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RESEARCH ARTICLE

OPTIMIZATION OF PROCESS PARAMETERS FOR THE PRODUCTION OF CELLULASES BY *TRICHODERMA REESEI* USING AGROWATES AS A CARBON SOURCE

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ABSTRACT

The purpose of this study was to investigate the effect of agro-waste substrates like banana peel powder and coir powder at varying environmental parameters of pH (4-9) and temperature (20-50ºC) on the cellulase production by *Trichoderma reesei*. The enzyme production was measured by the amount of glucose liberated in IU/ml by using dinitrosalicylic acid method. The substrates were pretreated with alkaline pretreatment and autoclaved. The maximum activity of the enzyme was assayed at varying pH with temperatures being constant and varying temperatures with pH being constant. The maximum activity of the enzyme was assayed at varying pH was documented at pH 6 for banana peel powder (0.57±0.03 IU ml^{-1}) and coir powder (0.42±0.02) and the highest activity of the enzyme at varying temperature was recorded at 40ºC for both banana peel powder (0.65±0.02) and coir powder (0.47±0.002) after 144h of incubation. Thus the maximum amount of the enzyme was reported at pH 6 and temperature 40 ºC for both banana peel powder and coir powder. Moreover among the two substrates used for the production of cellulases by *Trichoderma reesei* banana peel powder exhibited maximum enzyme activity at 1.5% concentration (0.69±0.02 IU/ml) than coir powder as substrate.

INTRODUCTION

Cellulose is the most abundant organic compound in the biosphere and the major polysaccharide constituent of plant cell walls (1,2). It is a linear chain of approximately 8,000 to 12,000 glucose residues linked to one another by β-1, 4 glycosidic bonds (3). The cellulose fibers consist of crystalline and amorphous regions in alternate fashion. The crystaline regions are very rigid, formed by the parallel configuration of linear chains, which results in the formation of intermolecular hydrogen bonds, conducive to cellulose's insolubility and making it more unsusceptible to enzymatic hydrolysis. The amorphous regions are formed by cellulose chains with weaker organization, being more susceptible to enzymatic hydrolysis (4,5). With the increasing expansion of agro-industrial activities, large quantities of lignocellulosic residues are generated annually worldwide are treated as waste in many countries, and is disposed into environment without adequate treatment, raising environmental damage (6,7). Biotechnological innovations bring many significant and successful efforts to convert lignocellulosic residues into valuable products for biotechnological and industrial applications (8,9). Use of biowaste as a raw material give more flexibility and a broader application range because it would (a) Rely on biodegradable products that create less pollution and have fewer harmful environmental impacts (b) It adds to develop novel products that are not available from petroleum sources (c) Use of less expensive raw materials and (d) Reduce the dependence on fossil fuels. The cellulose content of biowaste materials has to be converted into sugars such as glucose that can be used as starting compounds in the biosynthesis of many useful bio-products. Therefore, the use of agro-industrial residues as carbon source for fungal growth and enzyme production has been reported in many studies as a way of significantly reducing process cost (10).

Although a large number of microorganisms are capable of degrading cellulose, only few of these produce significant quantities of cellulases, a multi component system capable of completely hydrolyzing crystalline cellulose *in vitro*. Fungi are the main cellulase producing microorganisms, although a few bacteria and actinomycetes have also been reported to yield cellulase activity (11,12). Filamentous fungi particularly *Trichoderma* and *Aspergillus* are the most distinguished producers of enzymes involved in the degradation of lignocellulosic materials, and the search for new strains displaying high potential enzyme production is of great biotechnological importance. Recently, research has been focused on the potential use of microbial cellulases for the degradation of lignocellulosic materials, aiming at the releasing of fermentable sugars that can be converted to second generation ethanol (13,14). Furthermore, the crystalline structure of cellulose prevents the action of microbial enzymes (15). In order to facilitate the enzymes

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access to the cellulose, several pretreatments of the lignocellulosic materials have been proposed, with the intention of disorganizing the plant cell wall structure and lignin removal (16). However, many physical, chemical and microbial pre-treatment methods for enhancing bioconversion of cellulosic materials have been reported (17,18,19,20). Several agro-industrial wastes are commonly used as raw material, such as sugarcane bagasse, wheat bran, corn cob and straw, rice straw and husk, soy bran, barley and coffee husk (7), but the studies related to the coir powder and banana peel powder as the major biowastes in coir industry and agriculture as a carbohydrate source on cellulase production is scanty. Therefore the present study was carried out to study the influence of banana peel powder and coir powder on *Trichoderma reesei* at varying pH with constant temperature and varying temperature with constant pH.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from ten different locations of the Kaza and Mangalagiri villages of Guntur district, Andhra Pradesh, India. Samples were collected from 6-10 cm depth and transported to the laboratory in sterile bags for further microbiological analysis.

Isolation

The soil was enriched by pretreating soil sample with 1% cellulose and incubated for 30 days at ambient temperature of 30ºC. The treated soil sample was suspended in sterile distilled water (1g in 100 ml), homogenized by vortexing and 0.1 ml of serially diluted sample (10^{-5} dilution) was spread over the surface of czapek-dox agar supplemented with streptomycin (25 µg/ml). After incubation of the plates at 28ºC for 7 days, typical fungal colonies selected on morphological basis were picked out, purified and preserved on potato dextrose agar medium at 4 ºC.

Screening for cellulase enzyme

The purified strains were screened for cellulolytic activity by inoculating the strains separately over modified Mandel's mineral agar medium[21] composed of (g/l) yeast extract-0.2g, CMC-10g, peptone-1g, $(NH_4)_2SO_4$-4g, KH_2PO_4-2g, Urea-0.3g , $MgSO_4.7H_2O$-0.3g, $Cacl_2$-0.3g , $FeSO_4.7H_2O$-0.5g , $MnSO_4.4H_2O$-0.16 g, $ZnSO_4$-0.14g , $CoCl_2$-2g , L-Sorbose-6g , Congo red- 0.025g , Tween 80-0.1% , Triton X 100-0.1% and agar-20g at pH 5. The inoculated plates were incubated at 28° C for 4 days. After incubation selective colonies of fungi were identified based on the diameter of zone produced on Mandel's agar plates after flooding with 1M NaCl solution for 15-20 min. Fungal colony producing wide zone was identified by 18S rRNA analysis.

Production medium and Cultural conditions

The strain presenting large clear zone on modified Mandel's mineral agar medium was used for enzyme production. To assay the cellulase activity, the pure culture of the strain was inoculated in the production medium contains Mandel's mineral salts solution (21) consisting (gl^{-1}) of urea-0.3g , $(NH_4)_2SO_4$-1.4g , K_2HPO_4-2g , $CaCl_2.2H_2O$-0.4g , $MgSO_4.7H_2O$-0.3g , Peptone-1g , yeast extract-0.25g , Maize steep liquor-10g , Cellulose-2g , $FeSO_4.7H_2O$-5 mg , $MnSO_4.7H_2O$- 1.6 mg, $ZnSO_4.7H_2O$-1.4 mg , $COCl_2.6H_2O$-2 mg and the fermentation was carried at 28ºC for 7 days. The culture filtrate was collected at regular intervals of 24 h and assayed for the cellulolytic activity.

Cellulase assay

The enzyme assay was based upon the procedure described by Miller (22). The reaction mixture containing 1.5 ml of 0.5% CMC, in 50 mM sodium phosphate buffer (pH 5) and 0.5 ml of the enzyme extract were incubated at 30ºC for 30 min. The reaction was terminated by the addition of 2 ml of DNS reagent followed by boiling. After cooling the developed color was read at 540 nm using spectrophotometer. The amount of released sugar was quantified using glucose as standard; hence one unit of enzyme activity was defined as the amount of enzyme releasing 1µmol of glucose equivalent from substrate per minute.

Optimization of cultural conditions for cellulase enzyme production

Influence of initial pH and incubation temperature on cellulase activity

The effect of initial pH and incubation temperature on enzyme activity of the strain was determined by adjusting the pH of the production medium ranging from 4 to 9 and temperature ranging between 25 to 55ºC. The optimal pH and temperature achieved was recorded with commercial cellulose as substrate.

Pre-treatment of substrates

To lessen the moisture content and to make them vulnerable to crushing, the substrates were sun dried individually and crushed. Crushed substrates were sieved to collect in the form of powder. The substrates were then soaked individually in 1% sodium hydroxide solution (NaOH) in the ratio 1: 10 (substrate: solution) for two hours at room temperature. After which, they were washed for free of chemicals and autoclaved at 121°C for one hour. The treated substrates were then filtered and washed with distilled water until the wash water becomes neutral (17).

Influence of bio waste substrates as carbon source on cellulase activity

To investigate the influence of bio waste substrates on cellulase activity, the production medium was supplemented with pretreated banana peel powder and coir powder each at a concentration of 1% as the sole carbon source with the other ingredients of medium remained same. The culture conditions were optimized to study the effect of varying pH (4, 5, 6, 7, 8 and 9) with constant temperature and varying temperatures (25, 30, 35, 40, 45, 50 and 55ºC) with constant pH on the cellulase activity of the fungal isolate. Influence of the actual concentration of best bio waste substrate (0.5-2.5%) supporting optimal yields of cellulase was recorded.

Statistical analysis

Data obtained on cellulase enzyme production under different cultural conditions were statistically analyzed with one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

On the basis of morphology and microscopic observations and also the 18S rRNA sequencing studies the fungus exhibited

good cellulolytic activity was identified as *Trichoderma reesei* SUK-3. The observations of the cultures were based on the characteristic features described by Larone (23).

Influence of initial pH and incubation temperature on cellulase production

The influence of pH on cellulase production was recorded by cultivating the strain at different pH levels between 4 and 9 (**Fig.1**). The enzyme activity increased gradually from pH 4 (0.74±0.01IUml^{-1}) and maximum production of enzyme was observed at pH 5 after 7th day of incubation (0.82±0.02 IUml^{-1}). There was a considerable decrease in the enzyme activity with the increase in pH 6 (0.70 ±0.01 IUml^{-1}), pH 7 (0.64±0.03 IUml^{-1}), pH 8 (0.57 ±0.02 IUml^{-1}) and pH 9 (0.45 ±0.02 IUml^{-1}). Gomes *et al.*(24) reported that the maximum cellulase activity of *Trichoderma* sp SUK-3 at pH levels between 4.5-5.0. Yang *et al.* (25) recorded that maximum production of cellulase at pH 4.5. Gautam *et al.* (26) also reported that the production of enzyme was high after 6th day of incubation (27) which is suitable from the commercial point of view.

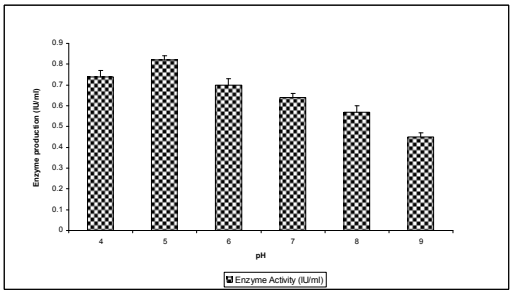

Fig 1: Effect of varying pH (4-9) on cellulase production by *Trichoderma reesei.* Data were statistically analyzed and found to be significant at 5%

Influence of temperature on cellulase production by the strain is presented in **Fig.2**. The strain cultured at different temperatures showed maximum yields of optimal temperature for cellulase production was reported at 30°C. The cellulase activity increased gradually from temperatures 25ºC (0.72±0.01IUml^{-1}) and reached maximum at 30ºC (0.87±0.02 IUml^{-1}). There was further decline in the enzyme activity with the increase in temperature 35ºC (0.78±0.02 IUml^{-1}), 40ºC (0.75±0.01IUml^{-1}), 45ºC (0.67±0.02 IUml^{-1}), 50ºC (0.55±0.01IUml^{-1}) and 55ºC (0.47±0.01IUml^{-1}) respectively. Many workers have reported different temperatures for maximum cellulase production either in flask or in fermentor studies using *Trichoderma* sp. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (28,29).

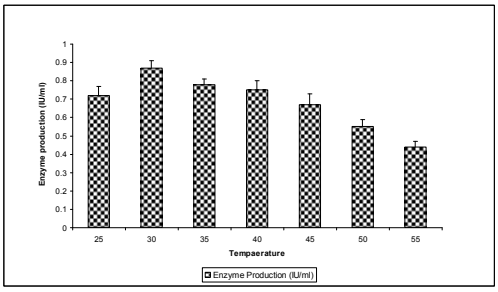

Fig 2: Effect of varying temperature (25-55ºC) on cellulase production by *Trichoderma reesei.* Data were statistically analyzed and found to be significant at 5%

In the present study 7 day old culture showed maximum enzyme activity when grown at pH 5 and temperature 30°C. The optimal incubation period for obtaining high yields of cellulases was found to be 144 h for *Trichoderma* sp. (30).

Influence of Coir powder on cellulase enzyme production

The influence of varying pH with constant temperature and varying temperature with constant pH on cellulase activity of the strain was determined after 7th day of incubation, with coir powder as substrate represented in **Fig. 3**. The cellulase activity recorded at varying pH 4, 5, 6, 7, 8 and 9 with constant temperature 30ºC was found to be 0.31±0.02, 0.39±0.03, 0.42±0.02, 0.35±0.03, 0.33±0.01, 0.27± 0.02 IU ml^{-1} respectively. Optimum pH for the cellulase activity was found at pH 6 (0.42±0.02) by using coir powder as substrate instead of cellulose. The activity of cellulase enzyme was optimized by incubating the flasks with the production medium at varying temperatures (20, 25, 30, 35, 40, 45 and 50º C) with constant pH 6. The cellulase activity was found to be 0.29±0.03, 0.34± 0.01, 0.38±0.03, 0.41± 0.01, 0.47±0.02, 0.32±0.03 and 0.25±0.02 IU ml^{-1} respectively **Fig. 4.** The optimum cellulase activity was found maximum at temperature 40º C with constant pH 6 after 7 days of incubation. Immanuel *et al.* (31) have reported that cellulase activity of *Aspergillus niger* was maximum at pH 5 (0.91IU/ml) with coir powder as the substrate but Mrudula and Murugammal,(32) have reported that the maximum activity of cellulase production was obtained at pH 6. These results are in agreement with the reports of Acharya *et al.*(33) and Devanathan *et al.*(34) who reported that the change in the physical parameters as pH will influence the enzyme production. In addition the Mrudula and Murugammal,(32) have reported that optimum temperature 30°C for the production of Cellulase by *Aspergillus niger*.

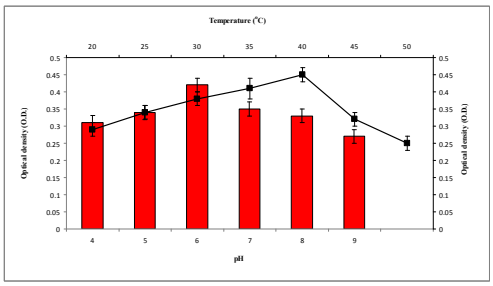

Fig 3: Effect of Coir powder on cellulase activity of *Trichoderma reesei* at varying pH with constant temperature and varying temperatures with constant pH. Data were statistically analyzed and found to be significant at 5%

Influence of Banana peel powder on cellulase enzyme production

The production of cellulase enzyme by *Trichoderma reesei* in the presence of banana peel powder as the carbon source showed maximum activity at pH 6 (0.57±0.03IU ml^{-1}) with temperature 30ºC constant after 7th day of incubation. The enzyme activity at pH 4 and 5 with temperature 30 ºC was (0.39± 0.01, 0.45± 0.02 IU ml^{-1}) showed decreased activity than at pH 6 , further increase in the pH above 6 showed reduced enzyme activity pH 7(0.51±0.01 IU ml^{-1}), pH 8 (0.49±0.03 IU ml^{-1}), pH 9 (0.43±0.02 IU ml^{-1}) (**Fig. 4**). In addition the activity of enzyme by the strain at varying temperatures using banana peel powder as the substrate exhibited maximum activity at 40ºC (0.65±0.02) (Fig 4) with

pH 6 constant after 7th day of incubation which was found to be optimum, further when the temperatures were increased 45ºC - 50ºC the enzyme activity decreased (0.55±0.02 and 0.51±0.03 IU ml^{-1}). Decrease in temperature below 40º C showed reduced enzyme activity at temperatures 20, 25, 30 and 35 ºC (0.37±0.02, 0.45±0.01, 0.51±0.03 and 0.60±0.02).

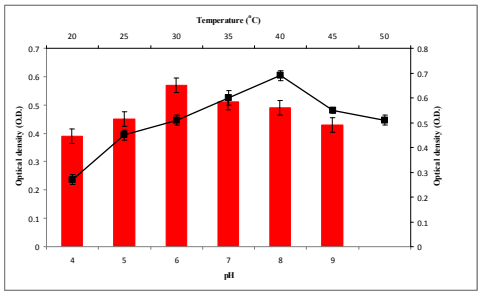

Fig 4: Effect of banana peel powder on cellulase activity of *Trichoderma reesei* at varying pH with constant temperature and varying temperatures with constant pH. Data were statistically analyzed and found to be significant at 5%

Baig *et al.* (35,36) and Omojasola *et al*. (37) have reported that the degradation of banana waste and pineapple peel by the cellulase produced by *Trichoderma lignorum and Trichoderma longibrachiatum* was maximum at pH 6. El-Hawary and Mostafa, (38) have also reported that the optimum temperature for the cellulase production by T*richoderma koningii* was found to be 25°C when CMC was used as the carbon source. But the production of celluase by *Trichoderma lignorum and Trichoderma longibrachiatum* was found to be 45°C as reported by Baig *et al.* [35,36] and Omojasola *et al.*(37) but Gautam *et al.*(39) have reported that the optimum temperature for enzyme production by *Trichoderma* sp. was 40°C.

Effect of substrate concentration on enzyme production

As banana peel powder emerged as the best carbon source for cellulase production by the strain, varying concentrations of banana peel powder (0.5%, 1%, 1.5%, 2% and 2.5%) tested to determine its optimal concentration, the maximum activity was observed at concentration of 1.5% (0.69±0.02 IU/ml). The enzyme production was low at concentration 0.5% (0.47±0.02 IU/ml), 1% (0.51±0.02 IU/ml), 2% (0.54±0.01 IU/ml) and 2.5% (0.41±0.03 IU/ml). As shown in Fig. 5, banana peel powder at 1.5% concentration exhibited optimal enzyme production.

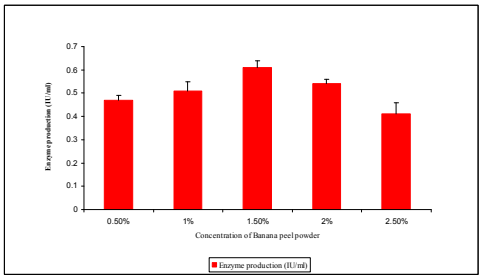

Fig 5: Effect of different concentrations of banana peel powder on cellulase activity of *Trichoderma reesei.* Data were statistically analyzed and found to be significant at 5%

The bio waste substrates such as banana peel powder and coir powder were amended individually in the production medium to determine their effect on cellulase enzyme production. Banana peel powder was found to be the best carbon source for cellulase production. However in the present work, maximum yields of cellulase were obtained with cheap and easily available carbon source which may be useful in the development of cost effective and high quality biotechnological processes for cellulase production. In the present study, *T.reesei* yielded maximum amount of cellulases in the medium containing banana peel powder as best carbon source with pH 6.0 and temperature 40ºC for 144h of incubation at 1.5% substrate concentration and further studies regarding the isolation and purification of cellulases produced by the strain are in progress.

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