacillus species*.

**Isolation and screening of cellulolytic Bacteria:**
0.1g of soil sample was taken and dissolved in 100ml of sterile water. From this 0.2 ml of sample was spread on the nutrient agar medium plates by spread plate method. The plates were incubated at 37º C for 2 days. From the colonies developed, the nutrient containing 1% CMC cellulose was added and incubated at 30º C for 48 hours. After 24 hours of incubation, the plates were flooded with 1% Congo red solution and left undisturbed for 15 minutes. The 1M Nacl solution was added in order to visualize the clear zone. The diagrammatic representation of the bacterial colonies were displayed in the **(Fig. 1).**

**Identification of Bacillus Species:**
The selected colonies were analyzed by Gram's staining method and by biochemical tests for the identification of the bacteria at species level. The diagrammatic representation of the identification of bacillus species was displayed in the **(Fig. 2).**

**Gram's staining:**
The gram's staining was done by usual method. This test was done to verify the difference between gram positive and gram negative bacteria. The gram positive bacteria retain the colour whereas the gram negative bacteria do not retain the colour.

**Methyl Red - Voges Proskauer Test:**
This test was done by usual traditional method. This test enables the microbiologists to determine

the pathway being used to ferment glucose, and in the process which helps us to determine the species of bacteria that is most likely present. MR/VP is actually two tests: The methyl red (MR) test determines whether or not large quantities of acid have been produced from mixed acid fermentation of glucose. The Voges-Proskauer (VP) test determines whether a specific neutral metabolic intermediate, acetoin, has been produced instead of acid from glucose. The diagrammatic representation of the methyl red positive was displayed in the **(Fig. 3).**

**Casein Hydrolysis Test:**
The selected bacterial colony was inoculated in casein agar plates and incubated at 37° C for 24 hrs and observed for the results. The diagrammatic representation of the casein hydrolysis test was displayed in the **(Fig. 4).**

**Catalase Test:**
This test was also done by usual method. This test is to detect the presence of the enzyme *catalase* which hydrolyzes $H_2O_2$ to produce $H_2O$ and $O_2$. The diagrammatic representation of the *catalase* test was displayed in the **(Fig 5).**

**Production of *cellulase* enzyme:**
Selected colony from the screening process was sub-cultured in the enzyme production medium. The culture flask was incubated at 37°C for 72 hours in a rotator shaker. Then the liquid culture was centrifuged at 1600 rpm for 20 minutes.

**Partial Purification of *cellulase* enzyme:**
20ml of the crude *cellulase* extract was partially purified by Ammonium Sulphate precipitation method followed by Dialysis. The cell free *protease* extract was saturated with 75% ammonium sulphate and it was left overnight at 4°C. The precipitate was collected by centrifugation at 12,500 rpm for 20 minutes at 4°C and dissolved in 10ml of 1M Acetate Buffer of pH 5. Then the solution was dialysed against the same buffer at 4°C for 8 hours with 3 changes of the dialysis buffer. The final extract was used for further analysis.

**Estimation of Protein:**
The amount of protein present in the test sample was estimated by the formal method. The amount of protein present in the partially purified cellulase extract was estimated spectrophotometrically by taking OD at 280nm in UV–Visible Spectrophotometer. The results were shown in the **(Graph 2)**.

**Assay for *cellulase* activity By DNS method:**
Three test tubes marked as Test 1 (T1), Test 2 (T2), Blank (B) were maintained. 1ml of CMC substrate was added to all the tubes. The tubes were kept in water bath of 50°C and 1ml of the crude enzyme extract was added to T1 and T2 tubes alone. The tubes were incubated in the water bath for 10 minutes. After completion of incubation 2.4ml of DNS reagent was added to all the tubes. Then the absorbance of the samples was read at 540nm in spectrophotometer.

**Analysis of effect of pH on *cellulase* activity**:
The above said assay was repeated by using Acetate buffers of various pH 3.0, 4.0, 6.0, 7.0.Other parameters maintained were temperature - 50°C, substrate concentration – 1%, incubation time – 10 minutes. The results were shown in the **(Table 3)**. The graphical representation of pH was shown on **(Graph 5)**.

**Analysis of effect of temperature on *cellulase* activity:**
Similarly 4 more reaction sets were prepared and maintained in water bath for varying temperatures 30°C, 40°C, 60°C, 70°C. Other parameters maintained here are pH – 5, substrate concentration – 1%, incubation time – 10 minutes. The results were shown in the **(Table 1)**. The graphical representation of pH was shown on **(Graph 3)**.

**Analysis of effect of substrate concentration on *cellulase* activity:**
Various concentrations of substrate CMC ranging from 0.25, 0.5, 0.75, 1.25 and 1.5%, were prepared and assayed as per the above said procedure. Other parameters maintained were pH - 5, temperature - 50°C, incubation time – 10 minutes. The results were shown in the **(Table 2)**. The graphical representation of pH was shown on **(Graph 4)**.

**Analysis of effect of incubation time on *cellulase* activity:**

Similarly, 6 more reaction sets were maintained and each set is maintained in water bath for different incubation periods. Reaction set 1 to 6 is maintained in water bath for various incubation periods ranging from 2, 4, 6, 8, 12, 14 minutes respectively. Other parameters maintained were pH - 5, temperature - 50°C, substrate concentration – 1%. The results were shown in the **(Table 4)**. The graphical representation of pH was shown on **(Graph 6)**.

**RESULTS AND DISCUSSION:** The isolated colonies present in the sample were screened under microscope and the colony formed with zone of clearance in the sample was identified by gram's staining. The results were discussed in the **Fig.1** and **Fig. 2.** From this test we came to knew that the *cellulase* producing microorganisms *Bacillus species* exhibits rod shaped bacteria.

**Diagrammatic representation of cellulolytic colonies with zone of clearance:**

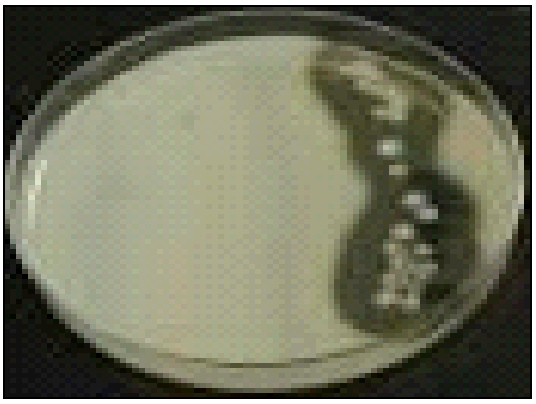


**FIG. 1: *CELLULOLYTIC* BACTERIAL COLONIES WITH ZONE OF CLEARANCE**

**Diagrammatic representation of gram positive – rod shaped *bacillus* organisms:**

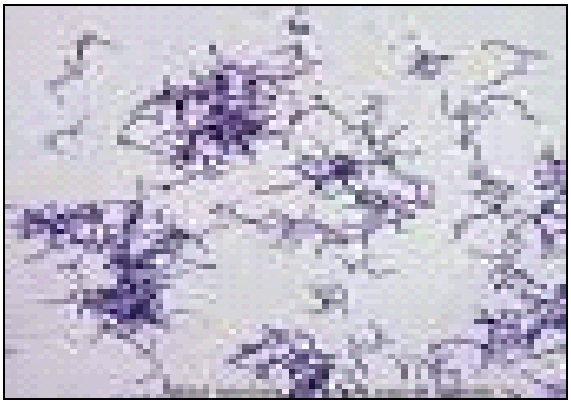


**FIG. 2: GRAM POSITIVE – ROD SHAPED**

***Bacillus* organisms:**

The biochemical tests which are analysed in this theses includes MR test, VP test, casein hydrolysis test, *catalase* test were discussed in the **Fig. 3, 4** and **5** respectively.

**Diagrammatic representation of methyl red negative:**

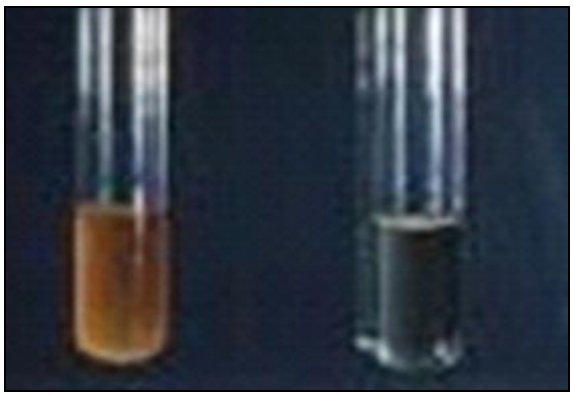


**FIG.3: METHYL RED NEGATIVE**

**Diagrammatic representation of VP test positive:**

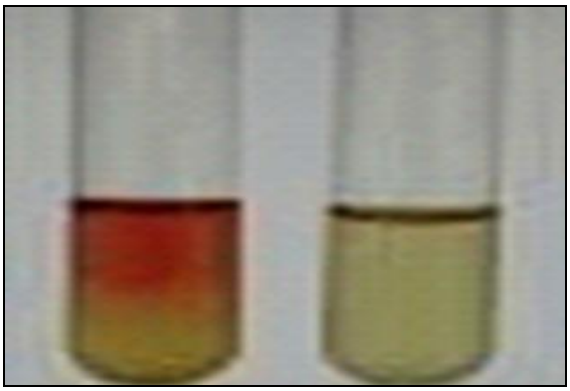


**FIG.4: VP TEST POSITIVE**

**Diagrammatic representation of Casein hydrolysis positive:**

**FIG.5: CASEIN HYDROLYSIS POSITIVE**

**Diagrammatic representation of *Catalase* positive:**

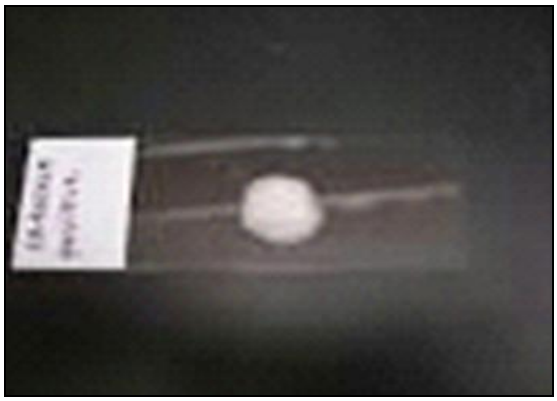
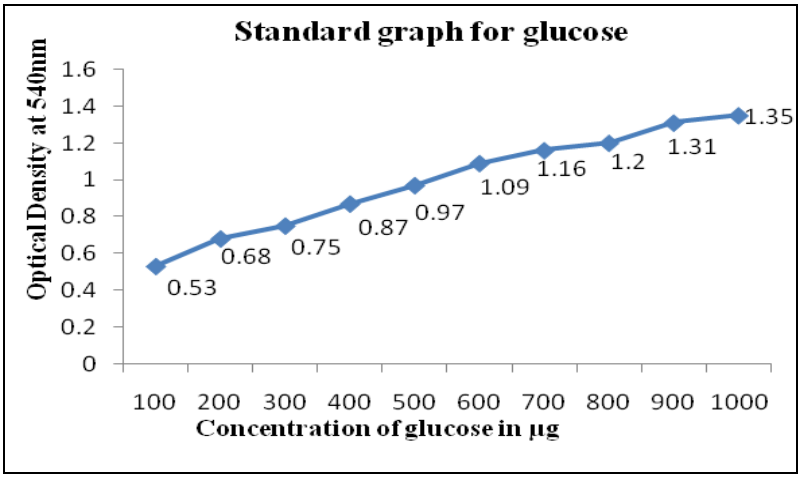


**FIG.6: *CATALASE* POSITIVE**

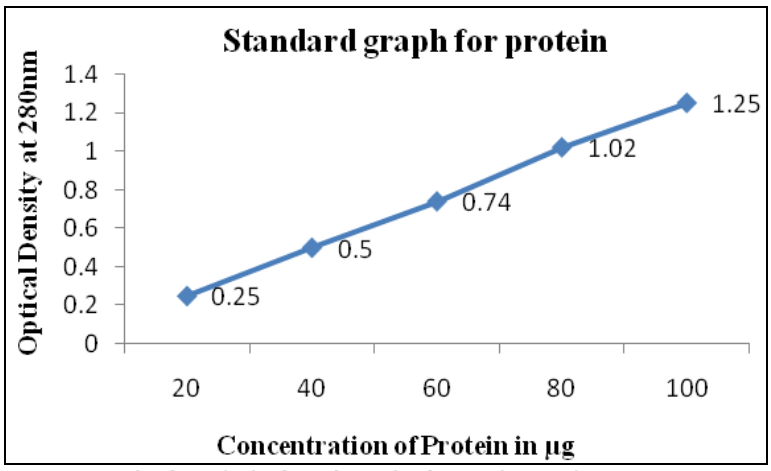
The above said biochemical tests were done in order to verify the fermentation capacity of the microorganisms to ferment glucose in the sample whereas the VP test determines the presence of acetoin which is the last intermediate in the butanediol pathway. The *catalase* test determines the amount of free radicals scavenging nature of the enzyme. The casein hydrolysis test declares that the casein, milk protein acts a nutrient supplement for the growth of the microorganism in the medium. The optimization protocols of enzyme includes standardisation of glucose concentration, standard graph for protein concentration, effect of temperature on *celluase* activity, effect of substrate concentration on *cellulase* activity, effect of pH on the *cellulase* activity, effect of incubation time *on cellulase* activity were briefly discussed on the **(Graph 1, Graph 2, Graph 3, Graph 4, Graph 5, Graph 6)** along with the data in their corresponding tables respectively.

**Graphical representation of standard graph for glucose:**




**GRAPH 1: STANDARD GRAPH FOR GLUCOSE CONCENTRATION**

**Graphical representation of standard graph for protein:**

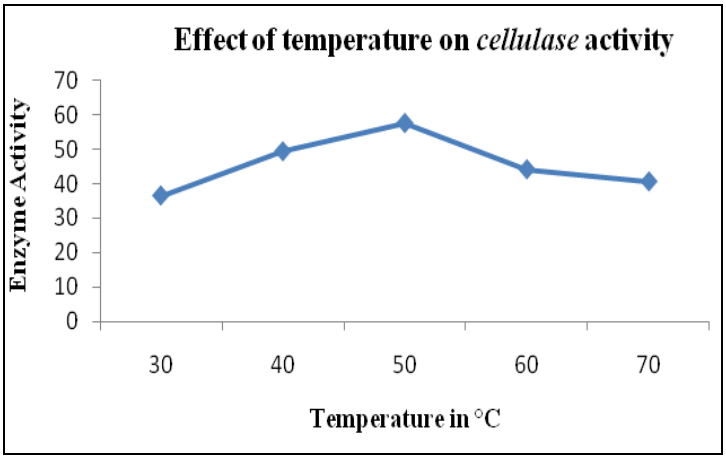



**GRAPH 2: STANDARD GRAPH FOR PROTEIN**

**TABLE 1: EFFECT OF TEMPERATURE ON THE ACTIVITY OF *CELLULASE***

| S. No. | Incubation Temperature in (°C) | OD at 540nm | Amount of glucose released in (µg) | Specific activity of the enzyme in units (µg of glucose produced/mg of protein/min.) |
|---|---|---|---|---|
| 1 | 30 | 0.62 | 182.35 | 36.47 Units |
| 2 | 40 | 0.84 | 247.05 | 49.41 Units |
| 3 | 50 | 0.98 | 288.23 | 57.64 Units |
| 4 | 60 | 0.75 | 220.56 | 44.12 Units |
| 5 | 70 | 0.69 | 202.94 | 40.59 Units |

## Graphical representation of temperature on *cellulase* activity:

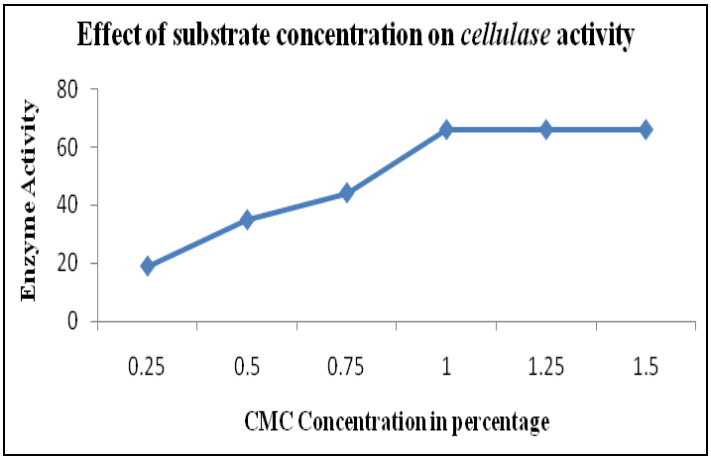



**GRAPH 3: EFFECT OF TEMPERATURE ON *CELLULASE* ACTIVITY**

**TABLE 2: EFFECT OF SUBSTRATE CONCENTRATION ON *CELLULASE* ACTIVITY**

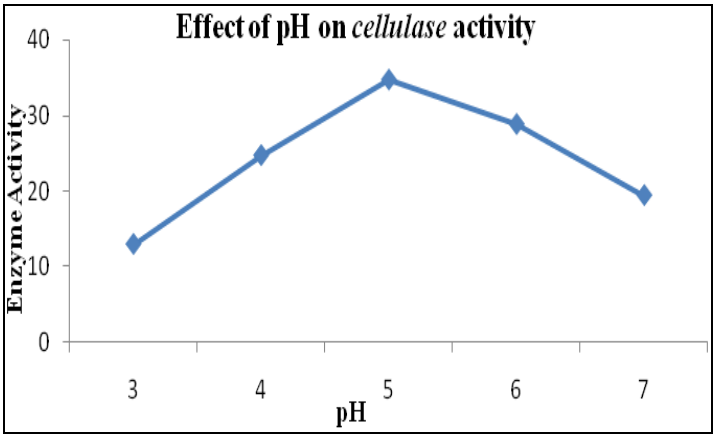
| S. No. | Concentration of CMC (Substrate) in (%) | OD at 540nm | Amount of glucose released in ( µg) | Specific activity of the enzyme in units (µg of tyrosine produced/mg of protein/min.) |
|---|---|---|---|---|
| 1 | 0.25 | 0.32 | 94.12 | 18.82 Units |
| 2 | 0.50 | 0.59 | 173.53 | 34.78 Units |
| 3 | 0.75 | 0.75 | 220.59 | 44.12 Units |
| 4 | 1.00 | 1.12 | 329.41 | 65.88 Units |
| 5 | 1.25 | 1.12 | 329.41 | 65.88 Units |
| 6 | 1.50 | 1.12 | 329.41 | 65.88 Units |

## Graphical representation of substrate concentration on *cellulase* activity:




**GRAPH 4: EFFECT OF SUBSTRATE CONCENTRATION ON *CELLULASE* ACTIVITY**

**Graphical representation on effect of pH on *cellulase* activity:**

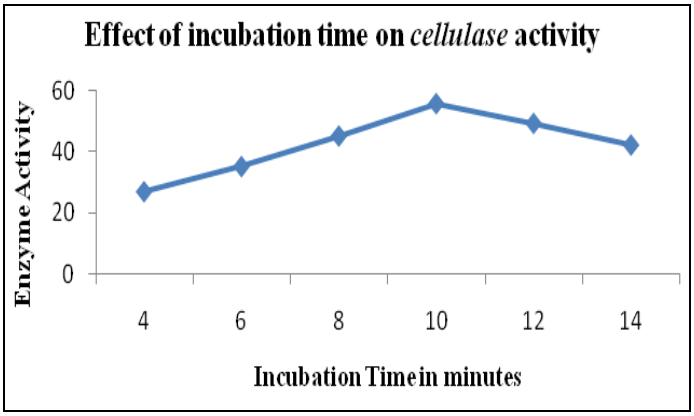



**GRAPH 5: EFFECT OF pH ON *CELLULASE* ACTIVITY**

**TABLE 4: EFFECT OF INCUBATION TIME *ON CELLULASE* ACTIVITY**

| S. No. | Incubation Period in (minutes) | OD at 540nm | Amount of glucose released in (µg) | Specific activity of the enzyme in units (µg of glucose produced/mg of protein/min.) |
|---|---|---|---|---|
| 1 | 4 | 0.46 | 135.29 | 27.06 |
| 2 | 6 | 0.60 | 176.48 | 35.30 |
| 3 | 8 | 0.77 | 226.47 | 45.30 |
| 4 | 10 | 0.95 | 279.41 | 55.88 |
| 5 | 12 | 0.84 | 247.05 | 49.41 |
| 6 | 14 | 0.72 | 211.76 | 42.35 |

**Graphical representation on effect of incubation time on *cellulase* activity:**




**GRAPH 6: EFFECT OF INCUBATION TIME ON *CELLULASE* ACTIVITY**

**CONCLUSION:** Degradation of cellulosic material is a complex process requiring participation by a number of microbial enzymes. Habitats that contain this substrate are the best sources in where to find these microorganisms. So the site for the sample collection were selected as those were rich in cellulosic biomass such as sugarcane cultivating soil, hence there were maximum possibilities to get potential *cellulase* producing bacterial strain. The cellulolytic activity shown by the isolated bacterial species was reported to depend on the source of occurrence in various natural environment enable them to be responsible for the degrading of cellulose. The extracellular *cellulase* produced by isolates were partially purified by ammonium sulphate precipitation and dialysis and the determination of total protein concentration of both crude and partially purified enzyme was carried out by taking OD at 280nm and their specific activity was

determined. Selected potent bacterial isolate were characterized for their morphological, culture and biochemical characteristic. The identified bacterial strain was *Bacillus sp.* Microbial *cellulases* have a number of commercial applications in industries like fruit juices, paper, bio fuels and textile. It has been reported that the production of extra cellular *cellulases* by different microorganisms depends upon the source and the culturing conditions.

Similarly the optimization of the isolated enzyme is an important task for the production of commercially stable enzymes for the industries. So, it becomes necessary to understand the nature of *cellulase* and their catalytic potentiality under different conditions.

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