

# Optimization of the Physical Process Parameters for the Endo-Glucanase Production by *Aspergillus Terreus* Grown on Soy Hull Based Substrate Through Solid State Fermentation.

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**Abstract** -Endo-glucanase, one of the three components of cellulase complex, has been proved as an industrially important enzyme for its various applications during cellulolysis. Low cost efficient production of such enzyme was the prime goal of this study. Soy hull, one of the major agro residues in India, was exploited as a solid substrate for the production of endo-glucanase by solid state fermentation (SSF). The influential physical process parameters viz. initial moisture content, initial medium pH, incubation temperature, fermentation period and effect of solid to flask volume ratio were optimized aiming the maximum enzyme production. The one parameter at a time approach revealed significant increase in yield and the maximum endo-glucanase yield ( $124 \pm 10$  IU/gds) was achieved after 96h of incubation at  $30^{\circ}\text{C}$ , 70% moisture level, initial pH:5.4ml of inoculums volume and with solid to flask volume ratio of 20:500 (w/v).

## 1. INTRODUCTION

Cellulose is the major component of plant biomass and its annual production is around  $4 \times 10^9$  tons which eventually makes it the most abundant biopolymer on the earth [1]. The Cellulosic biomasses have been regarded as the most important renewable resource for bioconversion to different value added products including chemicals, bio-fuels etc. Bioconversion, particularly enzymatic hydrolysis, of these cellulosic materials into simple sugars, has become a subject of intensive research [2]. Cellulose consists of 3500–10,000 glucose units linked together by  $\beta$ -1,4-glucosidic bonds and the glucose chains are tightly packed together by two types of non covalent interaction viz. van der Waals force and hydrogen bonds which also provide crystallinity to the cellulose structure[3,4]. Complete enzymatic hydrolysis of crystalline cellulose requires synergistic actions of three component enzymes of cellulase complex i.e., exoglucanase; EC 3.2.1.91, endoglucanase; EC 3.2.1.4 and  $\beta$ -d-glucosidase; EC 3.2.1.21) generally synthesized by potential microorganisms [5]. During cellulolysis, the endo-glucanases cut at internal amorphous sites of cellulose causing release of oligosaccharides of various chain lengths. Exo-glucanases act progressively on cellulose oligosaccharide chains, and liberate either glucose or cellobiose as major products. Finally,  $\beta$ -

glucosidases hydrolyzes cellodextrins and cellobiose into glucose [6]. Although cellulases find many industrial applications including pulp and fiber, food and feed, textiles, lignocellulosic ethanol etc, production of these enzymes has been indicated to be a bottle neck for its very high cost [7-10].

India is the second largest producer of agro industrial wastes (560 million tons annually) in Asia [11], causing significant environmental challenges. Solid state fermentation (SSF) finds greater application in solid waste management as well as it also allows the use of such low cost agro industrial residues as substrate for enzyme production which helps in reducing the cost of production. Particularly for the cellulase production SSF has been found to be most preferred way and considerable research is being carried out in this field [12-17]. Soy hull is one of such agro residues, consisting cellulose, protein and fibers, mainly utilized for animal feed can also be value added by SSF production of cellulase as the cellulose in it acts as an inducer to the source microorganism for the production of inducible enzymes as well as its physical make up is also supportive for the fungal growth during fermentation [18].

Cellulases can be produced by the bacteria, fungi or actinomycetes in which fungi has been found to be the superior but only few out of them has been pointed out for the secretion of significant amount of cellulase proteins [19-20].

In the present study, soy hull was used as the solid substrate for the production of endo-glucanase exploiting the fungal strain of *Aspergillus terreus*. The effects of different physical process parameters were evaluated during the SSF and subsequently optimized for maximum endo-glucanase yield.

## 2. MATERIALS AND METHODS

### 2.1. Microorganism:

*A.terreus* MTCC8661 was used in all experiments. The culture was maintained on Czapek Dox agar slants at  $4^{\circ}\text{C}$ , and sub cultured monthly. A homogeneous spore suspension, used as inoculum, was obtained from 7-day-old culture by dislodging the spores in sterile 0.85% saline containing 0.02% Tween-80. The spore concentration was estimated by haemocytometer and the number adjusted to  $4 \times 10^7$  spores/ml.

## 2.2. Enzyme assay:

Endo-glucanase activity was assayed following the method described by Miller (1959) with minor modifications [21]. Briefly, a reaction mixture composed of 1ml of suitably diluted enzyme sample, 1.5 ml of 2.0 % low viscosity sodium salt of carboxymethylcellulose (Sigma, USA. Product code: C 5678) in 0.05 mM citrate phosphate buffer (pH 4.8) and 0.5ml of the same buffer was incubated at 50 °C in a shaking water bath for 20 min. The reaction was terminated by adding 3 ml of DNSA reagent. The color was then developed by boiling the mixture for 6 min. The absorbance of the samples was measured at 540 nm. One unit (U) of enzyme activity was defined as the amount of enzyme, which liberates 1 $\mu$ mol of glucose per minute under the above assay conditions.

## 2.3. PRETREATMENT OF SUBSTRATE:

The substrate soy hull, collected from the local market, was under gone acid and alkali treatment to understand the requirement of pretreatment of the substrate. 1N HCl and 1N NaOH was used for the pretreatment studies. The native soy hull was placed in the acid and alkali solution and made slurry of 10% consistency. The slurries were kept overnight at room temperature. The pretreated solid were separated and washed with demineralized water till the pH became neutral. The materials were oven dried at 70°C for overnight prior to use in SSF. The washed and dried native soy hull without any treatment was also used as production medium.

## 2.4. Enzyme production by SSF:

SSF was carried out by placing 10 g of oven dried substrates in 500ml Erlenmeyer flasks and wetted with Mandles medium composed of (per liter of distilled water): Urea 0.3 g, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1.4 g, KH<sub>2</sub>PO<sub>4</sub> 2.0 g, CaCl<sub>2</sub> 0.3 g, MgSO<sub>4</sub>, 7H<sub>2</sub>O 0.3 g, protease peptone 1.0 g, FeSO<sub>4</sub>, 7H<sub>2</sub>O 5.0 mg, MnSO<sub>4</sub>, 7H<sub>2</sub>O 1.6 mg, ZnSO<sub>4</sub>, 7H<sub>2</sub>O 1.4 mg, CoCl<sub>2</sub> 2.0 mg. and pH 4.0, to obtain 50% moisture content. The contents were sterilized by autoclaving and were inoculated with 2ml spore suspension followed by incubation at 30°C at static condition for 120h.

## 2.5. Enzyme extraction:

The fermented moldy bran was extracted with 100ml of distilled water by shaking at 150 rpm in a rotary shaker for 2h at 30°C. The pasty material was then sieved through two fold muslin cloth and clarified by centrifugation at 10000 rpm at 4°C for 15min. the resultant brown colored cell free supernatant was used as enzyme source.

## 2.6. Optimization of physical process parameters:

One factor at a time approach was adopted in this present study to investigate the effects of the influential physical

process parameters during SSF production of endo-glucanase. The tested process parameters were, initial moisture content (IMC): 40-90%, initial pH: 3-7, incubation temperature (20°C-40°C), inoculum volume (1ml-5ml), substrate to flask volume ratio (5:500-25:500 w/v).

## 2.7. Time course study of the endo-glucanase production by SSF:

To investigate the effect of incubation time the SSF was carried out up to 164 h. The enzyme activity was assayed at different time intervals.

## 3. RESULTS AND DISCUSSION

### 3.1. Selection of substrate for the SSF production of endo-glucanase:

The native soy hull was found to be superior for the growth of the fungal strain used in this study. Compared to acid and alkali treated soy hull, the yield of endo-glucanase on the native soy hull was significantly higher (p<0.05) (data not shown). For rest of the studies untreated soy hull has been used as the substrate for the SSF.

### 3.2. Initial moisture:

Initial moisture content (IMC) had been reported as an important process parameter during the solid substrate enzyme production using fungal cultures because of its effect in substrate physico-chemical make up as well as on microbial growth which eventually affected enzyme yield [22,23]. High level of IMC used to account for the low gas transfer in the SSF system and low level of moisture resulted poor nutrient solubility. The result shown in Fig: 1.a. was also in line with the same phenomena. The maximum endo-glucanase activity (89 U/gds) was observed at IMC of 70%. There was a decreasing trend in enzyme activity noticed with the shift of IMC value from the optimal level. Subsequent experiments were conducted at 70% IMC level.

### 3.3. Initial pH:

As a regular practice of process development in solid substrate enzyme production only the initial pH could be possible to set at a desired set point but pH could not be controlled during the fermentation. With variation of initial pH the enzyme productivity was also found to be varied significantly<sup>24</sup>. In the present study, highest endo-glucanase activity (96 U/gds) was found at initial pH of 5 (Fig: 1.b.). In another study on the *A. terreus* endo-glucanase, pH-4.5 was reported as optimum [25]. Further optimization of other process parameters were done by conduction SSF at optimum pH.

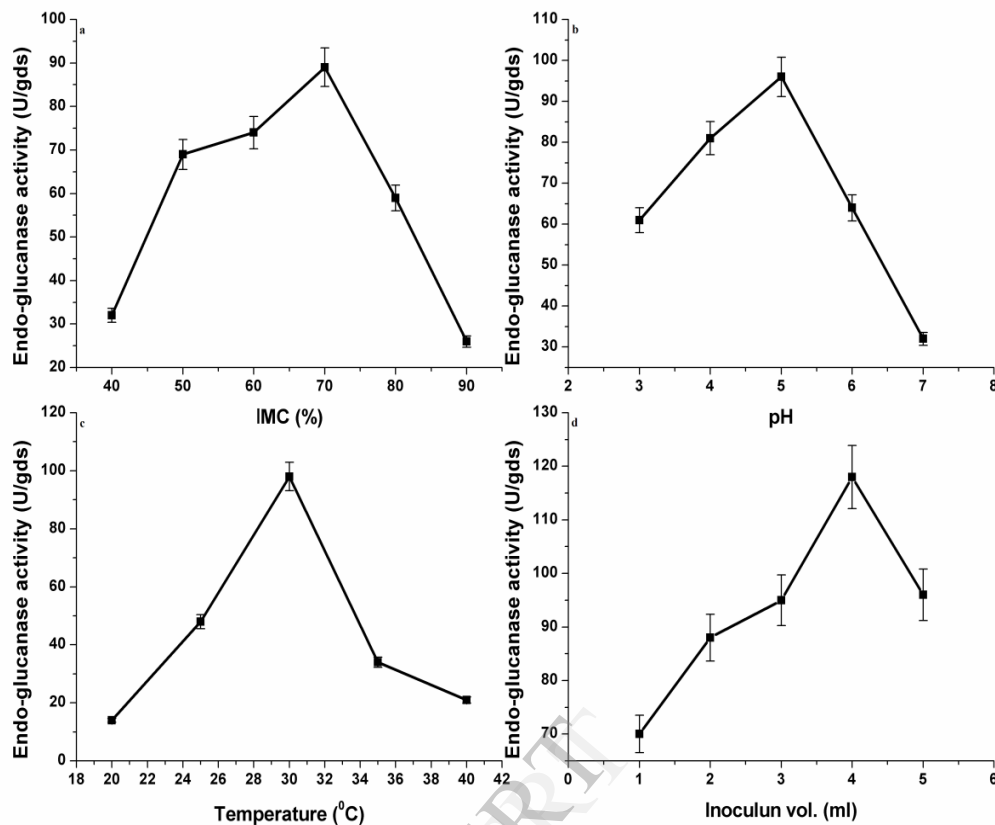


Figure.1. Effect of different environmental factors viz. IMC (a), initial pH (b), temperature (c) and inoculums vol. (d) on the production of endo-glucanase by *A.terreus* MTCC8661 during solid state fermentation on soy hull substrate.

### 3.4. Incubation temperature:

To reveal the effect of incubation temperature on the enzyme activity SSF experiments were carried out at different temperatures. It was found that increase in temperature beyond 30°C significantly reduced the enzyme yield ( $p < 0.05$ ) (Fig: 1.c.). With the temperature increase of 5°C the activity reduced more than 50% of the optimum value (98 U/gds). Similar condition for the production of hydrolytic enzyme by *Aspergillus* mold was also reported by Grover et al. 2013 [26]. The SSF were continued at 30°C for rest of the studies.

### 3.6. Effect of solid to flask volume ratio:

During the investigation of the effect of solid to flask volume ration on enzyme activity, it was found that maximum enzyme

### 3.5. Inoculum level:

Lower and higher volume of inoculum would cause slow growth of the culture and accelerated biomass synthesis followed by rapid nutrient depletion respectively. Both the conditions could result less metabolite development. A balance between the proliferating biomass and available nutrient would yield an optimum at which the enzyme synthesis would be maximum [27, 28]. It was observed in the present study that maximum endo-glucanase yield (118 U/gds) occurred at inoculums level of 4ml (Fig: 1.d.).

activity achieved when 20g of dry solid substrate was inoculated in 500ml flask (Table.1).

Table.1. Effect of different solid to flask volume ration on the production of endo-glucanase by *A.terreus* MTCC 8661 during SSF on soy hull substrate.

Solid to flask volume ratio (w:v)	End-glucanase activity (U/gds)
5:500	72
10:500	96
15:500	109
20:500	122
25:500	119
30:500	106

### 3.7. Time of fermentation:

The effect of incubation time was studied by cultivating the source organism up to 164h on soy hull. The enzyme yield was increased significantly with time up to 96h. After that gradual decrease in the enzyme yield was received. It might be

due to the nutrient depletion in the medium which in turn stressed the fungal system by inactivating the secretory system of the enzyme of interest [29-30]. The maximum endo-glucanase titre ( $124 \pm 10$  IU/gds) was obtained at 96h (Fig.2).

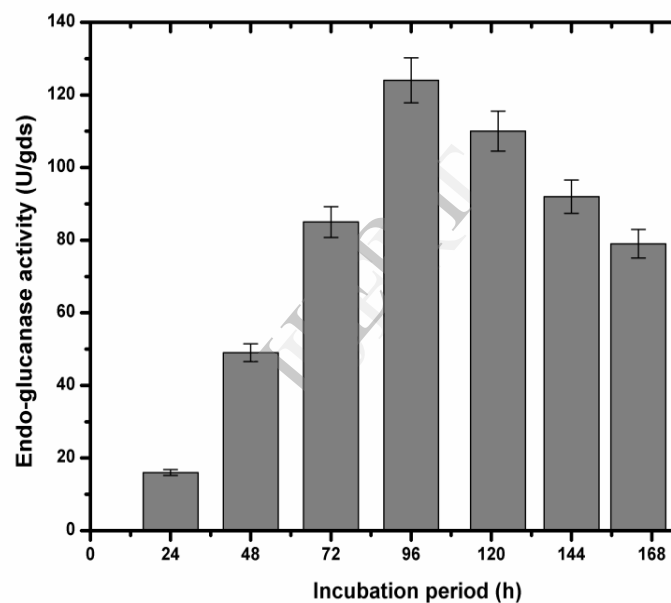


Figure.2. Profiling of incubation period during SSF based production of endo-glucanase by *A.terreus* MTCC8661

### 4. CONCLUSION:

It was evidenced from the present study that the native soy hull supported the growth and the enzyme production with out any acid or alkali aided pretreatment. The effect of physical process parameters were monitored and subsequently optimized for the maximum yield of endo-glucanase. With one parameter at a time approach ~1.4 times improvement in enzyme yield was achieved. For more improvement next part of this study has been planned with media engineering followed by biochemical characterization of the crude endo-glucanase source.

### 5. ACKNOWLEDGEMENT:

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