

Optimization of Fermentation Parameters and Purification of Cellulase with Cellulose (Paper) as Substrate

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Abstract

A study was taken up to evaluate the role of some fermentation parameters like incubation period, temperature, pH and carbon source on cellulase production by submerged fermentation using cellulose as the substrate and *Trichoderma reesei* in the form of slant culture. Cellulose in the form of filter paper was used as the substrate for the production of cellulase. Temperature of 32°C, incubation period of 8 days, pH of 7 and carbon source as 25% starch and 75% glucose were found to be the optimum parameters for cellulase production. Cellulase produced using optimum parameters was purified using alcohol precipitation and ion exchange chromatography. From this study, we were able to estimate the optimum fermentation parameters required for cellulase production and carry out its purification

Keywords: Cellulase; Submerged fermentation; *Trichoderma reesei*; Optimized fermentation parameters; Ion exchange chromatography

Introduction

India is an agricultural based country and produces a lot of agriculture waste per year. Examples of agricultural waste and forest waste in India include all lignocellulosic materials like rice husk, cotton waste, rice straw, sugarcane bagasse, paper and pulp waste etc. Lignocellulose is the most abundant renewable biological resource and thus the most promising feedstock for the production of enzymes, chemicals, food and energy. The bioconversion of cellulosic materials is highly economical and leads to the development of large-scale processes beneficial to mankind. Formation of soluble sugars from cellulose in paper/agricultural residues depends on the coordinated action of individual components such as -exoglucanase, -endoglucanase, -glucosidase of cellulase enzyme. The degradation of cellulose into glucose molecules by cellulase enzyme is detected by the presence of free carboxyl group (DNS assay). In this study, production of cellulase enzyme with cellulose in the form of filter paper or paper waste as the substrate was carried out and the fungal species *Trichoderma reesei* was used in the fermentation of the media components. *T. reesei* was used because of its physiological, enzymological and biochemical properties. Certain fermentation parameters such as temperature, incubation period, carbon source, pH etc play an important role in obtaining good cellulase yield. The present study was planned with the objective of optimizing the fermentation parameters for maximising cellulase production using *T. reesei* and also its purification.

Materials and Methods

Trichoderma reesei Rut C-30 obtained from Institute of microbial technology (IMTECH), Chandigarh, India was used in the study. plate as well as slant culture of *T. reesei* was prepared by inoculating the organism in a sterilized Petri plate and slant tube respectively containing Malt extract agar. Sterilization in an autoclave at 15 psi for 20 min and the inoculum was cooled before inoculation was carried out. Malt extract agar was prepared by dissolving 2 g of Malt Extract powder and 2 g of Agar-agar in 100 mL distilled water. Potato dextrose agar also proved to be effective. The plates (or tubes) were incubated in laminar air flow chamber for 48 hrs. Colonies of *Trichoderma* initially green in colour, and later brown, appeared in the plates.

A flask culture of *T. reesei* was prepared by inoculating from slant culture/plate culture. Malt extract medium was prepared by dissolving 2 g of malt extract powder in 100 mL of distilled water. The medium was sterilized and inoculated with 2% (w/v) of inoculum and left overnight in shaker at 100 rpm speed and 32°C.

A final production media having the following composition was prepared for the quantitative analysis. To 100mL of distilled water, 1.5 g Malt extract, 0.5 g ammonium nitrate or ammonium sulphate, 2.7 g glucose, 100 mg magnesium sulphate, 100 mg manganese sulphate, 100 mg calcium chloride, 100 mg zinc sulphate, 100 mg ferrous sulphate, 0.5 g di-potassium hydrogen phosphate and 0.1 mL Tween-80 were added and

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the media components were mixed well and autoclaved at 15 psi for 20 min, cooled to room temperature and inoculated with 10 mL of fungal culture per every 200 mL of the liquid medium and the flasks were kept in the rotary shaker at 32°C for 8 days. For optimization, 200 mL of media was taken in 5-6 different shake flasks and incubated at different fermentation parameter conditions. The samples were drawn from the flask under sterile conditions from 24 hrs onwards after inoculation and analysed for filter paper activity. The samples were centrifuged at 5000 rpm for 10 min and the supernatant was analysed for the enzyme activity. Un-inoculated liquid medium was used as blank solution. This method tests the presence of free carboxyl group, the so-called reducing sugar. This involves the oxidation of the aldehyde functional group present in glucose and ketone functional group in fructose and simultaneously 3,5-dinitrosalysilic acid (DNS) is reduced to 3-amino,5-nitrosalysilic acid under alkaline conditions. One mole of sugar react with one mole of 3,5-DNS. Because dissolved oxygen can interfere with glucose oxidation, sulphite, which itself is not necessary for the colour reaction is added in the reagent to absorb the dissolved oxygen. The type of side reaction depends on the exact nature of the reducing sugars.

Assay of cellulase enzyme from *T. reesei* was carried out using the following protocol: (DNS assay).

- The medium was centrifuged at 5000 rpm for 10 min.
- 3 mL of the supernatant was taken with 3 mL of citrate buffer (sodium citrate/ citrate phosphate-pH 6.5)
- 0.5 g of substrate (cellulase filter paper) was added and incubated for 1 hr at 50°C
- 3 mL of the solution was taken with 3 mL of DNS present in the reagent and heated at 95°C for 5 min.
- The solution was cooled and 1 mL of Rochelle's salt (40%) was added.
- The Optical Density values were measured at 540 nm with a solution of DNS and Rochelle's salt as blank.

Results

Optimum conditions were found to be: Temperature 32°C, Incubation period 7 days, pH 7 and carbon source 25% starch and 75% glucose.

Discussion

Effect of different operating temperatures is shown in Figure (1). The maximum enzyme production was found to be at 32°C as against maximum temperature of 35°C–45°C using *A. fumigatus* (Sherief *et al.*, 2010). As shown in Figure 1 cellulase production is typically temperature dependent. The rate of reducing sugar consumption is highest at 29°C and lowest at 32°C throughout the process. Cellulase production increased with increase in temperature from 25°C to 32°C upto 7 days after which it declined. The enzyme production decreased with further increase in temperature from 32°C. Thus, the optimum temperature for the fermentation of media components using *T. reesei* was found to be 32°C. At temperatures lower or higher than that of optimum, less enzyme production was observed. Decline in enzyme production at higher temperatures might be due to denaturation of

enzyme or its inactivation at higher temperatures. Therefore, the subsequent experiments were conducted at an incubation temperature of 32°C. *Trichoderma reesei* Rut C-30 was grown in liquid culture for 8 days using glucose and malt extract as the major carbon source. The enzyme activity increased regularly starting from day 1 and reached the highest on day 7 and started to decrease after 7 days i.e from day 8 of fermentation. Thus, the maximum enzyme yield was on the 7th day after inoculation. Therefore, subsequent experiments were carried out at incubation period of 7 days. But in other experiments where *A. fumigatus* was used, the maximum enzyme activity was obtained on the 2nd day (Sherief *et al.*, 2010).

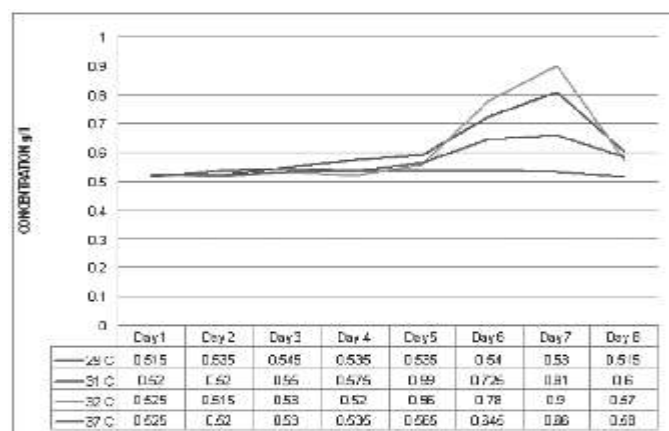


Figure 1. Effect of different temperature and incubation period on enzyme production

The effect of different pH conditions is shown in Figure (2). The maximum enzyme production was found to be at pH 7. As shown in Figure (2), cellulase production is pH dependent. Cellulase production increased with increase in pH from 5 to 7 up to a period of 7 days after which it declined. The enzyme production decreased with further increase in pH from 7. Thus, the optimum, pH for the fermentation of media components was found to be 7 with maximum enzyme yield on 7th day of incubation as against with optimum pH of 6 using *A. fumigates* (Sherief *et al.*, 2010). At pH lower or higher than that of optimum, less enzyme production was observed. Therefore, subsequent experiments were carried out with optimum pH of 7.

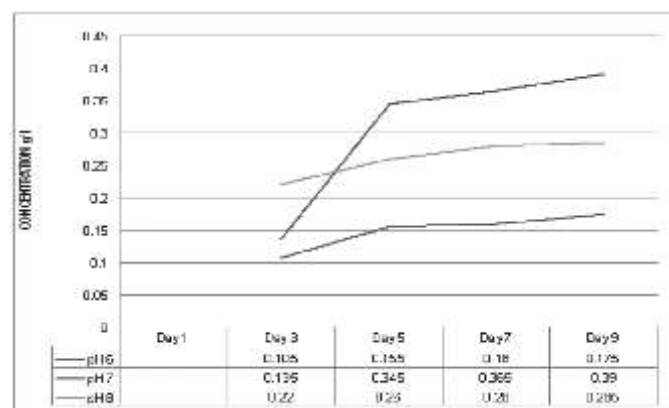


Figure 2. Effect of different pH on enzyme production

The effect of different media components on enzyme production is shown in Figure (3). The maximum enzyme production was obtained using the carbon source glucose in combination with starch. As shown in Figure (3), cellulase production is dependent on the concentration of carbon source in the production media. Cellulase production using a combination of glucose and starch as the carbon source increased upto a period of 7 days after which it declined. For other sources of carbon (glucose, glucose+potato and glucose+barley) cellulase production was found to be less when compared to the glucose+starch combination. Thus, the optimum, carbon source for the fermentation of media components using *T. reesie* was found to be glucose in combination with starch with maximum enzyme yield at 7th day of incubation. For other carbon combinations other than that of optimum, less enzyme production was observed. Maximum production of enzyme using glucose and starch as the carbon source may be due to immediate uptake of energy.

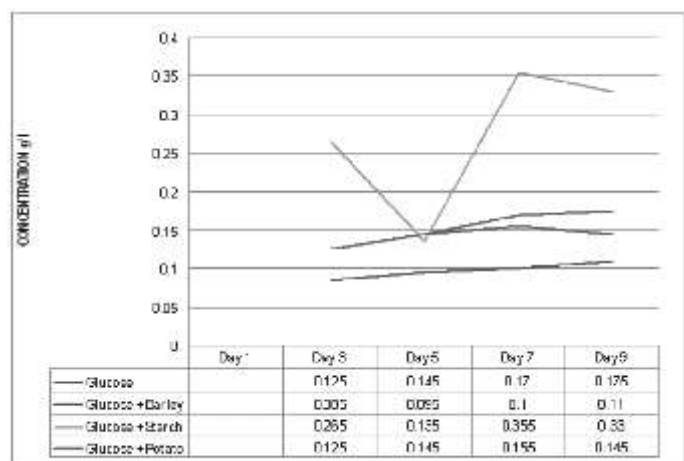


Figure 3. Effect of different carbon source on enzyme production

Effect of different concentrations of the carbon source on enzyme production is shown in Figure (4). The maximum enzyme production was obtained using the carbon source 75% glucose in combination with 25% starch. As shown in Figure (4) cellulase production is dependent on the concentration of carbon source in the production media. Cellulase production using the combination of 75% glucose and 25% starch (in the ratio 3:1) as the carbon source increased upto a period of 7 days after which it declined. For other combinations of glucose and starch, cellulase production was found to be comparatively less. Thus, the optimum carbon source for the fermentation of media components using *T. reesie* was found to be glucose 75% in combination with starch 25% with maximum enzyme yield at 7th day of incubation. For other carbon concentrations other than that of optimum, less enzyme production was observed.

Trichoderma reesie Rut C-30 was grown in liquid culture for 8 days using 75% glucose and 25% starch as the major carbon source and the other optimised parameters. The enzyme activity increased regularly starting at day1 to day7 with maximum at day 7 and started to decline after day 7 add for alcohol precipitation before 100 mL of crude enzyme was mixed with 500 mL of ethyl alcohol and precipitated for an hour. After an hour the precipitate was centrifuged at 5000 rpm for 10 min and the precipitated enzyme was refrigerated at 4°C. For ion exchange chromatography, the column was equilibrated with 50 mM Tris-HCl buffer (pH 7.4). 8.5 mL of clear crude extract was loaded onto a DEAE-

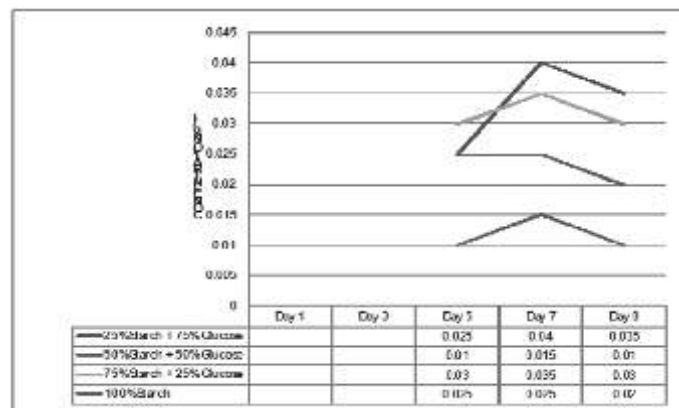


Figure 4. Effect of different concentration of carbon source on enzyme production

Sephacrose Fast Flow anion-exchange column. The cellulase was eluted in 50 mM Tris-HCl buffer (pH 7.4) with a 0.1–0.5 M NaCl gradient. Fractions (1.5 mL) were collected and cellulase activity was tested by detecting its overall hydrolytic activity over selected substrates.

Conclusion

From this study, we were able to establish that lignocellulosic waste such as paper) which have not been exploited commercially for any industrial application and are poorly disposed could effectively be used as substrate for cellulase production through the process of fermentation. could be scaled up to a pilot scale or commercial fermenter level thereby making the process more cost effective.

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