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Evaluation of a yeast co-culture for cellulase and xylanase production under solid state fermentation of sugarcane bagasse using multivariate approach

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ABSTRACT

Sugarcane bagasse (SB), an abundantly found lignocellulosic material in many countries, has been exploited for the production of industrially important enzymes by filamentous fungi and bacteria. Yeasts have not been reported to utilize SB, particularly under solid-state fermentation (SSF). In this work, two yeast strains, MK-157 and MK-118 were co-cultured to produce a multienzyme preparation comprised of endoglucanase (EG), β-glucosidase (BGL) and xylanase (XYL). Two Plackett-Burman Designs (PBDs) were executed separately to screen the factors affecting SSF of acid-pretreated and alkali pretreated SB for the production of multienzyme. The analysis depicted that SSF of alkali pretreated SB yielded higher titers of multienzyme than acid pretreated SB and three of the factors (temperature, inoculum size and incubation period) were exerting significant effect on the process. Consequently, the three factors were optimized by employing Box-Behnken Design (BBD). Under optimum conditions, i.e. inoculation of 0.5 ml g^{-1} yeast co-culture in alkali pretreated SB and cultivation at 35 °C for 94 h gave the EG titers of 9.81 IU mL^{-1} that was comparable to the predicted value of 9.61 IU mL^{-1}. Gravimetric analysis of untreated, pretreated and fermented SB showed that cultivation of the yeasts resulted in decrease in the quantity of cellulose, indicating utilization of this component. Moreover, alkaline pretreatment caused more delignification and disruption of the structure that was evident from scanning electron (SE) micrographs. SE microscopy also revealed that there were more changes in the structure of SB when cultivated with co-culture than with mono-culture. Furthermore, the fermented SB by co-culture adsorbed more dye (congo red) than the other SB indicating formation of pores in SB by the yeasts.

1. Introduction

Lignocellulosic (LC) biomass, a renewable source of chemicals and energy, is the major component of waste generated by agricultural activities. The availability of carbohydrates in huge quantities in LC biomass and its renewability have rendered attention of researchers to exploit the material for the production of industrially important enzymes (Romero-Gomez, 2014). Several agro-industrial wastes have been reported as potential feedstock including straws, brans, peels and bagasse, the choice of which depends on local agricultural practices (Ferreira et al., 2016). One of such waste generated from sugar industry, sugarcane bagasse (SB), has extensively been studied for its suitability as raw materials for biorefinery purposes.

The major proportion of annual production (328 Tg) of *Saccharum officinarum* (SC) is shared by Asian and South American countries (Kim and Dale, 2004), hence, SB offers tremendous potential as raw material in these regions. SB is generated as fibrous residue after crushing and extraction of juice from SC (Sindhu et al., 2016) and usually remains under-utilized, either used as fuel for factory boilers or for chip-board making. It is estimated that annually, 15 million tons of SB is produced in Pakistan (Arshad and Ahmed, 2016). With 50–75% fermentable sugars, high moisture level and less ash content, SB can serve as an excellent raw material for many fermentation industries.

Recalcitrant nature of SB necessitates effective pretreatment methods which are aimed to remove lignin seal (Sun et al., 2016; Binod et al., 2011) in order to make fermentable components available to microorganisms. Several pretreatment methods have been studied for the delignification of SB, where the use of alkali (Sindhu et al., 2014) and formic acid found to be more effective. Subsequent to pretreatment, SB can be converted to simple sugars by the activity of cellulolytic and xylanolytic organisms.

A large body of literature describes the production of cellulases and xylanase from filamentous fungi (Andersen et al., 2016; Do Vale et al., 2014) and bacteria (Gilbert and Hazlewood, 1993) using crude LC substrates. Whereas, the potential to produce cellulase or xylanase is not commonly found among yeast strains. Earlier, out of more than 350

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yeast strains, Gomes et al. (2015) reported cellulolytic and xylanolytic activities in only 67 and 154 strains, respectively. The major genera included *Cryptococcus, Fellomyces, Myriangiale* and *Ocultifer*. Very few of the strains could elaborate cellulolytic and xylanolytic activities, simultaneously. However, the exploitation of yeasts for the production of plant cell-wall degrading enzymes (PCWDE) can offer several advantages. For instance, yeasts can be used for the co-production of PCWDE, single cell protein, ethanol and few other related value added products in a relatively shorter period of time. Unlike filamentous fungi, they do not produce hyphae or pellets and hence do not cause rheological problems during fermentation (Ferreira et al., 2016). It is also noteworthy that suitability of yeast for consolidated bioprocessing or as an alternative to *Trichoderma* sp. has not been established so far.

Microbial strain that can be employed for the consolidated processing of biomass has yet to be isolated or developed. Therefore, metabolic engineering has been exploited for the development of the strains that can utilize most of the fragments of LC biomass, particularly, xylose from xylan. However, such strains suffer with the inhibition pose by chemicals generated during pretreatment of LC biomass and hence, these need to be engineered with the pathways to tolerate toxic compounds (Chen and Duo, 2016). Alternatively, co-culture based LC fermentation can be carried out to avoid engineering complex pathways to a strain. Various strains of filamentous fungi have been co-cultured on LC substrates especially under solid state fermentation (SSF) to produce industrially important enzymes (Rehman et al., 2014). Indeed, hyphal mode of growth with their good tolerance to low water activity (a_w) and high osmotic pressure make filamentous fungi suitable organisms for bioconversion of crude substrates under SSF (Raimbault, 1998). Whereas, yeasts based SSF processes have less frequently been reported. The present work describes utilization of SB under SSF by a co-culture of yeasts. The factors affecting multienzyme preparation containing cellulase and xylanase activities by the co-culture were optimized using statistical tools.

Statistical tools for design of experiment (DoE) employing multivariate analysis have become integral part of optimization of various bioprocesses (Pirzadah et al., 2014). The tools focus to minimize expenses incur during optimization processes by bringing down the number of experiments and keeping statistical relevance intact (Wadle et al., 2016). These tools predict about better response from a process by investigating the influence of different types of parameters and the interaction between them and by providing the optimum combination of parameters (Radhika and Raghu, 2014). DoE, including Plackett-Burman followed by Response Surface Methodologies (RSM) are popularly used to avoid limitations of traditional optimization process.

2. Materials and methods

2.1. Yeast strains and cultivation

Two yeast strains (*Saccharomyces cerevisiae* MK-157 and *Candida tropicalis* MK-118) were obtained from the culture collection of the Department of Microbiology, University of Karachi. The strain MK-157 was previously studied for the production of EG (endoglucanase) on commercially purified substrates (Shariq et al., 2018) while MK-118 was known to produce EG, BGL (β-glucosidase) and XYL (xylanase). The cultures were purified and maintained on Sabouraud's Dextrose Agar (SDA). The compatibility of the two strains to grow in a co-culture was studied by cross-streaking on SDA plates and observing the growth after incubation for 4 days 30 and 40 °C.

For enzyme production, inoculum was prepared by separately inoculating an isolated colony of MK-157 and MK-118 in SDB (Sabouraud's dextrose broth) and incubating the cultures at their suitable temperature (30° for MK-157 and 40 °C for MK-118) for 48 h with shaking at 150 rpm. The density of the cultures was determined by taking optical density (OD) at 600 nm and maintained at 1.0 OD for transfer to the production medium. For mono-culture, 0.25 or 0.5 ml g^{-1} of the inoculum of the single strain was used whereas, for co-culture inoculums of the two strains were mixed in equal quantities.

2.2. Preparation of the substrate

Sugarcane bagasse (SB) was obtained locally and chopped into 1–2 cm pieces. It was washed excessively with tap water to remove chemical contaminants. After solar drying, it was dried at 60 °C for overnight and ground to 100 µm mesh size. Pretreatment was carried out separately with 1% sulfuric acid or 1% sodium hydroxide by loading SB at the rate of 50 ml g^{-1} at room temperature for 24 h in acid or alkali. The residual acid or alkali was removed by washing the slurry with tap water until pH of filtrate became same as that of tap water. The slurry was oven dried at 60 °C and clumps were broken down by grinding.

2.3. Fermentation

Solid-state fermentation (SSF) was conducted in 100 ml conical flask containing 1 g of untreated or pretreated SB. The substrate was sterilized by autoclaving; inoculated with 0.5 ml of the mono- or co-culture and was moistened to 50 or 80% using 1% (w/v) peptone or yeast extract medium as a nitrogen source. The flasks were incubated under conditions stated in statistical designs.

The crude enzyme preparation was extracted by keeping the fermented SB in presence of 10 ml of 50 mM sodium citrate buffer containing 0.05% (v/v) tween 80 in an orbital shaker at 100 rpm for 1 h followed by centrifugation at 3000 × *g* for 20 min. Cell free culture supernatant (CFCS) was assayed for the activity of EG, BGL and XYL.

2.4. Statistical design

In the first step of optimization, Plackett-Burman design (PBD) was employed for screening of significant factors (see below) that affected the production of EG, BGL and XYL from co-culture of the yeasts MK-157 and MK-118 under SSF of SB. Two separate PBDs consisting of 16 different experiments were generated using Minitab17 software, one for acid pretreatment and the other for alkali pretreated SB. Seven factors were screened at two levels including temperature (30, 35 °C), pH (5, 7), source of nitrogen (Yeast extract, peptone), inoculum size (0.25, 0.5 mg mL^{-1}), incubation time (48, 72 h), pretreatment (with acid/alkali or untreated) and moisture contents (50, 80%). After performing experiments, CFCS were obtained and assayed for EG, BGL and XYL activities and IU mL^{-1} of the enzymes were taken as response. After analysis of all the experimental runs of PBD, three factors appeared as significant factors (temperature, inoculum size and incubation time) affecting the production of EG and BGL by the yeast co-culture under SSF of alkali pretreated SB. These significant factors were optimized by adopting Box-Behnken Design (BBD) as a RSM approach. The design consists of three factors (see below) at three levels was executed in 15 experimental runs as prescribed by Minitab 17 software. XYL was not included in the RSM because none of the factors found to be significant for its production.

All the experiments were conducted in triplicate and the mean values were used in calculations. For PBD as well as for BBD, the enzyme activities in CFCS were taken as response.

2.5. Enzyme assays

EG, BGL and XYL activities in CFCS were measured by adding 25 µl of crude enzyme, separately, to each test tube containing 0.5% (w/v) carboxymethyl cellulose (CMC), salicin or beechwood xylan, respectively. The reaction mixture was incubated at 37 °C for 15 min. After incubation, 150 µl DNS reagent was added and boiled for 5 min and then cooled down on ice for 10 min. It was then followed by addition of 720 µl distilled water and OD_{550} was measured against blank and