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Review

Semi-continuous production of high-activity pectinases by immobilized *Rhizopus oryzae* using tobacco wastewater as substrate and their utilization in the hydrolysis of pectin-containing lignocellulosic biomass at high solid content

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HIGHLIGHTS

- Tobacco wastewater can provide sufficient nutrients and inducers for PGs production.
- Production time was shortened using immobilized *Rhizopus oryzae*.
- Semi-continuous PGs production was achieved though repeated-batches mode.
- Enzyme productivity was highest among all studies reported to date.
- Hydrolysis by cellulase was improved by adding the crude enzyme.

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ABSTRACT

In this study, highly reactive endo- and exo-polygalacturonases (PGs) were produced from the tobacco industry wastewater using immobilized *Rhizopus oryzae*. Compared with free cells, immobilized cells increased enzyme activity 2.8-fold and reduced production time to 24 h by shake-flask production. Moreover, the immobilized cells enabled the semi-continuous production of enzymes through repeated-batch mode for seven consecutive cycles in a scale-up bioreactor. During the first five cycles, the average endo-PG and exo-PG activities reached 307.5 and 242.6 U/ml, respectively. The addition of crude enzyme for the hydrolysis of pectin-containing lignocellulosic biomass under high-gravity conditions increased glucose release 4.2-fold (115.4 vs. 29.0 g/L), compared with hydrolysis using cellulase alone. This process achieves the efficient production of pectin-degrading enzymes, provides a cost-effective method for tobacco wastewater treatment, and offers the possibility to obtain fermentable sugars with high-titer from pectin-containing lignocellulosic biomass, which has important potential for the commercial production of bio-fuels.

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1. Introduction

Pectinases constitute a large group of enzymes, including pectin lyase, endo-polygalacturonase (endo-PG), exo-polygalacturonase (exo-PG), and pectin esterase, which catalyze the biodegradation of pectin, a complex polysaccharide present in plant cell walls (Gao et al., 2014; Ortiz et al., 2017). Petinases currently constitute one quarter of the global food enzyme market and are widely used in different processes in the food, paper manufacturing, and oil extraction industries (Ortiz et al., 2017; Meneghel et al., 2014; Uzuner and Cekmecelioglu, 2015; Darah et al., 2015). In recent years, growing global energy and environmental issues have stimulated researchers all over the world to develop more efficient and green methods to convert renewable lignocellulosic biomasses into chemicals and fuels (Ferreira et al., 2016; Abdel-Rahman et al., 2013). Due to their ability to improve the access of cellulase to cellulose, pectinases also play a crucial role in the efficient hydrolysis of pectin-rich lignocellulosic biomasses, such as corn stover, corncob, and hemp hurds (Zhang et al., 2013; Pakarinen et al., 2012).

Tobacco (*Nicotiana tabacum* L.) is an important pectin-rich annual crop, that is widely planted around the world because of its high-value (Wang et al., 2013; Zheng et al., 2016). During tobacco manufacturing processes of tobacco, large quantities of pectin-containing wastewater is discharged. Generally, the production of 1 t of cigarettes will generate at least 60 t of tobacco wastewater (Wang et al., 2013). Due to the high content of organic matter and toxic substances, such as nicotine, tobacco wastewater treatment is complex, costly, and time consuming (Okunola et al., 2016). On the other hand, the presence of sugars, proteins, and soluble pectin in tobacco wastewater are considered as promising low-cost sources of carbon, nitrogen, and inducers for pectinase production, respectively (Meneghel et al., 2014; Zheng et al., 2016). Some pectinase-producing filamentous fungi, such as *Aspergillus oryzae* and *Rhizopus oryzae*, are capable of degrading nicotine and growing in tobacco wastewater (Meng et al., 2010; Battaglia et al., 2011), thus providing a possible means of exploiting tobacco wastewater as an inexpensive substrate for pectinase production.

However, submerged fermentation with filamentous fungi is associated with technical challenges. The highly branched fungal mycelia not only confers high broth viscosity, but also causes difficulty in mixing in conventional stirred-tank bioreactors (Tay and Yang, 2002), resulting in reduced enzyme production efficiency and high power consumption (Zhang et al., 2015; Meneghel et al., 2014). To overcome these problems, fermentation using immobilized fungal cells for enzyme production has attracted increased interest since they offer several advantages over free cells, such as reduced power consumption, higher enzyme activity, shorter fermentation time and increased operational stability (Taskin, 2013). Since oxygen supply is a crucial parameter influencing pectinase production, cell immobilization technology capable of enhancing oxygen transfer in a bioreactor may remarkably improve pectinase production from high-viscosity tobacco wastewater due to the presence of soluble pectin (Meneghel et al., 2014; Wang et al., 2010). Furthermore, repeated-batch fermentation using immobilized cells can achieve semi-continuous and efficient pectinase production by reusing cells without a separation step. This may further reduce production costs, simplify the production process, and minimizing fermentation time (Wang et al., 2010; Abdel-Rahman et al., 2013; El-Dalatony et al., 2016).

This research primarily aimed to develop an efficient and cost-effective process for pectinase production using tobacco wastewater as the sole substrate. Fermentation conditions for pectinase production using immobilized *R. oryzae* were first optimized in shake-flasks, followed by an evaluation of the stability of immobilized *R. oryzae* for semi-continuous enzyme production through repeated-batch mode operation in a scale-up bioreactor. Finally, pectinases produced from tobacco wastewater were investigated for their effectiveness in hydrolyzing a pectin containing lignocellulosic biomass for enhanced sugar release. To the best of our knowledge, this is the first demonstration of the semi-continuous production of high-activity pectinases from industrial wastewater in repeated-batch mode.

2. Materials and methods

2.1. Raw materials and chemicals

The tobacco wastewater used in this study was prepared by a traditional extraction process applied to tobacco waste. Tobacco waste (obtained from China Tobacco Chongqing Industrial Co., Ltd.) mainly contained tobacco powder, stem, and stalk tissue that remained without leaf material. Waste was found to be composed of 28.3% cellulose, 20.3% hemi-cellulose, 2.3% lignin, 8.8% protein, and 4.2% ash. Extraction procedures were as follows: (1) Tobacco waste was mixed with sterile water at a liquid-to-solid ratio of 10:1 at 90 °C; (2) after cooling, the slurry was filtered through sterile cheesecloth under vacuum to remove insoluble solids; and (3) the liquid extract was collected, and found to contain 5.28 g/L reducing sugar, 0.11 g/L glucose, 3.52 g/L soluble pectin, 3.25 g/L proteins, and 0.98 g/L nicotine using the method of Zhong et al. (2010). The pH of the liquid was 5.2. These values were in accordance with data for wastewater discharged from the China Tobacco Chongqing Industrial Co., Ltd, located in Fuling, Chongqing, China. The prepared liquid extract was thus used as equivalent tobacco wastewater in this study.

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