*IOSR Journal of Environmental Science, Toxicology and Food Technology (IOSR-JESTFT)*
*e-ISSN: 2319-2402,p- ISSN: 2319-2399.Volume 10, Issue 5 Ver. II (May. 2016), PP 68-73*
*www.iosrjournals.org*

# Optimization of Cellulase Enzyme from Vegetable Waste by Using *Trichoderma atroviride* in Solid State Fermentation

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***Abstract:*** *The present study deals with production of cellulase enzymes from filamentous soil fungus Trichoderma atroviride by using vegetable waste as substrate through the process of solid state fermentation. The enzyme production was further improved by optimizing a number of physical parameters such as incubation time, temperature, pH, inoculums size and nutritional factors (carbon source, nitrogen source and detergents). By optimization of different parameters, the maximum activities of cellulase synthesized by Trichoderma atroviride were observed after 5 days incubation at pH 6 and 30°C temperature with sucrose, yeast extract and Tween -80 as carbon, nitrogen and detergents supplements respectively. The high activity of cellulase produced by the fungus suggests its potential for commercial scale production for various industrial applications.*
***Keywords:*** *Cellulase, optimization, solid state fermentation, Trichoderma atroviride, vegetable waste.*

## I. Introduction

The need for utilizing renewable resources to meet the future demand for fuel has increased the attention on cellulose. Cellulose is the structural component of the primary cell wall of plant biomass. It is a polymer of β-1, 4 linked glucose units. It is considered as one of the most abundant renewable carbon source on earth and the dominating waste material from agriculture [1]. It represents about 1.5 x $10^{12}$ tons of the annual biomass production through photosynthesis and is considered to be an almost inexhaustible source of raw material. Its crystalline structure and insoluble nature represents a big challenge for enzymatic hydrolysis. Cellulose is generally degraded by multi-complex enzyme called cellulases [2]. Cellulase (E.C 3.2.1.4) refers to a class of enzyme that catalyzes the hydrolysis of 1, 4 β-D glycosidic linkages in cellulose component [3].

Cellulase enzymes play an important role in natural biodegradation processes in which plant materials are effectively degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa. In industry, these enzymes have its importance due to major role in the production of fermentable sugars and ethanol, organic acids and chemicals. The major other applications are biostoning of jeans and biopolishing of cotton and other cellulosic fabrics, paper recycling and as animal feed additives for improving the nutritional quality and digestibility [4]. It is also used for deinking of paper industries and to enhance pulp drainage in textile industries. It is used for bioremediation, waste water treatment and also for single cell protein [5]. It has importance in food sciences like food processing, drying of beans in coffee. Main function of cellulase enzyme in food industry is extraction, clarification and stabilization of fruit juices and vegetables.

Bioconversion of cellulosic materials mainly depends on the nature of cellulose component, sources of cellulase enzyme and optimal conditions for production of enzymes. Cellulase enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation [6]. However, fungi are well known agents of decomposition of organic matter due to their elongated hyphea that creates mechanical pressure on the cellulose structure, causing them to production of large amounts of cellulase enzymes [7]. Cellulase production from fungi is highly useful for the enzyme production as compared to other microorganisms. Fungi such as *Trichoderma sp., Aspergillus sp. and Penicillium sp.* are the most commonly cellulase producers. However, the most extensively studied cellulases are produced by efficient lignocellulose degrading fungi, particularly *Trichoderma sp*. [8].

Improvement of microbial strains for the over-production of industrial products can reduce the process cost and may also possess some specialized desirable characteristics. Several lignocellulosic materials are efficient substrates for white-rot fungi, which produce industrially important cellulolytic enzymes. Among processes used for enzyme production, solid state fermentation (SSF) using agro-wastes are an attractive and cost effective option because it presents higher productivity involving a simpler operation [9]. Solid state fermentation (SSF) is gaining interest as a cost effective technology for the production of higher yields of cellulase as compared to liquid culture. SSF has many advantages over other fermentation processes due to simple process, energy saving, less water consumption and less production of waste products. Production of cellulases by the fungal isolates requires optimal conditions for their growth which leads to the release of extracellular enzymes. The growth conditions as well as extracellular enzyme production conditions is likely to vary among isolates. Certain fermentation parameters such as temperature, incubation period, carbon source, pH etc. were found to be critically affecting the cellulase yield [10].

Therefore, the present study aims to enhance the production of extracellular cellulases by filamentous fungi *Trichoderma atroviride* under solid state fermentation using vegetable waste as substrate and optimizing cultural parameters favoring the maximal exploration of fungal capacity for overproducing of cellulase.

## II. Materials And Methods

### 2.1 Microorganism and Growth Conditions

*Trichoderma atroviride*, originally isolated from soil was used for this study. The culture was maintained on potato dextrose agar (PDA) slants at 4ºC and subcultured every 6 months.

### 2.2 Cellulase production from fungal strain in solid state fermentation

Solid state fermentation was carried out in 250 ml Erlenmeyer flasks containing mandel and weber media supplemented with 5 g of vegetable waste as substrate. Each flask was autoclaved at 121°C for 20 min. After cooling, each flask was inoculated with 2 ml of the spore suspension of *Trichoderma atroviride* prepared in Tween-80 from 5 days grown old slants, and the inoculated flasks were incubated in an incubator. Crude enzyme was extracted from fermented substrate by adding of 0.05 M citrate buffer and the contents were mixed for 1 hr at 180 rpm in an orbital shaker and filtered through a muslin cloth by squeezing. The extract was centrifuged at 10,000 rpm for 10 min and the supernatant was used for determination of enzyme activity. The enzyme activity was determined by carboxymethyl cellulase assay and filter paper assay.

### 2.3 Carboxymethyl Cellulase (CMCase) Assay

0.5 ml of crude enzyme supernatant was incubated with 0.5 ml of 1% (w/v) CMC (carboxymethyl cellulose) in 0.05 M citrate buffer solution of pH 4.8 for 30 minutes at 50ºC. The resulted reducing sugars were determined according to Miller's method with glucose as a standard [11].

### 2.4 Filter Paper (FPase) Assay

For FPase activity 0.5 ml of crude enzyme supernatant was incubated with 1 ml of 0.1M citrate buffer of pH 4.8 containing 50mg Whatman no. 1 filter paper. After incubation of 1 hour at 50ºC, obtained reducing sugars were estimated by Miller's method.

### 2.5 Optimization of culture conditions for cellulase production

To optimize the cellulase production, effects of fermentation conditions such as the incubation period, pH, temperature, carbon source, nitrogen source and detergents were studied.

#### 2.5.1. Effect of incubation period

Effect of incubation period on enzyme production was checked by incubating the fermentation medium for different incubation time (3 to 8 days) and enzyme assay was carried out at interval of 24 hours by DNS method.

#### 2.5.2. Effect of temperature

In order to determine the effective temperature for cellulase production by the *Trichoderma atroviride*, fermentation was carried out at 5°C intervals in the range of 25, 30, 35 and 40°C.

#### 2.5.3 Effect of pH

To determine optimal pH, fungus cultures were cultivated in a 250 mL flask containing optimized medium with different pH ranges from 3.0 to 8.0. The pH of the medium was adjusted by using 1 N HCl or 1 N NaOH. The flasks were kept at 30°C for 5 days of cultivation.

#### 2.5.4 Effect of carbon sources

Effects of various carbon compounds namely, sucrose, dextrose, starch and lactose were used for studying. The broth was distributed into different flasks and 1.0% of each carbon sources were then added before inoculation of the strain and after culture inoculation, the flasks were incubated for 5 days at 30°C.

#### 2.5.5 Effect of nitrogen sources

The fermentation medium was supplemented with organic and inorganic compounds (yeast extract, peptone, beef extract and sodium nitrate) and was incubated for 5 days at 30°C.

#### 2.5.6 Effect of different detergents

Four different surfactants for increasing of cellulase production such as Tween 20, Tween 80, SDS (sodiumdodecyl sulphate) and PEG-6000 (Polyethylene Glycol) were used.

**2.5.7 *Statistical Analysis***

Data presented on the average of three replicates (±SE) obtained from their independent experiments.

## III. Result And Discussion

### 3.1 Optimization of parameters for cellulase enzyme production

Medium parameters such as temperature, pH, incubation time, and carbon, nitrogen and detergent source play very important role in production of enzyme and greatly influence the enzyme activity. CMCase and FPase test were used to check the enzyme activity at variable parameters.

### 3.1.1Effect of incubation time

Time of fermentation has an important impact on the product formation. Enzyme production is related to time of incubation [12]. Time course for production of cellulase enzyme was investigated and fermentation for a period of 3 to 8 days were carried out. Enzyme activity was investigated after interval of 24 hours. The results showed that the maximum CMCase activity (77.39 U/g) and FPase activity (42.08 U/g) were observed at incubation time of 120 hours or 5 day was for *Trichoderma atroviride*. Decrease in cellulase activity was recorded with further incubation (Fig. 1). Beyond 5$^{th}$ day, a steep loss in enzyme production was observed. This trend of decreased enzyme activity may be due to the depletion of macro and micronutrients in fermentation medium with the lapse in time, which stressed fungal physiology resulting in the inactivation of secretary machinery of enzymes [13]. This result of incubation time for *Trichoderma atroviride* was found comparable with the reports of Karthikeyan et al. [14] who reported 120 hours to be optimum incubation time for the production of cellulase.

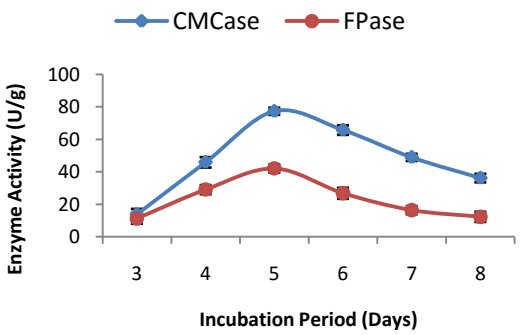


**Fig. 1:** Effect of incubation period on cellulase production.

### 3.1.2 Effect of temperature

Temperature is the important parameter that effects the cellulase production. Even slight changes in temperature can affect enzyme production. Presently, the optimal temperature for maximum cellulase production was studied by incubation of the inoculated media at different temperature ranging from 25 - 40°C and optimum temperature for CMCase and FPase activity was found to be 30° with FPase activity of 43.11 U/g, CMCase activity of 85.31 U/g. Enzyme activity was decreased at 35°C for (Fig. 2). These results of present study are comparable with the results of Maurya et al. and Shafique et al. [15, 16] who reported 30°C optimum temperature for cellulase production.

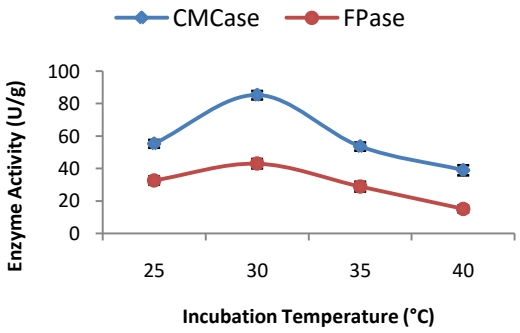


**Fig. 2:** Effect of incubation temperature on cellulase production.

### 3.3.3 Effect of pH

Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion [17]. The pH change observed during the growth of microbes also affects product stability in the medium. *Trichoderma atroviride* was allowed to grow in media of different pH ranging from 3.0 to 8.0. Maximum enzyme production was observed in medium of pH 6 (Fig 3). *Trichoderma atroviride* showed maximum CMCase (88.91 U/g) and FPase (50.96 U/g) production at pH 6 during fifth day of incubation at 30°C. Reduction in enzymes activities after 6.0 pH may be ascribed to the fact that change in pH may change the three dimensional structure of the enzymes. These results are in agreement with the observations of Baig *et al.* [18] wherein *Trichoderma lignorum* favoured a pH of 6.0 as optimum for maximum CMCase production from using banana waste. Our results are also similar with Jaradat *et al.* [19] who reported that the cellulase enzyme from the active isolate J2 was active over a pH range of 4–7 with maximum activity at pH 6.

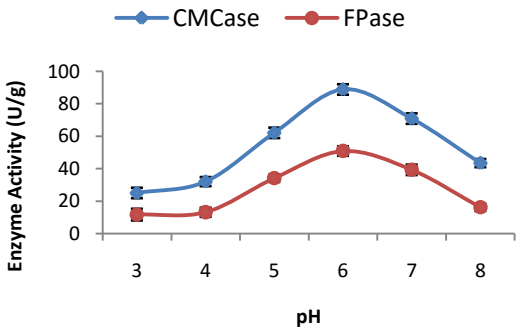


**Fig. 3:** Effect of pH on cellulase production.

### 3.3.4 Effect of carbon sources

Carbon sources play a crucial role in the cell metabolism and synthesis of cellulase. *Trichoderma atroviride* was grown on various carbon sources at 1% concentration to determine their potential to support cellulase enzyme activities. Various sources of carbon such as sucrose, dextrose, starch and lactose were used in growth media. Results obtained showed that *Trichoderma atroviride* in presence of sucrose brought about the maximum CMCase (120.81 U/g) and FPase (86.15 U/g) production compared to other carbon sources (Fig 4). Gautam *et al*. [20] also observed that sucrose (1.0%) was found to be the main carbon source for cellulase production by using *Trichoderma sp*..

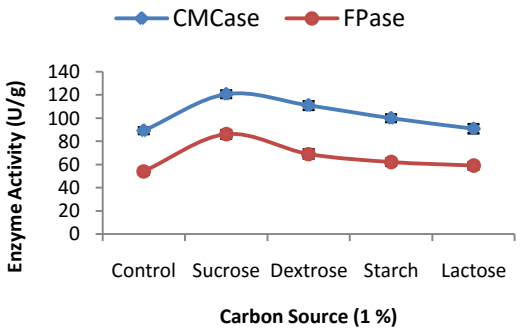


**Fig. 4:** Effect of carbon source on cellulase production.

### 3.3.5 Effect of nitrogen sources

The nitrogen source is important for industrial fermentation medium designing to meet maximum enzyme production. The effect of nitrogen sources on enzyme production of *Trichoderma atroviride* by incorporating various nitrogen sources at 1% concentration into Mandel and Weber media was studied and the results are shown in Fig. 5. The maximum activities of CMCase and FPase by *Trichoderma atroviride* using yeast extract as best nitrogen source were 176.29 and 93.46 U/g, respectively. Whereas minimum CMCase (91.06 U/g) and FPase (53.05 U/g) is recorded in control. Yeast extract was found to be the best organic source in enhancing cellulase production probably because this complex nitrogen source contains more elements that

are necessary for the metabolism of fungi. The results of present study regarding best nitrogen source were found to be similar with the results of the previous studies where yeast extract was the best nitrogen source for the production of cellulase by *Trichoderma.*

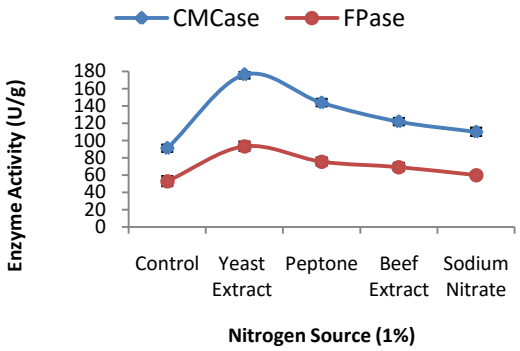


**Fig. 5:** Effect of nitrogen sources on cellulase production.

**3.3.6 Effect of detergents**

Surfactants alter cell permeability of microorganisms which lead to increased protein secretion or surface effects on cell-bound enzymes. The detergents had various effects on different enzymes and most commonly used detergents were Tween 20, Tween 80, Polyethylene glycol (PEG-6000) and sodium dodecyl sulfate (SDS) used in this study. In control experiment, no detergent was added. Maximum CMCase (178.92 U/g) and FPase (98.89 U/g) activities by *Trichoderma atroviride* was observed with Tween 80 as shown in Fig. 6. It was observed that medium containing Tween 80 had the highest positive influence on production of cellulase compared to other surfactants. The addition of Tween 80 increased the cellulase production by several folds. Similar observation was earlier reported by Liu *et al.* [21] for cellulase and xylanase production in SSF method by *Trichoderma viride*. The result of this study therefore, revealed that Tween 80 at a concentration of 1% was the best surfactant for optimum production of the cellulase.

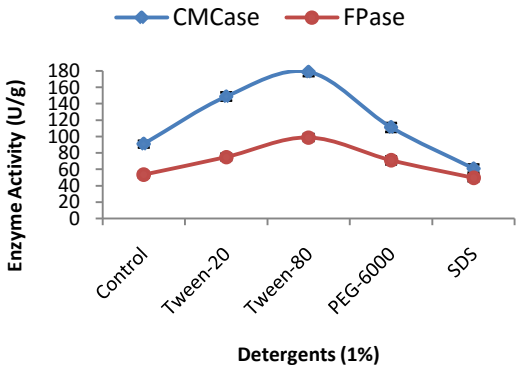


**Fig. 6:** Effect of detergents on cellulase production.

In the present study temperature of 30°C, pH 6, incubation time of 5 days, sucrose as carbon source and yeast extract as nitrogen source and Tween -80 as detergent source were found to be optimum for *Trichoderma atroviride* as maximum cellulase enzyme production was observed with these parameters.

## IV. Conclusions

The cost-effective technologies are needed for economical production of cellulases using vegetable waste as substrate. Cellulase yields appear to depend on a complex relationship involving a variety of factors like incubation time, pH value, temperature, presence of carbon, nitrogen and detergent sources. Major parameters affecting the fermentation process for enzyme production were studied and optimal levels were identified. Presently our studies investigated that the fungi belonging to *Trichoderma atroviride* effectively produced cellulase under laboratory conditions and can be used for various industrial applications.

## Acknowledgement

The author gratefully acknowledges support by getting Senior Research Fellowship from the University Grant Commission (UGC) and lab facilities from Deptt. of Environmental Sciences, M.D.University, Rohtak.

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