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Solid-state fermentation of lignocellulosic substrates for cellulase production by *Trichoderma reesei* NRRL 11460

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Cellulase production studies were carried out using the fungal culture *Trichoderma reesei* NRRL 11460 using four different lignocellulosic residues (both raw and pre-treated) by solid-state fermentation. The effect of basic fermentation parameters on enzyme production was studied. Maximal cellulase production obtained was 154.58 U/gds when pre-treated sugarcane bagasse (PSCB) was used as substrate. The optimal conditions for cellulase production using PSCB were found to be initial moisture content - 66%, initial medium *p*H-7.0, incubation temperature -28°C, NH_4NO_3 at 0.075 *M*, and 0.005 *M* cellobiose. The optimal incubation time for production was 72 h. Results indicate the scope for further optimization of the production conditions to obtain higher cellulase titres using the strain under SSF.

Keywords: cellulase, endoglucanse, *Trichoderma*, solid-state fermentation, lignocellulosic residues, sugarcane bagasse

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Introduction

Cellulose is the most abundant and renewable biopolymer on earth. It is the dominating waste material from agriculture and constitutes the wastes generated from both natural and man-made activities[1]. The need for utilizing renewable resources to meet the future demand for food and fuel has increased the attention on cellulose as the only foreseeable sustainable source of fuel available to humanity[2]. The major obstacle to the widespread utilization of this important resource is the absence of economically feasible technologies for overcoming the recalcitrance of cellulosic biomass. Hence, there is a considerable economic interest in the development of processes for effective pre-treatment and utilization of cellulosic wastes as inexpensive carbohydrate sources. A promising strategy for efficient utilization of this renewable resource is the enzymatic hydrolysis of lignocellulosic waste and fermentation of the resultant reducing sugars for production of desired metabolites or biofuel. The growing concerns about shortage of fossil fuels, the emission of greenhouse gases and air pollution by incomplete combustion of fossil fuel has also resulted in an increased focus on production of bioethanol from lignocellulosics[3-4] and especially the possibility to use cellulases and hemicellulases to perform enzymatic hydrolysis of the lignocellulosic material[5-6].

The major bottleneck in the development of such technologies is the cost of cellulase preparations, which are currently prohibitive to be economically used for the hydrolysis of lignocellulosics[7]. Consequently, there is a perpetual interest in finding hyperactive enzymes, the improvement of existing enzymes and the optimization of strategies for cost effective production of cellulases. The ability to secrete large amounts of extracellular cellulases is characteristic of certain fungi of the genus *Trichoderma* and they are regarded as most suited for the production of higher levels of extracellular cellulases. The most extensively studied fungus is *Trichoderma reesei* and the cellulase complex of *T. reesei* has been the most studied cellulase system as it converts native cellulose as well as derived cellulose to glucose[2]. Majority of the reports on microbial production of cellulases by *T. reesei* utilize the submerged fermentation (SmF) technology. Solid-state fermentation (SSF) for the production of cellulases is rapidly gaining interest as a cost-effective technology for the production of enzyme and higher yields of cellulase is reported under SSF compared to liquid cultures[8-9].

The substrates used by many researchers for cellulase production are pure forms of cellulose such as Avicel, Solka Floc, and Cotton[10-11], all of them are

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expensive feedstock for the large-scale production of cellulases. The cost of raw material is the limiting factor in developing an economic process for cellulase production[12-13]. The economy of cellulase production could be improved by the use of cheaper cellulosic substrates for enzyme production[5-10]. Agro-industrial residues and wastes are potent sources of cellulose and as such, can support the growth of cellulolytic microorganisms and the production of cellulases. This makes such residues the preferred substrates in SSF for the production of enzyme. The current study has been attempted to use agro-industrial waste as raw material for the production of cellulose using cellulose hyper producing fungus, *T. reesei* NRRL 11460, and the influence of various parameters were evaluated to design a suitable SSF process for cellulase production.

Materials and Methods

Solid Substrates and Pre-treatment

Four different agro-industrial residues-cassava bagasse, sugarcane bagasse, wheat bran and rice straw were tried as substrates for cellulase production with or without pre-treatment. The substrates were pre-treated using a 0.1 N NaOH solution for 12 h wherever indicated.

Culture Conditions

Trichoderma reesei NRRL 11460 was obtained from USDA Northern Regional Research Laboratories (Illinois, USA) and was maintained on potato dextrose agar (HiMedia, India) slants, at 30°C and subcultured every week. Fully sporulated cultures obtained after 7 d were either preserved at 4°C or were subcultured onto fresh PDA slants. For inoculum preparation, fully sporulated cultures in PDA slants were used. Distilled water (5 mL) was added to the slants and the spores were dislodged using an inoculation needle under aseptic conditions. The suspension was appropriately diluted and was used as the inoculum.

Solid-state Fermentation and Optimization of Parameters for Enzyme Production

The solid substrate (5 g) was weighed in 250 mL Erlenmeyer flasks and wetted with a mineral salt solution containing (g/L): $(NH_4)_2SO_4$, 10; KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.5; $CaCl_2.H_2O$, 0.5; and distilled water was added to obtain the desired initial moisture content. The contents were sterilized and were inoculated with 1.4×10^9 spores per flask. The flasks were incubated for 120 h at 30°C and initial moisture content of 60% unless otherwise mentioned. Samples were withdrawn every 24 h as whole flasks and enzyme extraction was performed using simple contact method. Phosphate buffer (0.2 *M*, *p*H 7.0) was added to the fermented substrate to a total volume of 100 mL and mixed for 1 h on a rotary shaker. The suspension was filtered and centrifuged and the supernatant was used as the crude enzyme preparation for assay of enzyme activity.

For optimization of process parameters, the composition of the wetting solution was varied to the desired levels of components or the incubation conditions were changed and enzyme production was studied under the varied conditions. The parameters studied were type of substrate-raw or pre-treated cassava bagasse (CB/PCB), sugarcane bagasse (SCB/PSCB), wheat bran (WB/PWB), and rice straw (RS/PRS), initial moisture content (45.2, 56.1, 66.4, 74.2 and 85.4%), *p*H (5, 6, 7, 8 and 9), incubation temperature (28, 30, 35 and 37°C), N source [NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$, and $NaNO_3$], and inducer (cellobiose) concentration (0.001, 0.005, and 0.01 *M*).

Analytical Methods

Cellulase activity was analyzed by the filter paper assay. Fifty milligrams of Whatman No. 1 filter paper (~6×1 cm strip) was incubated with 0.5 mL of the enzyme preparation and 0.5 mL of phosphate buffer for 1 h at 50°C. The glucose liberated was estimated by DNS method[14]. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1 µg of glucose per minute under the standard assay conditions and was expressed as units per gram dry substrate (U/gds).

Results and Discussion

Initial screening of the various substrates for their potential to support cellulase production indicated that among the tested substrates, pre-treated PSCB was found to be the best (154.58 U/gds), followed by pre-treated wheat bran (Fig. 1). However, it was noted that untreated SCB also supported appreciable levels of cellulase production after 96 h of fermentation (102.65 U/gds) and the cellulase yield remained fairly constant over the 72-120 h incubation period in the SCB substrate. Since the enzyme yield at 72 h and 96 h was comparable when PSCB was used as substrate and since the initial moisture content may affect the incubation time required for maximal enzyme produc-

tion, an optimization of incubation time was performed at different initial moisture levels for PSCB to find out the optimum initial moisture level. The results indicated that better enzyme yields are obtained at 96 h of incubation and an initial moisture content of 66.4% (Fig. 2). It is known that the water activity of the medium significantly affects the productivity and behaviour of an SSF and the control of this parameter could be used to control and modify the metabolic activity of the microorganism[15]. Based on these results, further optimization studies were performed with PSCB as substrate, initial moisture, 66.4% and incubation time, 96 h.

Optimization of Initial Medium *p*H and Incubation Temperature

The influence of medium *p*H was studied by adjusting the *p*H of salt solution used to wet the substrate. It was observed that an initial medium *p*H of 7.0 was found to be ideal for enhanced cellulase production (Fig. 3). Different ranges of initial *p*H from 2.8-6.0 was studied previously[16] in SSF using xylose industry waste and *p*H ranges 4-0 was studied for cellulase production on banana wastes[17]. The latter study also reported that an initial medium *p*H of 7.0 is most suited for cellulase production for the given substrate.

Incubation temperature is another important factor, which affects the enzyme production in SSF. Maximal enzyme production of 56.5 U/gds was recorded at 28°C, and production was reduced at temperatures higher than 28°C (Fig. 3). The temperature normally employed in SSF is in the range of 25-35°C and it depends mostly on the growth kinetics of the microorganism used. Nevertheless, it was observed that the activity was found to be low when incubation was carried out at 30° compared to 35°C. This could not be correlated to the growth of the fungus since the total soluble protein showed a declining trend along with increase in temperature. Perhaps the higher activity obtained upon incubation at 35°C compared to 30°C was due to the induction of some of the components of the cellulase system which could not be verified in the current study.

Effect of N Sources on Cellulase Production

N requirement of microbes used in SSF process are met from the substrate itself in some instances, whereas supplementation of additional nitrogen in organic or inorganic form is often resorted to. Supplementation of organic N sources like yeast extract and peptone though are generally not required while using complex media, some components present in these nutrients stimulate the growth of individual strains[2]. In the present study $(NH_4)_2SO_4$ in the base medium was replaced by one of the three different

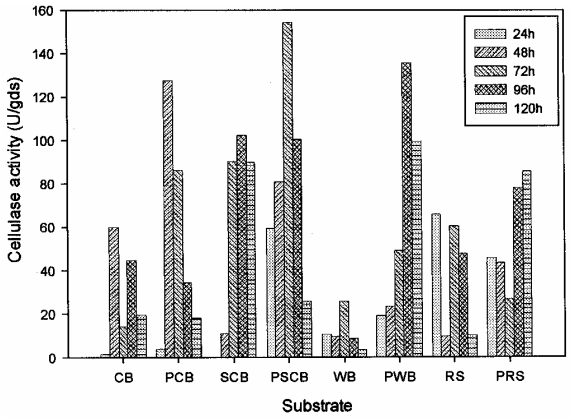

Fig. 1—Screening of agro-industrial residues for cellulase production

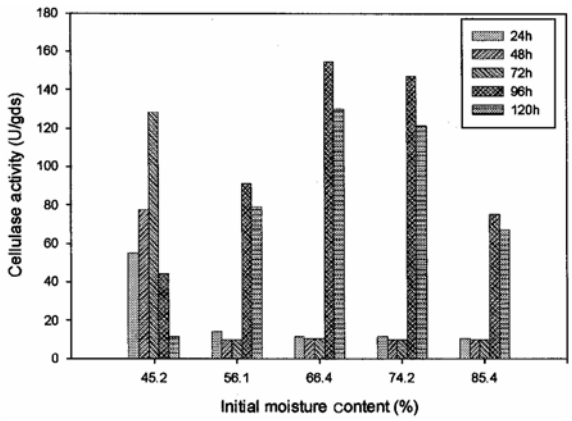

Fig. 2—Optimization of initial moisture content

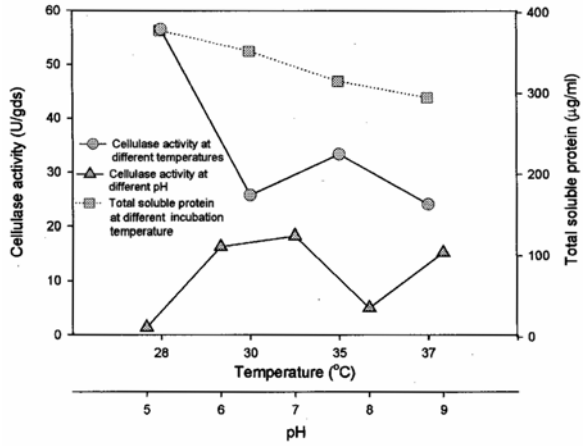

Fig. 3—Optimization of incubation temperature and initial medium *p*H

inorganic N sources NH_4NO_3, $NaNO_3$ or NH_4Cl at 0.075 *M* level to test their ability to support better cellulase production. Supplementation of NH_4NO_3 as the nitrogen source had the highest impact on cellulase production as indicated in Fig. 4. Supplementation of the medium with NH_4NO_3 supported the production of 154.67 U/gds cellulase activity; whereas the control gave only 25.76 U/gds cellulase.

Optimization of Concentration of Inducer (Cellobiose)

The cellulase system of *T. reesei* is inducible and a considerable amount of studies have been directed towards understanding cellulase induction and to find the natural inducer of cellulase complex[2]. Cellobiose, an oxidation product of cellulose hydrolysis, is widely regarded as a natural inducer of cellulase system and it also has been considered as a regulator of the system since at higher concentrations cellobiose can inhibit cellulase production[18]. Hence in the present study, three different concentrations of cellobiose were studied for its efficacy to induce the cellulase system. Supplementation of cellobiose regardless of the concentration tried had a positive effect on cellulase production compared to the control (Fig. 5). However, maximal activity was obtained at 0.005 *M* cellobiose concentration. Apparently, there exists an optimal concentration of the inducer for this system, since at the higher concentration of cellobiose tried; the activity was less indicating a possible repression mechanism. The results indicated that an optimization of the inducer levels could result in significant improvement of productivity.

The major bottleneck of the bioconversion of lignocellulosic wastes to useful metabolites is the cost of cellulase preparations to be used in enzymatic saccharification of the substrate. 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. The present study evaluated the application of different agro industrial residues as substrates for cellulase production. Sugarcane bagasse was found to be a potent substrate for the SSF production of cellulase employing the fungus *T. reesei*. Major parameters affecting the fermentation process for enzyme production were studied and optimal levels were identified. Results indicated scope for further studies on the fermentation process for improved and economically viable cellulase production if tried along with strain improvement strategies.

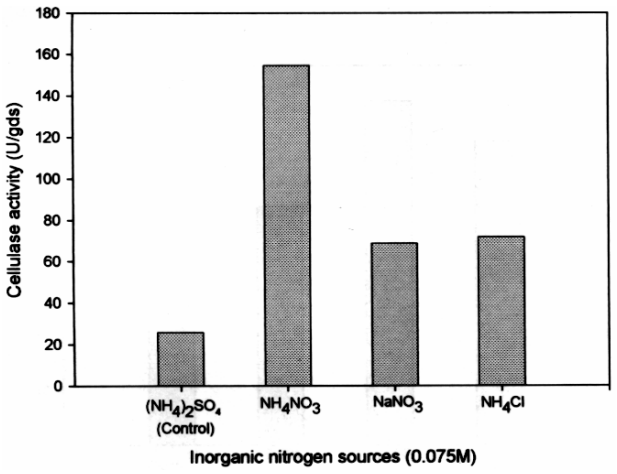

Fig. 4—Effect of inorganic nitrogen source on cellulase production

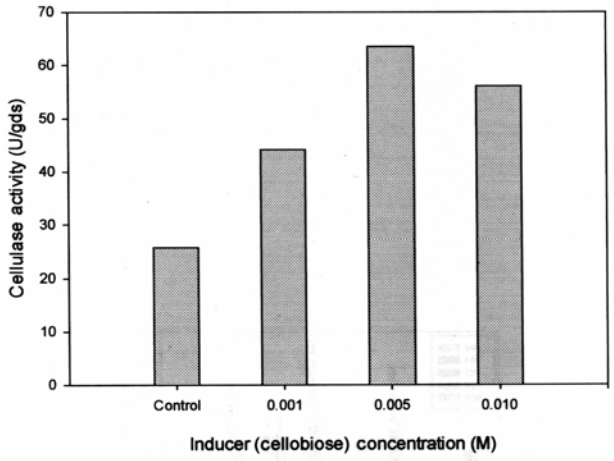

Fig. 5—Optimization of inducer concentration for cellulase production

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