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# Utilization of Banana Waste for Effective Production of Cellulase by Rhizopus Sp

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## ABSTRACT

Cellulase production by Rhizopus sp. at varying substrate concentration, pH, temperature and period of incubation was studied using Potato Dextrose (PD) as the basal medium. The cellulose source was supplemented with the banana waste. The result of the study reveals that the production of cellulase increased as the period of incubation increased upto 6 days at substrate concentration 1.5g/100mL and on further increase in the period of incubation no increase in cellulase was recorded. The pH of the medium had a significant effect on the production of cellulase and the optimum pH for maximal production was found to be 5.0.Similarly, the effect of temperature on cellulase production was studied and it was inferred that the cellulase production was maximal at a temperature of 28$^{o}$C.Purification of cellulase was carried out using ammonium sulphate dialysis and acetone precipitation. Enzyme level and protein content were determined in the purified cellulase.

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## INTRODUCTION

Agricultural products such as barley, rice, corn, soyabean and food processing industrial residues mixed with various mineral salts and additives are used as the traditional substrates for solid substrate fermentation [15, 20, and 37]. These agro residues can be used for the production of microbial enzymes such as xylanases, cellulases, amylases, pectinases, proteases and lipases [1-3, 10, 17, and 31]. Agricultural wastes and in fact all lignocellulosics can be converted into products that are of commercial interest such as ethanol, glucose and single cell protein [28].

Lignocellulosics are abundant source of carbohydrate, continually replenished by photosynthetic reduction of carbon dioxide by sunlight energy [8]. Thus they are the most promising feed stock for the production of energy, food and chemical [28, 38].

The bioconversion of cellulosic materials has been receiving attention in recent years, as it is now a subject of intensive research for the development of a large scale conversion process beneficial to mankind [8, 14] suggested that bioconversion would help to alleviate shortages of food and animal feeds, to solve modern waste disposal problem and diminish man's dependence on fossil fuels by providing a convenient and renewable sources of energy in the form of glucose. Since the production of cellulose enzyme is a major factor in the hydrolysis of cellulosic materials, it is important to make the process economically viable. Although much work has been done on the production of cellulose from the lignocellulosics [7, 13, 28] emphasis has not been made on utilization of banana waste [22].

Banana being a major cash crop with an average production of 53.95t/ha. After the harvest of crop, besides, the pseudo stem all the parts of the plant body are left behind. This agro-waste can be effectively utilized for the production of cellulases, which can be utilized for the production of cellulases, which can be utilized in the saccharification of these wastes. The production and characterization of cellulases by Trichoderma lignorum on banana wastes had been reported earlier [5].

Our study aims at optimization of medium components, the culture conditions and effective utilization of banana waste as substrate for efficient production of cellulose with the following objectives.

1. Isolation and enumeration of viable bacteria and fungi.
2. Utilization of banana waste.
3. To find out cheapest source of cellulase production.
4. To access the banana waste for high production of cellulase.
5. Effective utilization of Rhizopus sp. For production of cellulase.
6. To access the optimum substrate concentration for cellulase production.
7. To detect the optimum pH and temperature for cellulase production.
8. Partial purification of cellulose

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## MATERIALS AND METHODS

### MICROORGANISM

The organism used in the study was Rhizopus sp. obtained by isolation from soil collected at the cotton fields of TNAU, Coimbatore.

### PREPARATION OF CARBON SOURCE

### BANANA SOURCE

The substrate used for the study is banana waste which was collected from the agricultural field. It was washed in water, sun dried and oven dried overnight at 70$^{o}$C and milled to obtain powder form.

### DELIGNIFICATION

A quantity of 10g of banana waste powder was treated with 500mL of 0.1N Sodium hydroxide and sterilized at 15lbs for 15 minutes, water washed, neutralized with Hydrochloric acid and dried for 3 hours in oven and then stored until use.

### INOCULUM PREPARATION

100mL of PDA media (fermentation media) was inoculated with Rhizopus sp. And allowed to grow for 8 days and transferred to potato dextrose agar slants and pure culture was obtained and stored at 4$^{o}$C with regular sub-culturing. The inoculum was agitated continuously and was used for the fermentation process.

### DETERMINATION OF SUBSTRATE CONCENTRATION

100mL of sterilized media was dispensed into 4 conical flasks and the substrate (banana waste) varying from 0.5-2.0g was added to flasks, pH was adjusted to 5.0, allowed to cool and inoculated with Rhizopus sp. and incubated at room temperature for 10 days. The optimum substrate concentration for cellulase production was determined.

The optimum pH for cellulase production was determined by incubating the powdered banana waste to the PD medium at varying pH range of 3 to 7 at room temperature for a period of 10 days. In temperature dependence experiments, the basal medium was used and the pH was adjusted to 5.0 (optimum) before sterilizing and inoculated with 0.5mL of the organism and incubated at varying temperature of 26, 28 and 30$^{o}$C. The biomass was determined following incubation each day, using Whatmann No.1 filter paper.

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## ASSAY OF ENZYME ACTIVITY

The culture filtrate was centrifuged for 10 minutes at 10,000 rpm and the supernatant was used as the crude enzyme. Cellulase activity was determined using 1% carboxy methyl cellulose (CMC) as a substrate.

Assay mixture consisted of the following

4.5mL of 1% CMC in 55mM citrate buffer pH 5.0
0.5mL of crude enzyme solution

Following incubation at $4^{o}C$ for 30 minutes the amount of glucose liberated was measured by O-toludine method using glucose as the standard.

## ENZYME UNITS

The amount of enzyme liberated was expressed in terms of IU/L under assay conditions. All the experiments were carried out in duplicates and the data represent the mean value. Purification of cellulose was done by ammonium sulphate precipitation followed by dialysis. The specific activity of the enzyme was determined and expressed as IU/L

## RESULTS

Table 1 reveals the total viable bacteria and soil fungi encountered in the cotton field. Eleven fungal strains were isolated and Lactophenol staining was performed and one strain was selected for the present study namely Rhizopus sp.

**TABLE 1: ENUMERATION OF TOTAL VIABLE BACTERIA AND FUNGI**

| DILUTIONS | TOTAL VIABLE BACTERIA | FUNGI |
|---|---|---|
| | CFU/ g OF SOIL SAMPLE | |
| $10^{-4}$ | 85 X $10^{-4}$ | 3 X $10^{-4}$ |
| $10^{-5}$ | 11 X $10^{-4}$ | NIL |

Figure 2 reveals that the maximum production of cellulase was obtained on the $6^{th}$ day with the value of 1.60 IU/mL. It is also evident that maximum mycelial growth was on the tenth day of incubation.

ISSN: 0975-8585

**Figure 2: Production of Cellulase at Varying Period of Incubation and Substrate Concentration**

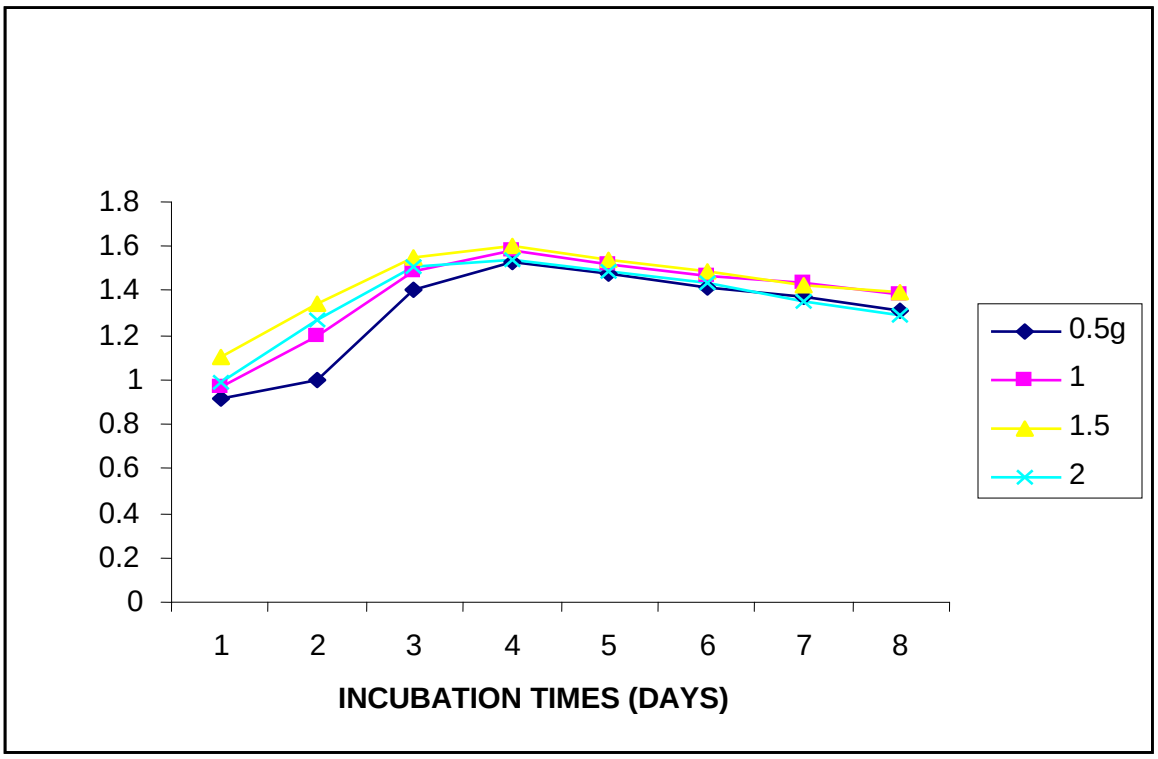
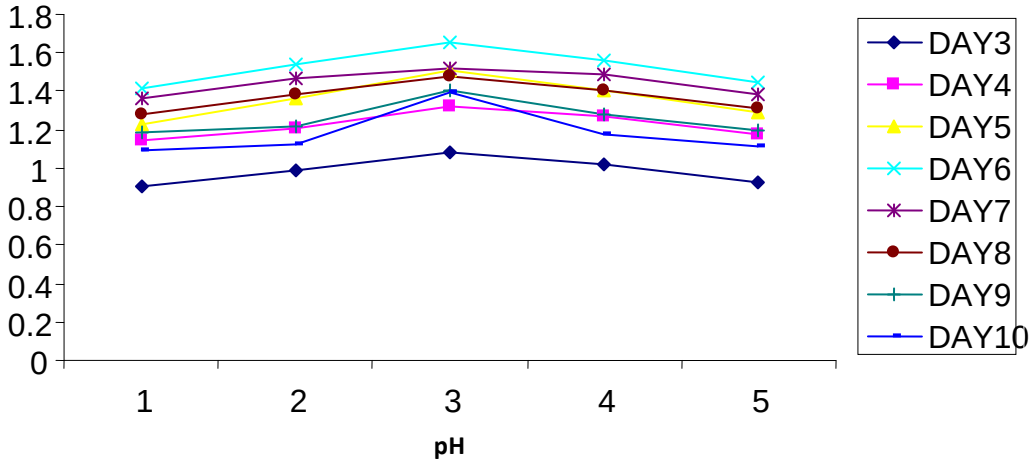



Figure 3 reveals the production of cellulase by Rhizopus sp. at varying pH and maximum production of cellulase was achieved at a pH of 5.0 (1.65 IU/mL).

**Figure 3: Influence of Varying Ph on Cellulase Production**



ISSN: 0975-8585

**Figure 4: Influence of Temperature on Cellulase Production**

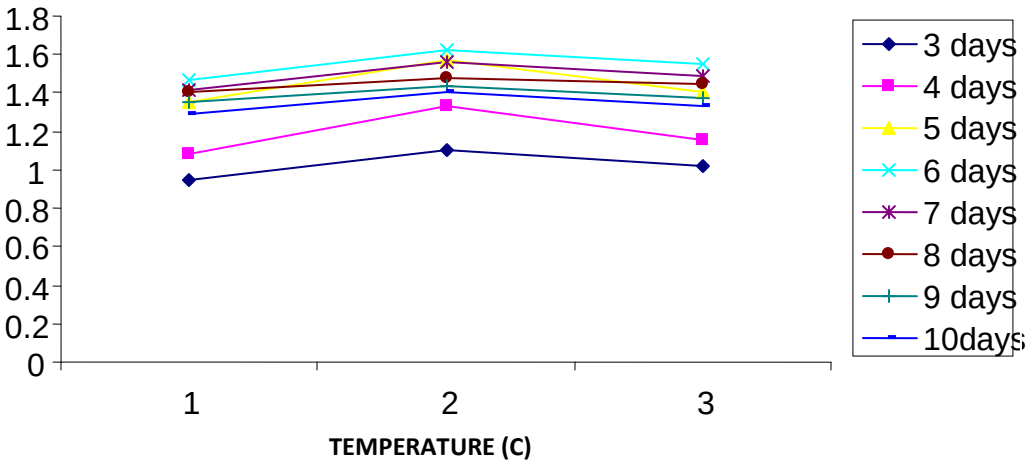



Figure 4 depicts the production of cellulase at varying temperature (26-30$^{o}$C) maximum cellulase of 1.62 IU/mL production was achieved at a temperature of 28$^{o}$C

**TABLE 5: Level of Reducing Sugar at Varying Substrate Concentration**

| SUBSTRATE CONCENTRATION (g) | REDUCING SUGAR CONTENT (mg/Ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | INCUBATION TIME (DAYS) | | | | | | | |
| | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 0.5 | 0.87 | 1.01 | 1.29 | 1.37 | 1.28 | 1.19 | 1.09 | 0.96 |
| 1.0 | 0.92 | 1.06 | 1.31 | 1.40 | 1.32 | 1.27 | 1.15 | 1.09 |
| 1.5 | 0.94 | 1.10 | 1.39 | 1.48 | 1.41 | 1.35 | 1.27 | 1.23 |
| 2.0 | 0.93 | 1.07 | 1.35 | 1.43 | 1.37 | 1.29 | 1.18 | 1.11 |

**TABLE 6: Protein Content of the Crude Enzyme at Varying Substrate Concentration**

| SUBSTRATE CONCENTRATION (g) | PROTEIN CONTENT (mg/Ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | INCUBATION TIME (DAYS) | | | | | | | |
| | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| 0.5 | 0.46 | 0.49 | 0.58 | 0.70 | 0.62 | 0.56 | 0.44 | 0.35 |
| 1.0 | 0.51 | 0.56 | 0.62 | 0.74 | 0.68 | 0.59 | 0.51 | 0.47 |
| 1.5 | 0.68 | 0.72 | 0.79 | 0.83 | 0.76 | 0.71 | 0.65 | 0.59 |
| 2.0 | 0.53 | 0.65 | 0.69 | 0.77 | 0.69 | 0.63 | 0.56 | 0.43 |

Table 5 and 6 reveals the level of reducing sugar and protein in the crude enzyme extract. Results of purification studies are summarized in table 7.

ISSN: 0975-8585

**TABLE 7: Partial Purification of Cellulase**

| DESCRIPTION | CELLULASE ACTIVITY (IU/mL) | TOTAL PROTEIN (mg/mL) | SPECIFIC ACTIVITY |
|---|---|---|---|
| CRUDEEXTRACT | 0.51 | 0.49 | 1.04 |
| 30% NH4SO4 | 0.54 | 0.45 | 1.20 |
| DIALYSED | 0.60 | 0.39 | 1.54 |
| 50% ACETONE | 0.69 | 0.24 | 2.87 |

## DISCUSSION

Cellulases are inducible enzymes and its production on a commercial scale induced by growing the fungus on solid cellulose or by culturing the organism in the presence of a disaccharide inducer such as lactose. Both the methods of induction results in high costs. Carbon source in majority of commercial cellulase fermentations are cellulosic biomass including straw, spent hulls of cereals and pulses, rice or wheat bran, bagasse, paper industry waste and various other lignocellulosic residues [6, 9, 25, 26, 35]. Though majority of the processes are batch processes there has been attempts to produce cellulase in fed batch [6] or continuous [12, 27] mode which helps to override the repression caused by accumulation of reducing sugar. The major technical limitation is the increased fermentation time with low productivity [29].

Solid state fermentation for production of cellulases is rapidly gaining interest as a cost effective technology, not only for the production of the enzyme but also for the bioconversion of lignocellulosic biomass employing cellulolytic microorganisms. [30] Indicated that there was about a tenfold reduction in the production cost in solid state fermentation than submerged fermentation. The large scale commercial processes are still using the proven technology of submerged fermentation [29].

Cellulolytic microbes are primarily carbohydrate degraders and are generally unable to use proteins or lipids as energy sources for growth [19]. Cellulolytic microbes notably the bacteria Cellulomonas and Cytophaga and most fungi can utilize a variety of other carbohydrates in addition to cellulose, [23, 24] while the anaerobic cellulolytic species have a restricted carbohydrate range, limited to cellulose or it's hydrolytic products [21].

The challenges in cellulase production involves

1. Developing suitable bioprocess.
2. Media for cellulase production.
3. Identification of cheaper substrates and inducers.

Cellulases were initially investigated several decades back for the bioconversion of biomass which gave way to research in the industrial applications of the enzyme in animal feed, feed, textiles and detergents and in the paper industry [39]. With the shortage of fossil fuels and the arising need to find the alternative source for renewable energy and fuels, there is

renewal of interest in the bioconversion of lignocellulosic biomass using cellulases and other enzymes [29].

Apart from these common applications cellulases are employed in formulations for removal of industrial slime, in research for generation of protoplast, [36] and for generation of antibacterial chito-oligosaccharides, which could be used in food preservation [16] immunomodulation and as a potent antitumour agent [29].

Baig studied the carbon source in the medium affects considerably in the synthesis of the cellulolytic enzymes by Trichoderma lignorum in liquid cultures. Glucose, sucrose, glycerol, cellobiose, CMC, paper dust, banana leaves and pseudo stem waste were effectively utilized as carbon source with slight differences in the production of cellulases. Trichoderma lignorum showed the highest activity on the dried leaf powder closely followed by pseudo stem waste. Thus inexpensive medium can be designed by using banana biomass as source of carbon containing simple constituent [4].

The studies carried out by Baig [4] using Trichoderma lignorum on banana waste showed maximum cellulase production on $8^{th}$ day of incubation. In the coculture condition using Aspergillus terreus and Trichoderma viride on groundnut shell waste, maximum activity was observed on the $14^{th}$ day of incubation [22, 32] observed that the increased cellulase activity was observed at about $12^{th}$ hour for all lignocellulosic materials. [28] has worked on optimization of cellulase production by Aspergillus flavus on bagasse and observed that the enzyme could be harvested about $12^{th}$ hour, when activity is highest.

Aspergillus terreus showed higher endoglucanase activity of 2.7821 IU/mL and exoglucanase of 0.356 FPU/mL. Coculture of Aspergillus terreus and Trichoderma viride showed higher endoglucanase activity of 4.536 IU/mL and exoglucanase of 0.457 FPU/mL, compared to the combinations of Aspergillus terreus + Aspergillus nidulans and Aspergillus nidulans Trichoderma viride [32]. Aspergillus flavus grown on saw dust gave the highest cellulase activity of 0.0743 IU/mL respectively [22].

The optimum pH for the production of cellulase by Trichoderma lignorum in banana agro-waste based medium was in the range 5.6-5.8; acidic pH was favorable for the enzyme production [4]. The optimum production of cellulase by T. reesei on corn straw was 5.0 [34].

The optimum temperature for the Trichoderma reesei was $28^{o}C$ [34]. The production of enzyme activity gradually increased from $20^{o}C$ upto $45^{o}C$ and there after a drastic reduction in the yield of cellulase was observed. Thus, $45^{o}C$ was optimum temperature for Trichoderma lignorum on banana agro waste [11].

In the present study banana waste was used as the substitute for cellulose. The maximum activity (1.60 IU/mL) was observed at a concentration of 1.5g/mL, on the $6^{th}$ day of

incubation. The optimum pH for cellulase activity was at a pH 5.0 (1.65 IU/mL), and the optimum temperature for cellulase activity was at 28$^{o}$C (1.62 IU/mL).

Since not much work has been done with Rhizopus sp. with banana waste, it has been aimed with two way approach of utilizing the banana waste and novel production of cellulase using Rhizopus sp. thus a simplified medium containing banana waste proved to be cost effective substrate for cellulase production, when compared with that of other conventional substrates.

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