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Waste office paper: A potential feedstock for cellulase production by a novel strain *Bacillus velezensis* ASN1

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ABSTRACT

This paper reports the cellulase (FPase) production by newly isolated *Bacillus velezensis* ASN1 using waste office paper (WOP) as feedstock and optimization of production conditions through two level factorial design, steepest ascent/descent and second order response surface methodology (RSM). Various fermentation parameters, like chemical factors (potassium dihydrogen phosphate, potassium chloride, yeast extract, magnesium sulphate, sodium nitrate, Tween 80, and waste office paper), physical factors (temperature, pH and time) and biological factor (inoculum size) were examined using two level full factorial design to check the key factors significantly affecting the cellulase production. The central composite design (CCD) was used to optimize the vital fermentation parameters, such as carbon (WOP), nitrogen, pH, and inoculum concentration in the medium for achieving higher cellulase production. The optimum medium composition was found to be WOP (9 g/L), sodium nitrate (0.35 g/L), inoculum size (6.56%) and pH 4.72. The model prediction of 2.46 U/mL cellulase activity at optimum conditions was verified experimentally as 2.42 U/mL.

1. Introduction

The increased exhaustion of fossil fuel is drawing attention for the development of alternative resources to produce biofuels from fermentable sugars (Sukumaran et al., 2010). Lignocellulosic biomass is considered as the largest renewable biological resource and consists of forestry waste, agro-industrial wastes, municipal solid waste, food processing and other industrial waste (Alzate et al., 2006; Kumar et al., 2009). Lignocellulosic biomass is composed of 40–50% cellulose, 25–30% hemicellulose and 15–20% lignin in which glucose and xylose are the major sugars derived (Zhou et al., 2017). Lignocellulose wastes are accumulated with a yearly production of $\sim 200 \times 10^9$ tons, causing severe environmental impacts (Zhang, 2008; Mussatto and Teixeira, 2010). Currently, lignocellulosic biomass feedstocks are constantly explored for the production of a number of value-added products, such as fuels, food additives, organic acids, pesticides, enzymes, aromatic compounds, pharmaceuticals, detergents, fertilizers and plastics among others (Maki Naik et al., 2010; Naik et al., 2010; Rathore et al., 2014; El-Shishtawy et al., 2015; Gunny et al., 2015). The selection of the substrate for the enzyme production mainly depends on the availability and cheap cost (Pandey et al., 2000). Waste paper is one among the lignocellulosic biomass, considered as one of the relatively abundant and low cost (average $52/ton) municipal-industrial solid waste which is generated to approximately 400 million tons around the world annually (Annamalai et al., 2018).

Waste paper is mainly composed of 40–80% cellulose, 5–15% of hemicellulose and minor amounts of lignin (Sun and Cheng, 2002). The saccharification of cellulose content of the waste paper relies on the participation of cellulase-producing microorganisms and their cellulase enzymes. Reports on the utilization of waste office paper (WOP) for the production of cellulolytic enzymes using bacteria are limited. Therefore, utilization of WOP which can be easily digested without aggressive physical or chemical pretreatments (Wang et al., 2013) to produce cellulase not only reduces the cost of production but also solves the problem of disposal of solid wastes in landfills which contaminates the ground water and cause the emission of greenhouse gases (Lo et al., 2009; Brummer et al., 2014). However, the efficient conversion of waste paper still remains as a challenge because of its recalcitrant structure, in

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which cellulose, hemicellulose, and lignin are locked, making it difficult to depolymerize into fermentable sugars (Zhang et al., 2007).

Cellulases, hydrolyze the β-1,4-glycosidic linkages of cellulose and produce glucose, cellobiose, and cello-oligosaccharides as primary products (Kuhad et al., 2016). It is comprised of three major categories of enzymes such as endoglucanases (E.C. 3.2.1.4), exoglucanases (E.C. 3.2.1.176) (E.C. 3.2.1.91) and β-glucosidases (E.C. 3.2.1.21) (Sukumaran et al., 2005; Khianngam et al., 2014; Gaur and Tiwari, 2015). Cellulase has an extensive range of applications in various industries, such as textile, food, animal feed, fuel, chemical industry, detergents, pulp, and paper industry and waste management (Kuhad et al., 2011, Juturu and Wu, 2014). Therefore, searching for a potential cellulase-producing native microbial strains for lignocellulosic bioconversion reduces the production cost (Shil et al., 2014). But, the major factors that affect the enzymatic hydrolysis of lignocellulose are the structural characteristics of the cellulose, end product inhibition, decrease in the enzyme reaction rate, ratio of enzyme to substrate concentration, pretreatment of biomass, etc. (Wingren et al., 2003; Sukumaran et al., 2009; Juturu and Wu, 2014; El-Shishtawy et al., 2015). Furthermore, optimization of different factors mainly the substrate (WOP), nitrogen sources, pH, temperature, incubation time and inoculum density would greatly influence the cellulase production from waste paper.

Response surface methodology (RSM) is now being routinely used for optimization of medium components for cellulase production as it serves the purpose by determining the optimal conditions in any given system by a set of independent variables over a specific region of interest by establishing the relationship between more than one variable and a given response (Beg et al., 2003; Devi and Kumar, 2017). The aim of this study is to isolate a novel strain that can utilize WOP, a cheaper lignocellulosic raw material, to produce cellulase and then the optimization of the fermentation to improve its production through RSM. This is the first report on the production of cellulase by *B. velezensis* ASN1 using WOP as a substrate.

2. Materials and methods

2.1. Isolation and identification of cellulase producing bacteria

For isolation of cellulase producing bacteria, the soil samples were collected from the animal farm house from Barka, Oman and were serially diluted and plated on carboxymethyl-cellulose (CMC) agar plate (g/L): yeast extract- 0.5; KH_2PO_4-1; $MgSO_4$- 0.5; CMC- 10; NaCl- 1; $NaNO_3$-1; KCl- 1; Agar 15; pH 7 and incubated at 37 °C for 48 h. The plates were flooded with Congo red solution and destained with 0.1% sodium chloride (w/v). The colony which produced highest clear zone was considered as a potential bacterial strain and used for further studies.

The potential cellulase producing bacterium was identified through morphological, physiological and biochemical characteristics. Physiological properties were determined according to the procedures outlined in Bergey's manual of systematic bacteriology (Garrity et al., 2004). Standard protocols were used to assess carbon source utilization, oxidase activity, degradation of gelatin and nitrate reduction (Tindall et al., 2007; Ge et al., 2016). Further confirmation was done by 16S rRNA sequencing using universal primer 1492 R (5′ TACGGYTACCTTGTTACGACTT 3′) and 27F (5′ AGAGTTTGATCMTGGCTCAG 3′). PCR reactions were performed with the following conditions: 35 cycles consisting of 95 °C for 1 min and 72 °C for 5 min, followed by a final extension of 5 min at 72 °C. The purified PCR product was sequenced. The resulting 16S rRNA gene sequence was analyzed with other related sequences from the National Center for Biotechnology Information (NCBI) database (by BLASTn) and the phylogenetic analysis was performed using the MEGA7.

2.2. Preparation and pretreatment of WOP substrate

WOP collected from Sultan Qaboos University was shredded into small pieces (2 × 5 mm) using a mechanical shredder (Atlas, China). The shredded WOP was soaked with distilled water (5% w/v), grinded and dried at 55 °C for 24 h. Further, the dried pulp was milled again and used for further study. Pretreatment was carried out by soaking WOP (5% w/v) in 1% sulfuric acid at 50 °C for 1 h. The solid residues were collected by filtration and washed thoroughly with distilled water until the pH becomes neutral. The pulp dried at 60 °C for 24 h was milled again and used as a substrate for cellulase production.

2.3. Statistical design and analysis for cellulase production

2.3.1. Screening of significant variables by Plackett-Burman

The Plackett-Burman (PB) design is an effective technique used to select factors that significantly influenced the cellulase production (Ferreira et al., 2009; Singh et al., 2011). According to the PB factorial design, each factor was evaluated at two levels (Jiang et al., 2016), low and high, denoted by (−) and (+) signs, respectively.

The design was based on the first-order linear model as represented by the following equation:

$$Y = \beta_0 + \sum \beta_i X_i \quad i = 1, 2, 3.... k$$

where Y denotes the response (cellulase production), β_0 is the model's intercept, β_i is the linear coefficient and X_i is the level of the independent variable (Peng and Chen, 2011). Eleven variables in which chemical factors (KH_2PO_4, KCl, yeast extract, $MgSO_4{\cdot}7H_2O$, $NaNO_3$, Tween 80, and WOP), physical factors (temperature, pH and time) and biological factor (inoculum size) were examined with one center point to check the key factors significantly affecting the cellulase production. Thirteen experiments were done in this design (Table 1). All the runs were carried out in triplicate, and the average was taken as a response.

2.3.2. Path of steepest ascent/descent

The significant factors at 95% level ($p < 0.05$) in PB design were selected and further optimized using steepest ascent/descent method. Experiments were performed along the steepest ascent/descent with defined intervals, which was estimated by coefficient ratio and by practical experience (Jiang et al., 2016). Four factors (pH, WOP, $NaNO_3$ and inoculum size) were selected for steepest ascent/descent experiment.

Table 1
Variables and their levels employed in PB design.

Factor	Variables	Levels	
		−1	+1
Waste office paper (g/L)	A	5	20
$NaNO_3$ (g/L)	B	0.5	1.5
KH_2PO_4 (g/L)	C	0.5	1.5
Time (h)	D	24	72
pH	E	5	9
Inoculum (%) (v/v)	F	1	5
Temperature	G	30	40
Tween80 (g/L)	H	0.2	1
$MgSO_4$ (g/L)	J	0.2	0.7
Yeast extract (g/L)	K	0.2	1
KCl (g/L)	L	0.05	0.15