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Comparison Of Cellulase Production In Trichoderma reesei (NCIM – 1052) And Aspergillus niger (NRRL – 322); Media Optimization And Enzyme Characterization Of Cellulase From Trichoderma reesei With Lyophilization

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Abstract: Cellulase (E.C.3.2.1.4) production levels were compared in *Aspergillus niger (NRRL – 322)* and *Trichoderma reesei (NCIM – 1052). T. reesei* was used for media optimization studies and the cellulase activities were calculated at various assay pH and temperatures. The maximum cellulase activity was 217.17 U/ml at pH 2 and temperature 50 ºC for the media containing 2% groundnut, 1% soya bean meal. The lyophilized cellulase powder had 22.5 U/g for sucrose based formulation and 12.166 U/g for dextrose based formulation. % loss on drying was also calculated.
Keywords: Cellulase; *Trichoderma reesei; Aspergillus niger*; lyophilization .

1. INTRODUCTION:

Enzymes are biological catalysts. A catalyst is any substance which makes a chemical reaction proceeds faster, without itself being changed [1]. Cellulases are enzymes which break down cellulose to β – glucose [2]. Cellulases are one of the most useful enzymes in wide range of industrial applications [3]. Cellulases are generally produced by fungi, bacteria or actinomycetes but the most common producer for industrial application is fungi [4, 5]. The cost of cellulases is high due to the high cost of substrates used in production and the slow growth rate of fungi. Bacteria, which has higher growth rate as compared to fungi has good potential to be used in cellulase production. However, the application of bacteria in producing cellulase is not widely used. Bacterial cellulase usually lacks one of the three cellulase activities, i.e. FPase.

Cellulases are used in the textile [6], detergent [7], pulp and paper industries [8] etc. [9-12] Today, these enzymes account for approximately 20% of the world enzyme market, mostly from *Trichoderma* and *Aspergillus* species.

In this work, the cellulase production in *Aspergillus niger (NRRL – 322)* and *Trichoderma reesei (NCIM – 1052)* was reported. Also, the media optimization for the maximum enzyme production and the enzyme characterization were studied. Lyophilization of the crude cellulase enzyme was also carried out.

2. MATERIALS AND METHODS

2.1 Micro-organisms:

The micro – organisms used in the experiments were *Trichoderma reesei* (NCIM – 1052) obtained from National Collection of Industrial Microorganisms (NCIM), Pune, India and *Aspergillus niger* (NRRL – 322) obtained from Agricultural Research Service (ARS), USA.

2.2 Chemicals and reagents:

Potato dextrose agar (HIMEDIA, India), Yeast extract (MERCK, India), 3, 5 – Dinitrosalicylic acid (MERCK, India), citric acid (Fisher scientific, India), sodium acetate (MERCK, India), di-sodium hydrogen phosphate anhydrous (HIMEDIA, India) of analytical grade were used.

2.3 Shake flask studies:

Stock cultures of *Trichoderma reesei (NCIM 1052)* and *Aspergillus niger (NRRL 322)* were stored on potato dextrose agar, incubated at 26 °C for 120 h [13, 14]. A loopful of inoculum from the potato dextrose agar slant was used for further level production studies. Final production level was carried out in 1L flask containing 300mL production media with 10% inoculum, incubated at 150 rpm, 30 °C for 120 h.

2.4 Enzyme Extraction:

After incubation for 120 h, the culture was filtered using Whatman No. 1 filter paper and the filtrate was further centrifuged at 3000 rpm for 20 min. The insoluble materials and cell debris were separated and the crude enzyme was obtained. If required the crude enzyme was again filtered using cellulose nitrate filter paper (pore size 0.45 µm) [15]. The supernatant which contains the crude cellulase enzyme was stored at 4 °C and was used for further assay studies.

2.5 Enzyme Assay:

Filter paper activity (FPase) of cellulase in the culture filtrate was determined with a modification to the method described [16]. A 40 mg strip of filter paper was added to 0.1 ml of culture filtrate (crude enzyme extract) in 50 mM citrate buffer (pH 4.5). The reaction mixture was incubated at 50 °C for 1 h. The hydrolysis was terminated by addition of 1 mL DNS solution, followed by 10-15 min boiling. After cooling, 5 mL of distilled water was added and the absorbance was measured at 540 nm using glucose as standard.

One unit of cellulase (U) is equal to 1µg of reducing sugar released in terms of glucose per minute.

2.6 Media Optimization:

Four different media containing one of the following carbon source: wheat bran, cellulose, dextrose, groundnut were selected. The media components of each were: media (1) Wheat bran - 1%, Di-sodium hydrogen phosphate – 0.5% , Sodium chloride – 0.2%, Magnesium sulphate – 0.1%, dextrose – 0.1%; (2) cellulose – 1%, Di-sodium hydrogen phosphate – 0.5% , Sodium chloride – 0.2%, Magnesium sulphate – 0.1%, dextrose – 0.1%; (3) Dextrose – 0.6%, soya bean meal – 1%, Sodium chloride – 0.2%, Magnesium sulphate – 0.1%; (4) Groundnut – 2%, Di-sodium hydrogen phosphate – 0.5% , Sodium chloride – 0.2%, Magnesium sulphate – 0.1%, dextrose – 0.1%, cellulose – 0.2%.

2.7 Protein Assay:

Soluble protein was estimated by Lowry's method using bovine serum albumin (BSA) as standard [17].

2.8 Enzyme activity parameters:

2.8.1 pH:

Enzyme activity of cell – free crude enzyme extract was measured in various pH values (2, 4.8, 6, 8 and 10.5). Potassium chloride – Hydrochloric acid buffer for pH 2, Citrate – phosphate buffer for pH 4.8, Phosphate buffer for pH 6, 8, Carbonate–bicarbonate buffer for pH 10.5 were used.

2.8.2 Temperature:

For various temperature ranges (20, 40, 50, 60 and 80 °C) the enzyme activity was measured.

2.9 Lyophilization:

Lyophilization was carried out for the crude enzyme extract with drying parameters -50 °C and 0.1 mbar. The samples were pre-freezed at -20 °C overnight and were used for drying.

Two different formulations were made and the enzyme was lyophilized.

A. Sucrose 10%, Sodium chloride 3%, Ammonium sulphate 4%
B. Dextrose 15%, Sodium chloride 5%, Ammonium sulphate 4%

3. RESULTS:

The cellulase production in *Aspergillus niger* (NRRL – 322) was 23.3 U/mL whereas for *Trichoderma reesei* (NCIM – 1052) 38.75 U/mL was obtained. In media optimization, highest enzyme activity of 217.17 U/mL was obtained for groundnut containing media (media – 4) at 50 °C and pH 2. The lyophilized enzyme had an activity of 22.5 U/g and 12.166 U/g for sucrose (formulation A) and dextrose (formulation B) containing media respectively, at standard assay conditions.

4. DISCUSSION:

Enzyme activity of cellulase was analysed in both *A. niger* and *T. reesei*, which had 23.3 U/mL and 38.75 U/mL respectively in commercial media (potato dextrose) (Figure 1).

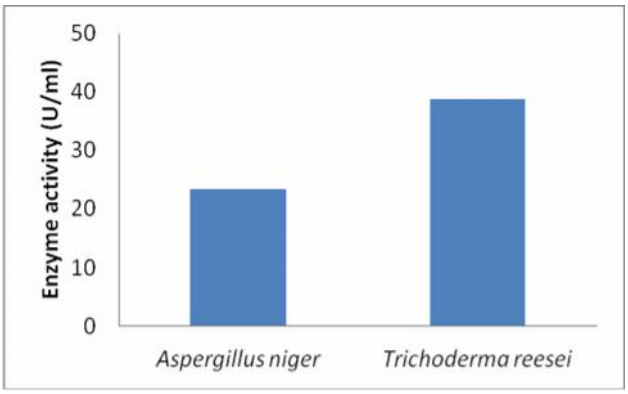

Figure 1: Cellulase production (Enzyme activity) of *Aspergillus niger* vs *Trichoderma reesei*

This result was contrary to the results obtained for Lee *et al*. where enzyme activity was 3.4 U/g and 2.2 U/g for *Aspergillus niger* and *Trichoderma reesei* respectively. This may be due to the changes in media composition where Lee *et al.* had used sugarcane bagasse and palm cake substrates [18].
In this experiment, since *T. reesei* had higher production (enzyme activity) than *A. niger*, *T. reesei* was taken up for further media optimization and enzyme production studies. Among different media chosen, groundnut containing media (media – 4) had the maximum enzyme activity of 43.067 U/mL (Figure 2).

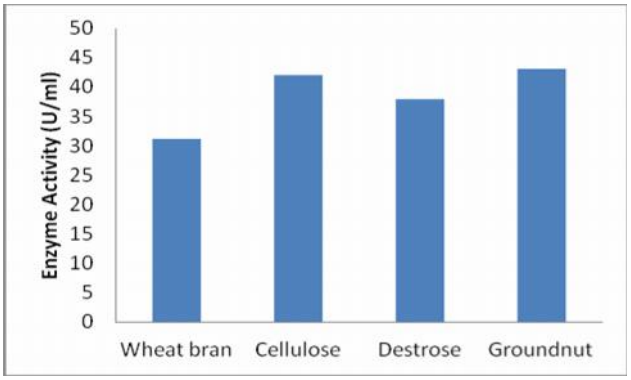

Figure 2: Cellulase activity of *Trichoderma reesei* in various substrate media

The enzyme activity of cellulose containing media (media – 2) was also almost equal to that of media – 4. But media – 2 was not taken for further studies since the cost of cellulose was higher compared to groundnut and also cellulose is a substrate to the production enzyme because of which more enzyme flux would be wasted in the growth phase itself that might lead to lower enzyme production quantities when used in large-scale levels. For cellulose based media, Guatam *et al*. had reported an activity of 1.99 U/mL for 1% substrate concentration [19]. Deshpande *et al*. had discussed that wheat bran had produced cellulase activity of 0.41 U/mL [20] while enzyme activity of 0.681 U/mL had been obtained for dextrose [19].
The enzyme activities of the media – 4 crude enzyme extract were assayed at various pH and temperature were studied. The maximum enzyme activity was obtained at pH 2 and the minimum was obtained at pH 6. Temperature profile (20 – 80 ºC) of cellulase activity for various pH ranges (2 – 10.5) was also studied (Table 1). Andrade *et al.* had reported an enzyme activity of 0.564 U/mL for 4% wheat bran media at temperature of 60 ºC and had an optimum pH of 3 [21].

Table 1: FPase activity for media – 4 enzyme extract at various pH and temperatures

pH	Temperature (ºC)				
	20	**40**	**50**	**60**	**80**
2	189.500	41.634	217.167	184.000	200.500
4	137.334	42.567	213.833	164.334	144.834
6	102.834	24.034	87.667	113.667	82.834
8	130.000	30.467	122.330	180.166	116.666
10.5	139.000	30.567	157.667	175.833	135.834

Optimum enzyme activity was obtained at temperature 50 ºC, pH 2 and lowest cellulase activity was observed at temperature of 40 ºC and pH 6. The reason for lowest activity at pH 6 may be due to the buffer used which might interfere with the enzyme and substrate reactions. But the exact reason for the same was not clear. The enzyme activity is more at lower and higher pH ranges whereas the enzyme activity declines at higher temperatures.
Lyophilized enzyme activity was higher for sucrose added formulation (22.5 U/g) whereas for the enzyme with dextrose the residual activity was very low (12.166 U/g) compared to sucrose and the loss on drying was 27.027% and 60.54% for formulation A and B respectively. There was considerable loss of enzyme activity after lyophilization for which further studies on additives has to be studied to prevent activity loss.

5. CONCLUSION:

From the results obtained, *Trichoderma reesei* (NCIM – 1052) was proved to be one of the maximum production organisms and groundnut based media can be used for cheap and efficient enzyme productions. Further large-scale studies have to be done for studying the stability and applicability of the cellulases and to maximize the yield of cellulases.

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