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Optimization of concomitant production of cellulase and xylanase from *Rhizopus oryzae* SN5 through EVOP-factorial design technique and application in Sorghum Stover based bioethanol production

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ABSTRACT

Current study deals with the production of cellulases and xylanases from the *Rhizopus oryzae* SN5 isolated from composed soil of Himalayan pine forest, in order to meet the challenges of lignocellulosic biomass based biorefineries. Culture parameters for concomitant production of cellulase and xylanase were optimized through EVOP-factorial design technique under solid state fermentation. And maximum yield of cellulase and xylanase were obtained 437.54 U/gds and 273.83 U/gds, respectively at 30 °C and pH 6.0 after 5 days of incubation. On applying these enzymes for the saccharification of the dilute acid pretreated Sorghum Stover (SS), 0.407 g/g sugar was yielded. This hydrolysate on fermentation, yielded 0.411 g/g ehanol with *Saccharomyces cerevisiae* (NCIM 3288), which could be considered a good conversion. Therefore, *Rhizopus oryzae* SN5 was found as potent strain for the production of the cocktail of lignocellulosic biomasss hydrolytic enzymes and would be promising tool in the area of lignocellulose based bio-refineries.

1. Introduction

Renewable biomass is the cheapest and readily available alternative feedstock for alternative fuel to meet out the global energy demand of the future as well as to diminish the fossil fuel generated environmental issues [1–3]. Lignocellulosic biomass consists of the complex network of cellulose, hemicelluloses and lignin [2,3]. The first step in the lignocellulosic ethanol production is the pretreatment of the biomass which involves the deconstruction of lignocellulose complex association to make complex sugar free. In the second major step is the saccharifications of the complex sugars to fermentable sugars by acidic or enzymatic methods [4].

In acidic saccharification generates various unwanted fermentation inhibitory compounds such as furfural, hydroxymethylfurfural (HMF), phenolics and organic acids, therefore the enzymatic saccharification is the preferred over acidic method [5,6]. The enzymatic method requires two major enzymes such as cellulase and xylanase for the saccharification of cellulose and hemicelluloses into fermentable hexoses and pentoses. The cost of the enzyme creates an excess burden on the biorefineries to meet up with the people's demand in comparison to the existing fuel prices [3]. Therefore the present bioethanol researchers are targeting to find the cost effective method for enzyme production.

Cellulases are a group of hydrolases, including endoglucanase (1,4-β-D glucanohydrolase, exoglucanase (exo 1,4-β-D-glucan cellobiohydrolase), and β-glucosidase) [7,8]. Based on the source of the production, the cellulases possess different catalytic potential for the breakdown of cellulose. Hence now a day the research has been focused on the screening of new potential strains and development of optimized processes for efficient cellulase production [9]. Xylanase is the second most important enzyme used for the saccharification of the biomass, it hydrolyses the beta-1,4 glycosidic bond present in the xylan and produces fermentable sugars [10].

Solid state fermentation (SSF) is most preferred technique for the production of saccharification enzymes than submerged fermentation (SmF), because of its high efficiency and requirement of the less operational cost [8,11–13].

The process parameters for the extracellular enzymes production greatly influence their yield, therefore it must be optimized in order to search the optimum level by making the optimization process fast and convenient [8,14]. Conventional approach of

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optimization i.e. "One factor at a time approach" fails to integrate various variables at a time [9], therefore various statistical methods such as Response surface methodology (RSM) [3], Artificial Neural Network (ANN) [15], Evolutionary operations EVOP [8,14] have been exploited by many researchers to obtain the optimum level of the process.

In present study, an efficient cellulase and xylanase producing fungal strain had been isolated and characterized by 18S r-RNA method. The isolated strain was used for concomitant production of Cellulase and Xylanase in common reactor system through SSF and the process parameters were studied and optimized statistically by evolutionary operations (EVOP). The resulted crude enzyme was used for the saccharification of the Sorghum Stover (SS) and further the hydrolyzate obtained was used for the ethanol fermentation *by Saccharomyces cerevisiae* NCIM 3288.

2. Materials and method

2.1. Isolation, screening and characterization of cellulase producing fungal strains

Pine waste compost (PWC) was aseptically collected from the Ranikhet district, Uttarakhand, India, where pine forest waste was being used after composting, for isolation and screening of cellulase and xylanase producing microorganisms. One gram of PWC was serially diluted from 10^{-1} to 10^{-7} dilutions by using sterile distill water. Hundred micro liter of each diluted samples was spread plated over the Potato Dextrose Agar Plates and incubated for 120 h. The morphologically different fungal strains were inoculated on Cellulase selective media (g/l): $NaNO_3$ 2.5 g; KH_2PO_4 2 g; $MgSO_4$ 0.2 g; NaCl 0.2 g; $CaCl_2 \cdot 6H_2O$ 0.1 g; Agar, 20, pH-6.0 and to this add 1% Carboxymethyl Cellulose (CMC) was added. These Petri plates were incubated for 5 days at 28 ± 2 °C. After 5 days of incubation all the plates were flooded with 0.1% Congo red dye for 15 min [16]. The clear zone forming fungi were selected and screened for their cellulase producing efficiency. The most efficient cellulase producing fungal strain was characterized by 18S r-RNA sequencing method from Bioserve Biotechnologies Pvt. Ltd., Hyderabad, India.

2.2. Production of cellulase and xylanase in solid state fermentation (SSF)

2.2.1. Microorganism and inoculum preparation

The clear zone forming fungal strains were streaked over Potato Dextrose agar slants and allowed to sporulate for 5 days at 28 ± 2 °C. After 5 days of incubation 20 ml of sterile distilled water was added in each slants and the slanted growth surface was gently scratched with sterile inoculation loop. Each slant was vigorously shaken for homogenous spore suspension formation.

2.2.2. Production of crude cellulase by solid state fermentation (SSF)

Five gram of wheat bran moistened by modified Czapex-Dox inorganic medium ($NaNO_3$-2.0 g, KCl-0.5 g, $FeSO_4 \cdot 7H_2O$-0.01 g, $MgSO_4 \cdot 7H_2O$, KH_2PO_4-4.5 g in 1000 ml) in 250 ml Erlenmeyer flasks (in 1:2 w/v ratio) and autoclaved. Then inoculated with fungal spore suspension (with spore count 1.8×10^8 spores/ml) and incubated at 28 ± 2 °C for 5 days under static condition. Uninoculated soaked wheat bran was served as control in this study [11].

2.2.3. Extraction of cellulase and xylanase

After 5 days of incubation 50 ml of sodium citrate buffer (50 mM, pH 4.8) was added in each flask and the contents were mixed properly by shaking at 30±2 °C for 3 h at 120 rpm. The solid residues were separated by filtration through double layered muslin cloth, followed by centrifugation at 1000 rpm for 15 min at 4 °C [12], Supernatant obtained were treated as crude enzyme extract. All experiments were carried out in triplicate and presented values were mean values.

2.2.4. Optimization of variables by EVOP factorial design

The EVOP factorial design was applied to select the optimum levels of three physicochemical parameters (pH, temperature and incubation time) in different experiments for *Rhizopus oryzae* SN5. Firstly, the control experimental conditions (A1 and A6) were selected based on the results of earlier investigations on the effect of individual parameters on the production of cellulase and xylanase by SSF [12]. Secondly, new experimental conditions were selected +1 and −1 levels of parameters compared to the control or search level. Reactions were carried out for two repeated cycles. Cellulase and xylanase activities were estimated following the procedure described earlier and recorded for cycles I and II. Differences between Cellulase and xylanase activities of cycle I and II, as well as the average of the Cellulase and xylanase activities were calculated to estimate the effects and error limits.

2.2.5. Decision making procedure

Effects were compared with error limits and change in main effects that direct desired direction of change to improve the response. If any of the effects are greater than the error limits, the alteration in the variables could be achieving better results. In present study three variables; pH, temperature and incubation time were considered for optimization through EVOP-factorial design [12,14].

2.3. Pretreatment and saccharification of Sorghum Stover

Dilute acid pretreatment of Sorghum Stover (SS) was carried out in 1 L Erlenmeyer flasks with solid to liquid ratio of 1:9 (w/v). Fifty grams of SS was mixed with 450 ml of 0.5% H_2SO_4 and autoclaved at 121 °C for 45 min [6]. The reaction was and supplemented with a surfactant PEG-6000 in 1% (w/w) concentration [3]. After completion of pretreatment the contents was immediately cooled down at room temperature. Solid fraction was separated by filtration through double layered muslin cloth. The liquid portion was centrifuged at 10,000*g* for 10 min. The pH of the prehydrolyzate was adjusted to 7.0 with 1 M H_2SO_4 and 1 M NaOH. The solid residues were also neutralized by washing with hot distilled water and dried at 45 °C for 12 h.

2.4. Application of the crude enzyme in saccharification of pretreated SS

The enzymatic saccharification of pretreated SS was carried out in 500 ml Erlenmeyer flask. The filter paper activity of the crude enzyme preparation (with Cellulase activity: 437.54 U/gds and Xylanase activity: 273.83 U/gds) was calculated prior to be used in the saccharification reaction and it was found to be 21.76 FPU/ml.

The pretreated SS was mixed with 50 mM sodium citrate buffer (pH 4.8) at 5% (w/v) biomass loading and the crude enzyme was added at the loading of 50 FPU/g of the pretreated biomass. The reaction mixture was supplemented with 0.1% Tween-80 and incubated at 50 °C on an orbital shaker for 72 h at 150 rpm. To avoid any microbial growth 0.3% w/v sodium azide was also added in the reaction mixture. After completion of the saccharification process, residues were separated by centrifugation at 10,000 × g for 10 min and the hydrolyzate obtained were analyzed by 3, 5-dinitrosalicylic acid method [17].