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Optimization of solid state fermentation conditions for the production of cellulase by *Trichoderma reesei*

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Abstract

Cellulase production studies have been carried out using the fungal strain *Trichoderma reesei* NCIM 992 by using three different lignocellulosic materials by solid state fermentation (SSF). The effect of basic fermentation parameters (pH, temperature, moisture content, particle size of substrate and moistening agent) on enzyme production was studied. Maximum cellulase production was 2.63 U ml^{-1} using wheat bran as substrate. The optimal conditions for cellulase production for wheat bran were found to be: initial moisture content-70%, initial medium pH-5.0, temperature-30°C, moistening agents (MSS) and particle size of substrate (500 µm).The optimal incubation time for production was six days. Results indicate the scope for further optimization of the production conditions to obtain higher cellulase titres using the strain under SSF.

Key words

Cellulase, *Trichoderma reesei*, Solid state fermentation, Lignocellulosic material, Wheat bran

Introduction

Cellulose is a natural bio-polymer and a potentially important source for the production of industrially useful materials such as fuels and chemicals. Cellulosic materials can be directly converted into biofuel by using chemicals, enzymes or by the combination of both (Van Zessen *et al.*, 2003; Knauf *et al.*, 2004). Chemical method is unfavorable and uneconomical in comparison to the enzymatic hydrolyses because it produces more byproducts at high temperatures and due to environmental problems (Singhania *et al.*, 2006).

There are so many micro-organisms which have capability of producing cellulases. Successful utilization of cellulosic materials as renewable carbon sources are dependent on the development of economically feasible process technologies for cellulase production and for the enzymatic hydrolysis of cellulosic materials to low molecular weight products such as hexoses and pentoses (Chahal *et al.*, 1992; Reczey *et al.*, 1996; Aristidou *et al.*, 2000).

Cellulase is a multi-enzyme which is formed by several proteins. It catalyzes the conversion of cellulose to glucose in an enzymatic hydrolysis-based biomass to ethanol process (Kotchoni *et al.*, 2003). Production of cellulase enzyme proteins in large quantities using the fungus *Trichoderma reesei* requires study of the dynamics of growth and enzyme production (Lejeune *et al.*, 1995). It has extensive applications in food, fermentation and textile industries (Aristidou and Penttilä, 2000). *Trichoderma reesei* is an efficient producer of cellulase protein. Various strains of bacteria (aerobic species such as *Pseudomonas* and *Actinomycetes,* facultative anaerobes such as *Bacillus* and *Cellulomonas* and strict anaerobes such as *Clostridium)* and fungi (*Trichoderma reesei*, *Aspergillus niger* and *Trichoderma viride*) produce cellulase (Lejeune *et al.*, 1995). But the sharply increased demand of fungal cellulase is due to its greater yield, facilitated crystallization and higher degree of purity. Solid state cultivation for production of cellulase using fungal strain of *Trichoderma reesei* has many advantages over submerged fermentation. Its advantages include

superior volumetric productivity, higher concentration of product to facilitate recovery, simplicity of process operation, easier contamination control, less solvent for recovery of the product which significantly reduce the cost of down stream processing (Esterbauer *et al.*,1991; Holker *et al.*, 2004).

The objective of the present study was to optimize various factors like moistening agents, level of moisture content in solid matrix, particle size of substrate, pH and temperature for maximum yield of cellulase in solid state fermentation (SSF) using cheaper renewable wheat bran as substrate by fungal strain *Trichoderma reesei* NCIM 992.

Materials and Methods

Maintenance of culture: *Trichoderma reesei* NCIM 992 used in this study were obtained from the National Collection for Industrial Microorganism, NCL Pune, India. It was grown and maintained at 28° and 4°C on potato dextrose agar slants. Inoculum (seed culture) was prepared by transferring cells from a fresh agar slant into 250 ml Erlenmeyer shake flask, containing 50 ml of the culture medium. Culture was maintained on agar slant at 4°C and sub-cultured twice a month. The pH of the medium was kept at 5.0 and sterilized at 121°C (15 psi) for 20 min, incubated at 28°C for at least 6 days depending upon the growth of the culture.

Solid state fermentation (SSF) and optimization of parameters for enzyme production: The seed culture from the slant was transferred into a 250 ml Erlenmeyer flask containing 50 ml medium inoculated from a fresh agar slant, and incubated at 30 ± 0.2°C an a rotary shaker at 150 rpm. The cells were grown for 48 hr (until the onset of the stationary phase), after which 5.0 ml of this culture was transferred to 100 ml medium of the same composition in 500 ml Erlenmeyer flasks. Similarly, the desired inoculum volume was developed by subsequent transfers (Chahal *et al.*, 1985; Correa *et al.*, 1997).

The medium used for inoculum preparation contained (g l^{-1}): KH_2PO_4, 28; $(NH_4)_2SO_4$, 19.6; Urea, 4.2; $MgSO_4.7H_20$, 4.2; $CoCl_2$, 4.2; $FeSO_4.7H_20$, 0.07; MnSO4. $7H_20$, 0.021; $ZnSO_4\ 7H_20$, 0.019; $CaC1_2$, 0.028; yeast extract, 7; and glucose, 15; pH 5.0 ± 0.2. The media were sterilized by autoclaving at 121°C pressure of 15 psi for 15 min. The culture was incubated and shaken at 30°C for 48 hr in an orbital shaking incubator at 150 rpm before transferring to the production medium (Duenas *et al.*, 1995).

Ten gram wheat bran moistened with 25 ml mineral salt solution (pH: 5 to 5.2) was taken in 500 ml Erlenmeyer flask and sterilized at 121°C at 15 psi for 15 min by autoclaving. The fermentation was continued for 7 to 8 days and flasks were withdrawn at regular interval of 1 day for further analysis. The enzyme was extracted from fermented bran by 50 ml of citrate buffer (pH 4.8). The pooled extract was filtered with muslin cloth and centrifuged at 3000-3500 rpm for further clarification and then used for enzyme assay (Singhania *et al.*, 2006).

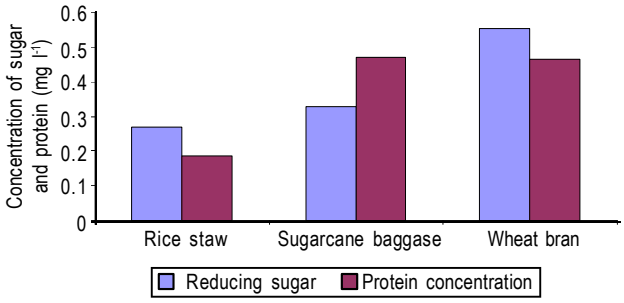

Fig. 1: Screening of agro-industrial residues for cellulase production at carbohydrate rather than protein to get free reducing sugar

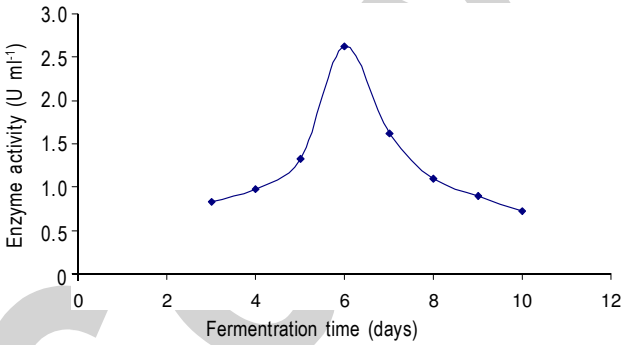

Fig. 2: Fermenting mixture consist 1:1 ratio of wheat bran and mineral salt solution. Maximum production of enzyme was obtained during 6 days incubation

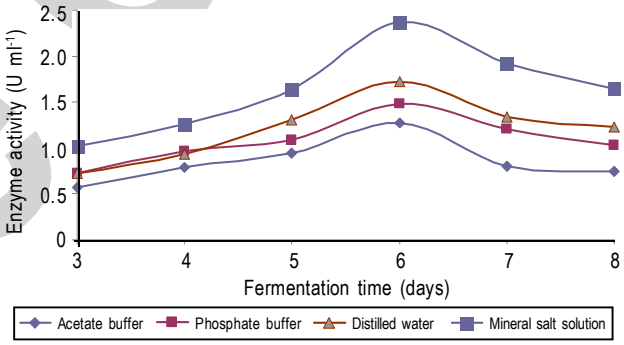

Fig. 3: Ingredient of mineral salt solution enhance the cellulase production at 30 °C and pH 5 because inorganic component actively participate in metabolic reaction of *Trichoderma reesei*

For optimization of process parameters, the composition of the wetting solution was varied to the desired levels of component or the incubation conditions where changes and enzyme production was studied under the varied conditions. The parameters studied were initial moisture level (40,50,60,70,80 and 90%), *p*H (3.0,4.0,5.0,6.0 and 7.0), incubation temperature (25,30,35,40 and 45°C), moistening agents (acetate buffer, phosphate buffer, distilled water and mineral salt solution) and particle size of substrate (1000,800,600,500 and < 500 μm).

Analysis: Cellulase activity was analyzed by the carboxymethyl cellulase (CMCase) assay. A 0.5 ml of 2% buffered carboxymethyl cellulose (CMCase) solution (sodium citrate buffer 0.2 M, pH 4.8) was incubated with 0.5 ml of the enzyme preparation and 0.5 ml of phosphate buffer for 30 min at 50°C. The liberated glucose was estimated by 3, 5-dinitrosalicyclic acid (DNS) method (Miller, 1959). The amount of glucose (reducing sugar) produced was estimated from the standard plot of glucose solution. One unit of cellulase activity was defined as the amount of enzyme required to liberate one µmole of glucose min^{-1} ml^{-1} under the standard assay conditions (Chaudhuri *et al.*, 1993).

Results and Discussion

Initial screening of the various substrates for their potential to support cellulase production indicated that among the tested substrates, wheat bran was found to be the best (protein content: 0.465 mg ml^{-1} and reducing sugar: 0.544 mg ml^{-1}). Rice straw was found to have least sugar content (reducing sugars), while it was highest for the wheat bran (Fig. 1).

The enzyme activity of the selected strains *Trichoderma reesei* NCIM 992, used for enzyme production is shown in Fig. 2. The result indicates maximum production of enzyme was obtained by SSF yielding 2.63 U ml^{-1} during incubation time of 6 days (Das *et al.*, 2008).

The wheat bran was supplemented with suitable moistening agents to meet the water requirement and additional nutrients to the growing cultures. The moistening agents such as distill water, acetate and phosphate buffer and mineral salt solution (starch, 5%; yeast extract, 1%; and $CaCl_2$. $2H_2O$, $CoCl_2$ 2 H_2O, $MgSO_4$.7 H_2O and KH_2PO_4 0.2% each) were used to supplement 10g of wheat bran. The Fig. 3 shows that the maximum enzyme production of 2.26 U ml^{-1} was obtained using mineral salt solution and enzyme production was influenced by additional proportionate amount of mineral ions with complex nutrients.

The moisture level of culture medium affects the physiology of the microorganism therefore it was desired to find out the optimum moisture level of the medium for the enzyme production. The enzyme

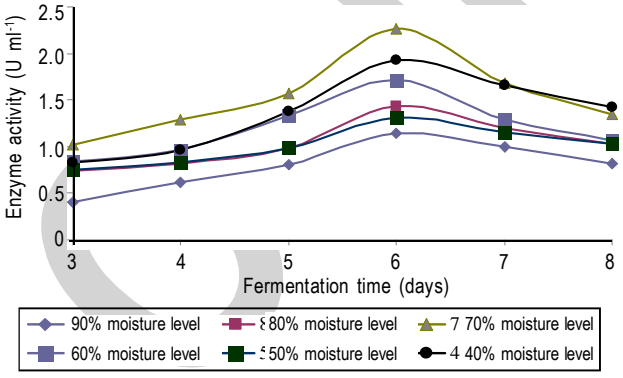

Fig. 4: Optimization of initial moisture level on cellulase production by *Trichoderma reesei* at 30 °C and pH 5 exhibit optimum cellulase productions

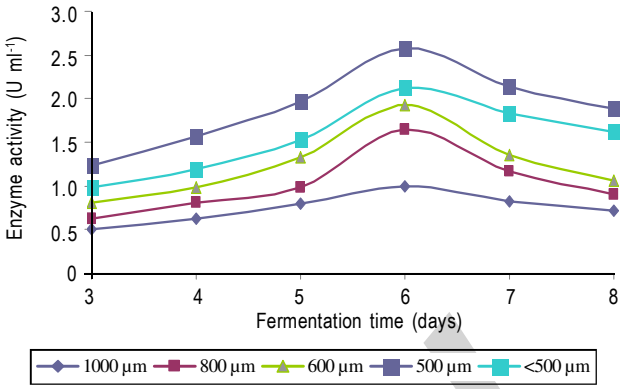

Fig. 5: Effect of particle size (500µm) show maximum cellulase production by *Trichoderma reesei* at 30 °C and pH 5

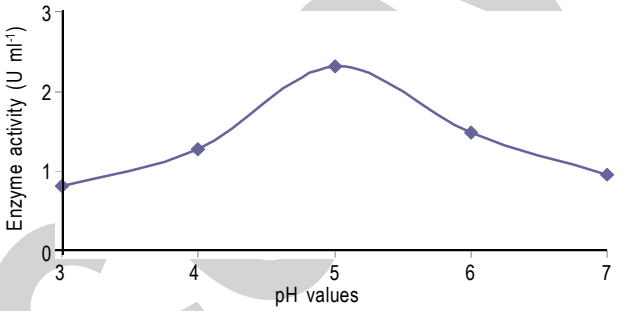

Fig. 6: Optimization of initial medium pH in SSF consisting 1:1 ratio of wheat bran and mineral salt solution at 30°C after 6 days incubation period show maximum cellulase activity at pH 5

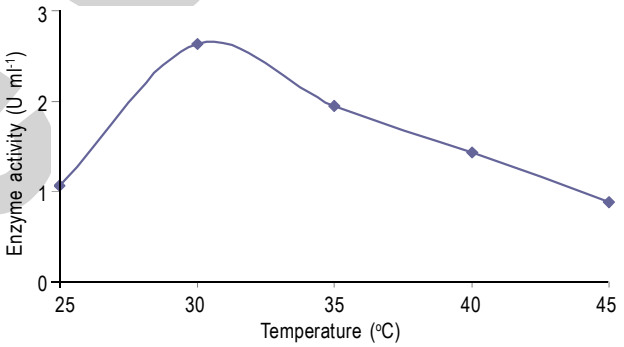

Fig. 7: Optimization of incubation temperature may be affected by cellulase production media. Higher and lower temperature above and below the optimum level either denature or deactivate the enzyme activity

production with moisture level varying from 40-90% in wheat bran medium moistened with mineral salts solution is shown in Fig. 4. The maximum yield of the enzyme 2.29 U ml^{-1} was obtained at 70% moisture level. Mekala *et al.* (2008) showed that at high moisture level (70%) the substrate prevents oxygen penetration and facilitates the contamination, whereas the low moisture level inhibits the growth, enzyme activity and accessibility to nutrients.

The particle size of wheat bran ranging from 500 to 1000 µm were used for enzyme production in solid state culture under identical conditions (Mekala *et al.*, 2008). The Fig.5 shows that the enzyme yield varied with wheat bran size and optimum enzyme

yield of 2.83 U ml^{-1} was obtained for particle size of 500 µm, whereas the enzyme yield was found to be reduced for larger particles (>500 µm) as well as for smaller particles (<500 µm). It also indicates that the smaller size bran provides large surface area which helps to mix bran with the microorganisms and other nutrients. Small particle size may lead to clumping of bran, resulting in reduced accessibility to nutrients anaerobic cultural conditions with lower yield of the enzyme. The similar observations on the effect of particle size on enzyme production in solid state cultures have been reported by Acebal *et al.* (1988).

The effect of pH on enzyme production was studied by adjusting the pH of the moistening agents (mineral salt solution) between 3 to 7 as shown in Fig.6. It indicates that the enzyme production was favored in acidic range of pH 4 to 6 with optimum enzyme yield of 2.32 U ml^{-1} at pH 5.2. Das *et al.* (2008) also observed cellulase activity was optimum at pH 4.8. The variation of pH from the optimum level causes denaturation of the enzyme and reduces enzyme synthesis ability.

The effect of temperature on enzyme production was investigated by incubating the flask at temperature between 25 - 45°C as shown in Fig.7. At 30°C, the maximum enzyme yield was 2.63 U ml^{-1}, where as the enzyme yield was reduced to 0.876 U ml^{-1} at temperature of 45°C with significant reduction in the microbial growth. Similarly, at lower temperature of 25°C the yield was reduced to 1.07 U ml^{-1}. Singhania *et al.* (2006) studied 28°C temperature was suitable for cellulase production. It indicates that cellulase is highly sensitive towards temperature and high temperature decreases the growth of micro organism and enzyme production.

The cost-effective technologies are needed for the production of enzyme and SSF is a suitable technology for economical production of cellulases using lignocellulosic residues as substrate. Major parameters affecting the fermentation process for enzyme production were studied and optimal levels were identified. It is concluded from the findings that the strategy to produce cellulase from wheat bran was successful as it resulted in a considerably good amount this enzyme produced by newly strain NCIM 992 under laboratory conditions. Furthermore, evolutionary operation factorial-design technique could be considerably effective in maximizing the yield of enzyme but all the parameter was optimized by one at a time method.

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