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Potential of apple pomace as a solid substrate for fungal cellulase and hemicellulase bioproduction through solid-state fermentation

Gurpreet Singh Dhillon[a], Surinder Kaur[a,b], Satinder Kaur Brar[a,*], Mausam Verma[c]

[a] INRS-ETE, Université du Québec, 490, Rue de la Couronne, Québec G1K 9A9, Canada
[b] Department of Mycology & Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University (BHU), Varanasi 221005, India
[c] Institut de recherche et de développement en agroenvironnement inc. (IRDA), 2700 rue Einstein, Québec (Québec) G1P 3W8, Canada

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ABSTRACT

The effect of different inducers on the cellulase and hemicellulase bioproduction by *Aspergillus niger* NRRL-567 using apple pomace as a substrate was investigated. Rapid production of different cellulase enzymes namely, FPase (filter paper cellulase), CMCase (carboxymethyl cellulase), BGL (β-glucosidase), and xylanase were observed with peak activity reaching between 48 and 72 h of fermentation period. The higher FPase and BGL activities of 133.68 ± 5.44 IU/gram dry substrate (gds) and 60.09 ± 3.43 IU/gds, respectively were observed while using $CuSO_4$ and veratryl alcohol after 48 h of incubation time. The higher CMCase activity of 172.31 ± 14.21 IU/gds was obtained with lactose after 48 h of incubation period. Similarly, higher xylanase activity of 1412.58 ± 27.9 IU/gds was observed with veratryl alcohol after 72 h of fermentation time. This study sheds light on the rapid bioproduction of fungal cellulase and hemicellulase using low cost waste, apple pomace as substrate when supplemented with different inducers.

1. Introduction

Cellulases have attracted worldwide attention due to their numerous applications, such as bioethanol production from abundant lignocellulosic biomass, textile industry, pulp and paper industry, formulation of animal feeds, extraction of fruit and vegetable juices and starch processing (Bhat, 2000; Zaldivar et al., 2001). Recently, cellulases find promising applications for non-specific hydrolysis of chitosan to form low molecular weight chitosan oligosaccharides (LMWCs) (Xia et al., 2008). The growing concerns about the potential consequences of worldwide depletion of fossil fuels, emission of green house gases resulting from the incomplete combustion of fossil fuel has caused an increased focus on the production of biofuels, such as bioethanol from lignocellulosic wastes (Zaldivar et al., 2001). In the context of green energy, cellulases and hemicellulases play an important role in enzymatic hydrolysis of the lignocellulosic material (Sun and Cheng, 2002). For a feasible and economical bioethanol production, low cost enzymes with balanced cellulase and xylanase activities play pivotal role. In addition, there is a general interest in obtaining new, more specific and stable enzymes having synergy with enzymes from different microorganisms.

Among different microorganisms known to produce extracellular cellulases, emphasis has been laid on the fungi because of their capability to produce copious amounts of cellulases and hemicellulases which are secreted extra cellularly in the medium for easy extraction and purification (Kim et al., 2003). Cellulase has three components, such as endoglucanase (endo-1,4-β-D-glucanase, EC 3.2.1.4), exoglucanase (exo-1,4-β-D-glucanase, EC 3.2.1.91), and β-glucosidases (1,4-β-D-glucosidase, EC 3.2.1.21). Along with cellulolytic enzymes, xylanases also play pivotal role in the efficient conversion of lignocellulosic substances having appreciable amounts of xylan (hemicellulose) residues to readily available sugars for feasible bioethanol production. For commercialization of bioethanol production from lignocellulosic wastes, xylanases are critical for the feasibility of the process (Mekala et al., 2008; Olofsson et al., 2008; Xiros and Christakopoulos, 2009; Brijwani et al., 2010).

The fungus *Aspergillus niger* has been shown to secrete large amounts of cellulolytic enzymes having optimal activities for efficient hydrolysis of lignocellulosic biomass. Cellulose saccharification requires the mutual action of endoglucanases which hydrolyze the cellulose polymer at internal sites exposing reducing and non reducing ends, and exoglucanases, which acts on the reducing and non reducing ends, releasing cellobiose and

Abbreviations: AP, apple pomace; BGL, β-glucosidase; CFUs, colony forming units; CMCase, carboxymethyl cellulase; CS, copper sulfate; EtOH, ethanol; FPase, filter paper cellulase; IU/gds, international unit/gram dry substrate; LAC, lactose; MeOH, methanol; *p*-NPG, *p*-nitrophenyl β-D-glucopyranoside; SSF, solid-state fermentation; VA, veratryl alcohol; WB, wheat bran.

* Corresponding author. Tel.: +1 418 654 3116; fax: +1 418 654 2600.
E-mail address: satinder.brar@ete.inrs.ca (S.K. Brar).

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cellooligosaccharides. Beta-glucosidase hydrolyzes the cellobiose which otherwise inhibits the action of exo- and endoglucanases and liberates fermentable sugars (Kaur et al., 2012). *A. niger* strains are also well known for higher production of β-glucosidase which is required for the efficient hydrolysis of cellulosic substrates (Brijwani et al., 2010).

Solid-state fermentation resembles the natural environment of the fungi and holds tremendous potential for the production of hydrolytic enzymes. Agro-industrial wastes being rich in carbohydrates and other vital nutrients are considered as promising substrates for culturing fungi in the SSF. The use of these low cost wastes as substrates helps to reduce the cost of the enzymes for which raw material translates into 40–60% of the production cost (Wen et al., 2005). Various agro-industrial wastes have been used as substrates for the cost efficient production of cellulases, such as apple pomace, kinnow waste, soybean hulls, sugarcane bagasse and rice and wheat straw among others (Gutierrez-Correa et al., 1999; Oberoi et al., 2010a,b; Brijwani et al., 2010; Kaur et al., 2012). The use of inducers can also render the process economically feasible by increasing the production of enzymes. As evident from the literature, apple pomace (AP) has been already utilized for the production of organic acids, protein-enriched feeds, mushrooms, bioethanol, aroma compounds, natural antioxidants and enzymes, such as cellulases, pectinases, pectin methylesterase, and lignocellulolytic enzymes (Vendruscolo et al., 2008; Gassara et al., 2010; Dhillon et al., 2011a,b,c; Kaur et al., 2012). Moreover, AP is available in abundance in the province of Quebec, Canada (Dhillon et al., 2011d).

Thus, the objectives of this study were to evaluate the potential of AP supplemented with rice husk (1%, w/w) for bioproduction of cellulases (FPase, CMCase, β-glucosidase) and xylanase by SSF with *A. niger* NRRL-567. *A. niger* NRRL-567 is a potent strain for citric acid production, and the present study was conducted to evaluate its efficiency for cellulases bioproduction. AP supplemented with rice husk has not been investigated so far for cellulases and hemicellulases production through SSF using *A. niger* NRRL-567 and thus has enormous potential as industrial fermentation substrate. The study also aimed to improve the cellulase activity by supplementing different inducers, such as veratryl alcohol (VA), $CuSO_4$ (CS), lactose (LAC), ethanol (EtOH), and methanol (MeOH), for achieving higher enzyme activity. The extraction of enzymes was also optimized using different shaking conditions and extraction buffer having varied pH and volume. To the best of our knowledge, VA and CS have not been utilized as inducers for cellulases bioproduction so far.

2. Materials and methods

2.1. Microorganism procurement and propagation

A. niger NRRL 567 strain, provided by Agricultural Research Services culture collection, IL-USA was selected as a suitable microorganism for cellulase and hemicellulase production using AP through SSF. *A. niger* NRRL 567 is well known producer of citric acid. Taking into account its ability to secrete high CA, it is imperative that *A. niger* secretes high titers of hydrolytic enzymes required for conversion of carbon source to metabolic by-products. The culture conditions, maintenance, and inoculum preparation have been already described by Dhillon et al. (2011b). Spore suspension having 1.0×10^7 spores/g substrate was used in the study.

2.2. Solid support substrate

AP waste (Lassonde Inc., Rougemont, Montreal, Canada) was used as solid substrates to evaluate its suitability for the production of cellulases and hemicellulases. Apple processing industries supplement the apples with rice husk (up to 1%, w/w) during juice extraction for better hold and efficient meshing. Therefore, the apple pomace used in this study was already supplemented with 1% (w/w) rice husk. The moisture of AP was analyzed using a moisture analyzer (HR-83 Halogen, Mettler Toledo, Switzerland). The physico-chemical characterization of AP waste is given in Table 1 (APHA, AWWA, WPCF, 2005).

Table 1
Physico-chemical characteristics of apple pomace waste.

Parameters	Values
Initial moisture (%, v/w)	71.28 ± 0.5
Initial pH	3.43 ± 0.1
Suspended solids (SS) (g/l)	202 ± 1.3
Total solids (TS) (g/kg)	257 ± 1.3
Volatile solids (VS) (g/kg)	231 ± 1.8
Total carbon (g/kg)	127.9
Total nitrogen (g/kg)	6.8
Sulfur (g/kg)	0.6
Lignin (W/dry weight)	23.5
Cellulose (W/dry weight)	7.2
Hemicellulose (W/dry weight)	–
Total carbohydrates (%)	48.0–62.0
Protein (%)	3.9–5.7
Fiber (%)	4.70–51.10
Ash content (%)	0.5–6.1
Reducing sugars (%, w/w)	10.8–15.0
Glucose	22.7
Fructose	23.6
Sucrose	1.8
Xylose	0.1
Galactose	–
Arabinose	–
Minerals (%, w/w)	
Phosphorous	0.07–0.076
Potassium	0.4–1.0
Calcium	0.06–0.1
Sodium	0.2
Magnesium	0.02–0.36
Copper (mg/kg)	1.1
Zinc (mg/kg)	15.0
Manganese (mg/kg)	3.96–9.0
Iron (mg/kg)	31.8–38.3

± represents standard error calculated using duplicate sets.

2.3. Solid-state cultivation

The diagrammatic representation of solid-state fermentation is shown in Fig. 1. Fungal growth and the production of enzymes were carried out in 500 ml Erlenmeyer conical flasks containing 40 g of dry substrate having particle size 1.7–2.0 mm. Sterilization was carried out at 121 ± 1 °C for 30 min. The substrate was inoculated with spore suspension of 1×10^7 spores/g substrate and inducers were added in different concentrations, VA (3 mM/kg), $CuSO_4$ (1 mM/kg), LS (1%, w/w), EtOH and MeOH (3%, v/w). Tween-80 was added at a concentration of 0.5% (v/w) in one treatment to evaluate its effect on the enzyme production. The initial moisture was adjusted to 75% with distilled water. After thorough mixing, cultures were incubated in environmental chamber at 30 ± 1 °C and 75% relative humidity and fermentation was carried out for 7 days. All the experiments were conducted in triplicates.

2.4. Enzyme extraction

Enzyme activities were analyzed at every 24 h intervals from fermented samples. One gram of sample was taken from each flask and mixed with 15 ml of 100 mM citrate buffer having pH 4.8. The samples were incubated for 30 min in shaker at 200 rpm followed by