Renewable Energy 98 (2016) 9–15

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene

Hydrolysis of pretreated rice straw by an enzyme cocktail comprising acidic xylanase from *Aspergillus* sp. for bioethanol production

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ARTICLE INFO

Article history:
Received 13 January 2016
Received in revised form
30 April 2016
Accepted 3 May 2016
Available online 24 May 2016

Keywords:
Acidic xylanase
Aspergillus sp.
Enzymatic hydrolysis
Enzyme cocktail
Rice straw

ABSTRACT

The most crucial enzyme involved in xylan hydrolysis is endoxylanase which cleaves the internal glycosidic bonds of xylan. The aim of this work was to study the production of extracellular xylanase by a locally isolated strain of *Aspergillus* sp. under solid-state fermentation (SSF) and to evaluate the potential of the enzyme in enzymatic hydrolysis of pretreated rice straw. Xylanase production reached maximum with incubation period (96 h), moisture level (80%), inoculum size (3×10^6 spores/mL), pH (4.8), temperature (25 °C), carbon source (wheat bran) and nitrogen source (yeast extract). Under optimized conditions, xylanase production reached to 5059 IU/gds. Crude xylanase was used for supplementing the enzyme cocktail comprising cellulases (Zytex, India), β-glucosidase (In-house) and xylanase (In-house) for the saccharification of alkali-pretreated rice straw to get the maximum reducing sugar production. The cocktail containing the three enzymes resulted a maximum of 574.8 mg/g of total reducing sugars in comparison to 430.2 mg/g sugars by the cocktail without xylanase. These results proved that the crude xylanase preparation from *Aspergillus* sp. could be a potent candidate for the enzyme cocktail preparation for biomass hydrolysis in lignocellulosic bioethanol program.

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1. Introduction

Lignocellulosic residues are the most abundant renewable resources available on earth which will play a pivotal role as an alternative bio-resource for bio-energy in near feature. Lignocelluloses are composed of cellulose, hemicelluloses and lignin [1]. These materials are accumulated in bulks every year without much usage in the form of agro-residues, forestry, etc. Large amounts of theses residues are bring burned in the fields causing pollution. Typical example is the burning of the most abundant residues such as rice straw and sugarcane baggase in Asian countries. The celluloses and hemicelluloses from the above said agro-residues can be acted up on by microbes using solid-state as well as submerged fermentation and produce value added compounds such as ethanol, food additives, organic acids, enzymes, etc.

Due to the recalcitrant nature of the lignocelloses, they are needed to be pre-treated in order to expose the celluloses and hemicelluloses [2]. Pretreatments are used to release cellulose from amorphous lignin and hemicellulose. Various improved pretreatment methods are being used among which acid and alkali pretreatment are used in common because of their simplicity and efficient performance. Acid pretreatments and alkaline pretreatments modifying cell walls selectively remove lignin portion [3]. During such pretreatments, solvation and saponification reactions take place and the biomass swells, enhancing the accession by saccharification enzymes to its inner space [4].

In order to convert lignocellulosic biomass into fuels and other products process effectively, it is necessary to improve the efficiency of enzyme production. Formation of enzymatic cocktail is one of the best methods to improve the efficiency. Since the cost of the enzymatic cocktails influences the viability of the process, utilization of enzyme produced by the microorganisms could be more significant. A broader suit of enzymes preparations are required to give high level of saccharification. Another choice is to produce the enzyme cocktails, which can act upon wide range of agro-residues in order to reduce the costs. Having a diverse library of cellulases along with lignocellulases can be used to enable the tailoring such cocktails. Xylanase supplementation in enzyme cocktail is an important strategy to increase sugar yields [5]. One of the most crucial enzyme involved in the hydrolysis is endoxylanase (EC 3.2.1.8) which cleaves the internal glycosidic bonds of the heteroxylan backbone which results in the formation of xylooligosacchrides [6,7]. As a result, the xylooligosacchrides are hydrolysed to xylotriose, xylobiose and xylose [8].

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http://dx.doi.org/10.1016/j.renene.2016.05.011
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Solid state fermentation (SSF) is particularly advantageous for the production xylanases at industrial level [9]. Reports indicates that SSF can be effectively used for enzyme production by filamentous fungi and enables the use of agro-industrial residues as solid substrate, acting as sources of both carbon and energy [10]. The aim of this work was to study the production of extracellular xylanase by a locally isolated strain of *Aspergillus* sp. under solid state fermentation (SSF) and to evaluate the potential of the enzyme in enzymatic hydrolysis of pretreated rice straw.

2. Materials and methods

2.1. Isolation and screening of microorganism

Aspergillus strains were isolated from the samples of vegetable decays. The isolation was done by dilution plate method. Beechwood xylan agar medium containing the following components (g/L) $NaNO_3$, 1.0; K_2HPO_4, 1.0; KCl, 1; $MgSO_4$, 0.5; yeast extract, 0.5; pH 4.8 was used for the isolation. The inoculated plates were incubated at 30 °C for 48 h. The clearing zones formed around the colony were visible when the plates were flooded with 0.1% (w/v) congo red solution. The pure cultures were maintained on xylan agar medium at 4 °C.

2.2. Inoculum

Pure culture of *Aspergillus* sp. was subcultured on beechwood xylan agar medium and incubated at 30 °C. Fully sporulated plates were obtained after 6 days. The sporulated plates were flooded using 20 mL of normal saline to harvest the spores and obtain the resulting spore suspension, which was used as inoculum in the subsequent experiments.

2.3. Xylanase production under submerged fermentation

The *Aspergillus* strains were cultured in 250 mL Erlenmeyer flasks containing 100 mL of mineral salts medium. The composition of the mineral salts medium was as mentioned in Section 2.12. The medium was then autoclaved at 121 °C for 15 min. After cooling, the flasks were inoculated with 1.0 mL of spore suspension containing 1×10^6 spores/mL. The inoculated flasks were incubated at 30 °C for five days. After incubation, the fermented broth was centrifuged at 10,000*g* at 4 °C for 10 min. The supernatant was used for enzyme assays.

2.4. Enzymes assays

2.4.1. Xylanase assay

Xylanase activity was determined by adopting the modified method of Bailey et al. [11] by mixing 500 μL of 1% (w/v) beechwood xylan prepared in 50 mM citrate buffer (pH 4.0) with 500 μL of appropriately diluted enzyme and the mixture was incubated at 50 °C for 30 min. After incubation, the reaction was terminated by the addition of 3.0 mL of 3,5-dinitrosalicylic acid reagent and the contents were boiled for 5 min [12]. After cooling, the colour developed was read at 540 nm. The amount of reducing sugar liberated was quantified using xylose standard. One unit of xylanase activity was defined as the amount of enzyme that liberates 1 μmol of reducing sugars (xylose) per minute under the assay conditions.

2.5. Filter paper activity assay

This was determined by the modified method of Ghose, 1987 [13]. For this, 0.5 mL of appropriately diluted enzyme was incubated with 50 mg (~1 × 6 cm strip) of Whatman No. 1 filter paper for 1 h in a total volume of 1.5 mL. At the end of reaction, 3.0 mL of DNS reagent was added to terminate the reaction. The reaction mixture was incubated in a boiling water bath for 5 min to allow the colour development. After cooling, the colour developed was read at 540 nm. The amount of reducing sugar liberated was quantified using glucose standard. One unit activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugars (glucose) per minute under the assay conditions.

2.6. Solid state fermentation for xylanase production by Aspergillus sp.

Ten gram of the solid substrate (wheat bran), taken in 250 mL Erlenmeyer flasks was well mixed with 47.5 mL of mineral salts solution containing (g/L) $NaNO_3$ 1.0, yeast extract 0.5, K_2HP0_4 1.0, $MgS0_4 \cdot 7H_20$ 0.5 (pH 4.8) and autoclaved at 121.5 °C for 15 min. The final moisture content during the start of the experiment was 80% and was maintained by incubating it in a humidity incubator. After cooling, the flasks were inoculated with *Aspergillus* sp. (1×10^6 spores/mL) and incubated at 30 °C for 120 h. Samples were withdrawn as whole flask and the contents were mixed with 100 mL of 50 mM citrate buffer (pH 4.0) on a shaker for 60 min, then filtered through cheese cloth. The filtrate was centrifuged at 10,000*g* at 4 °C for 20 min and the supernatant was used as crude enzyme extract. All the experiments were carried out in triplicate and the results have been present as the mean of three.

2.7. Optimization of process parameters for enzyme production

Various process parameters influencing enzyme production during solid state fermentation were optimized by adopting one parameter at a time approach. The strategy adopted was to optimize each parameter, independent of the others and subsequently optimal conditions were employed in all experiments [14]. The effect of process parameters on xylanase production were determined by incubating at varying incubation period (24–120 h), by using different carbon sources (1% w/v), different nitrogen sources, varying inoculum size (0.5×10^6–3.5×10^6) and the incubation temperature.

The effect of incubation time on enzyme production under solid state fermentation was studied by incubating the inoculated flasks at different time points (24–120 h). To study the effect of various carbon sources on xylanase production, solid state fermentation was supplemented with various simple and complex carbon sources at 1% level (w/v), such as rice straw (RS), cotton stalk (CS), Wheat bran (WB), Sugar cane bagasse (SCB), Sugar cane trash (SCT) Sorghum biomass (SM), Soya hull (SH). Fermentation was carried out at 30 °C at pH 4.8 for a period of 96 h. Different organic nitrogen sources and inorganic nitrogen sources were also used for enzyme production. The effect of inoculums size on enzyme production was studied by adjusting the inoculum size ranging from 0.5×10^6 to 3.5×10^6. The other conditions were kept constant. The effect of incubation temperature on enzyme production was studied by carrying out solid state fermentation at different temperatures (25–45 °C) with wheat bran as the substrate. The effect of initial pH of the medium on enzyme production was studied by adjusting the pH of the mineral salt solution to values ranging from 3.0 to 8.0 using different suitable buffers at concentration of 50 mM citrate buffer (pH 3.0–6.0, 1 M) and phosphate buffer (7.0–8.0). The other conditions were kept constant.

2.8. Effect of temperature and pH on enzyme activity

Optimum temperature for xylanase activity was determined by

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