Biochemical Engineering Journal 137 (2018) 247–254

Contents lists available at ScienceDirect

Biochemical Engineering Journal

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

Regular article

Leveraging pH profiles to direct enzyme production (cellulase, xylanase, polygalacturonase, pectinase, α-galactosidase, and invertase) by *Aspergillus foetidus*

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ARTICLE INFO

Article history:
Received 7 January 2018
Received in revised form 23 May 2018
Accepted 8 June 2018

Keywords:
Aspergillus foetidus
Cellulase
Xylanase
Pectinase
α-Galactosidase
Soybean hull

ABSTRACT

Aspergillus foetidus was found to have different optimal pH conditions for producing different carbohydrases including cellulase, xylanase, pectinase, α-galactosidase, polygalacturonase, and invertase. Designs to trigger these conditions sequentially using controlled pH gradients were evaluated for directing the culture through multiple production stages optimal for different enzymes. For production of enzyme mixtures with particularly high pectinase and α-galactosidase activities, for more effective hydrolysis of the complex carbohydrate in soybean meal, the best method tested was a pH gradient started at pH 7.0, decreased to pH 6.0 over 72 h, held constant for 24 h, and then decreased to pH 5 over 24 h.

1. Introduction

Effective utilization of renewable resources is a prerequisite to ensure a sustainable future for the continuously growing human population. The biorefinery concept that integrates fuel and chemical production from biomass contributes to this endeavor. Lignocellulosic biomass obtained as agricultural by-products includes an abundant and inexpensive group of renewable resources. Enzymatic processing of these resources to simple sugars can open up a wide range of biorefinery opportunities [1]. These enzymatic processes, however, have been found to rely on effective biomass pretreatment and optimal mixtures of multiple enzyme activities [2]. This requirement of multiple enzymes to hydrolyze all types of carbohydrates present in the biomass can make biorefinery expensive, compared to the petroleum-based processes [3]. Usually these enzymes are produced from different sources and then blended into cocktails and evaluated for hydrolysis effectiveness for particular biomass. If the optimal enzyme mixtures can be produced from a single fermentation using one strain/species and the enzyme composition can be adjusted to suit different biomass sources, the biorefinery process can be significantly more economical.

We have considered the enzymatic processing of soybean meal as an effective way of adding value to the soybean industry, by hydrolyzing carbohydrates to simple sugars that dissolve in aqueous hydrolysate and producing enriched protein that remains insoluble and is easily separable from the hydrolysate. Soybean meal is well-known for its value as a protein source. Along with its 50% protein content, it also has high carbohydrate content (about 30%) [4–6]. The carbohydrate includes polysaccharides (about 60%) and oligosaccharides (about 40%) [7,8]. The polysaccharides consist mainly of pectin, cellulose and hemicellulose. Presence of the carbohydrate reduces the value of protein in soybean meal [9–12]. It is therefore desirable to separate carbohydrate from protein. Not only does this separation increase the protein content but also improves the nutritional quality of soy protein products due to removal of indigestible carbohydrate [13]. This carbohydrate-protein separation can be achieved by enzymatic hydrolysis and resultant solubilization of carbohydrate while keeping protein largely insoluble in the hydrolysate [14]. In addition, the hydrolysate with largely monomerized sugars is more valuable as fermentation substrate for biofuel and biochemical productions [15–17].

Enzyme hydrolysis has been accepted as the most environmentally friendly technology for the conversion of carbohydrate in biomass into monomeric sugars. An enzyme mixture having multiple activities is required to achieve more complete hydrolysis of the complex carbohydrate present in lignocellulosic biomass. Moreover, lignocellulosic biomass of different origins have different

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https://doi.org/10.1016/j.bej.2018.06.008
1369-703X/

carbohydrate compositions and, therefore, require enzymes of different activities to achieve more effective and complete hydrolysis [18]. Such enzymes with multiple activities in defined proportions are difficult to obtain from a single source or production process. Therefore, in many applications, enzymes from multiple origins are blended to achieve desired degradation of carbohydrate in biomass [19,20]. Nevertheless, it can be highly economically advantageous to produce an effective enzyme mixture in a single fermentation, over the use of blended enzymes produced from multiple fermentation processes.

Development of soy-based biorefinery is an industrially important example that requires a complex mixture of enzymes to degrade all types of carbohydrate present in the soybean meal. In an earlier work, we studied the production of enzymes with cellulase, xylanase and polygalacturonase activities by *Trichoderma reesei* fermentation using soybean hull as substrate [21]. It was later found that complete hydrolysis of soybean meal carbohydrate would require enzymes containing at least cellulase (in terms of Filter-Paper Unit FPU), xylanase, pectinase (including polygalacturonase) and α-galactosidase [22]. More specifically, by modelling the enzyme hydrolysis of soybean meal carbohydrate, the half-maximum (Michaelis-Menten) constants K_m for these enzymes were estimated at 27.5 FPU/(g meal) for cellulase, 3.15 U/(g meal) for xylanase, 14.7 U/(g meal) for pectinase, and 1.16 U/(g meal) for α-galactosidase. These K_m values indicate that effective hydrolysis of soybean meal carbohydrate requires much higher activities of pectinase and α-galactosidase, along with cellulase and xylanase, than those produced by the *T. reesei* fermentation [22]. *Aspergillus* species have been reported to produce higher pectinase and α-galactosidase activities [23–25]. The objective of this study is to produce enzymes with improved proportions of cellulase, xylanase, pectinase, α-galactosidase and invertase for more effective hydrolysis of all carbohydrate types in soybean meal, using a single *A. foetidus* fermentation. There has not been any reported study for directed production of complex enzyme mixtures with specific activity ratios in a single fermentation process.

Enzyme production by *Aspergillus* species is sensitive to the nutrient and environmental conditions, and the reported optimal conditions vary significantly for different enzyme activities, substrates and/or species/strains used. Some examples are given here. In a study of cellulase production from coir waste by submerged fermentation and solid-state fermentation using an *A. niger* strain isolated locally from a coir retting ground, pH 6 and 30 °C were reported as the optimal conditions [26]. On the other hand, production of endoglucanase and β-glucosidase (major components of cellulase) by solid-state fermentation of *A. niger* MS82 on grass and corn based lignocellulosic materials was reported to be optimal at pH 4.0 and 30–35 °C and 25 °C, respectively, with the highest endoglucanase production occurring during the growth phase and β-glucosidase production during the stationary phase [27]. For xylanase production, the optimal condition was reported as pH 5.6 and 35 °C for submerged fermentation of an *A. niger* strain growing on ground wheat bran, corn cob and sugarcane bagasse, separately [28]. For polygalacturonase and pectinesterase production at 30 °C, the optimal pH was different at 4.1 and 6.5, respectively, for submerged fermentation of *A. niger* No. 300 (isolated from moldy citrus fruit peels) using pectin as substrate [29]. On the other hand, for pectinase production by solid-state fermentation of *A. niger* MTCC-281 on wheat bran and sugarcane bagasse, the optimal condition was reported as pH 5 and 40 °C [30]. For α-galactosidase production, the optimal pH was reported at 5–6 for solid-state fermentation of *Aspergillus foetidus* ZU-G1 at 28 °C using mixed wheat bran (83.3%) and soybean meal (16.7%) as substrate [31]. For invertase, effective production was reported at pH 5.0 and 30 °C by submerged fermentation of *Aspergillus fumigatus* (isolated from a sugarcane field in India) using fruit peel waste (orange, pineapple and pomegranate) as substrate [32].

Since the above literature reports suggested that *Aspergillus* produce different enzyme activities at different optimal pH, it was hypothesized that unique pH schemes may be designed in a single fermentation to direct production of enzymes with multiple activities in desired proportions. In this study, we achieved an effective strategy of controlling pH in a submerged fermentation to produce a desired enzyme mixture for hydrolysis of all types of carbohydrate in soybean meal. Patterns of production of different activities, including cellulase, xylanase, pectinase, α-galactosidase and invertase in fermentations controlled at different constant pH values were first established. The pH control schemes were then designed and the enzyme production under these schemes evaluated and further optimized by setting critical control points during the fungal fermentation.

2. Materials and methods

2.1. Culture, chemicals and equipment

The *Aspergillus foetidus* strain NRRL 341 used in this study was obtained from the United States Department of Agriculture (USDA) Agricultural Research Service (ARS) Culture Collection. Unless otherwise specified, chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). Proteose peptone was from Remel Microbial Products (division of Thermo Fisher Scientific). Soybean hull and flour were provided by Archer Daniels Midland Company (Decatur, IL, USA). The main equipment used included a UV–vis spectrophotometer (Shimadzu UV-1601, Colombia, MD); an orbital shaker (Thermo Scientific SHKA5000-7); 2 fermentors with controls for pH, dissolved oxygen concentration (dO2), agitation and temperature (BioFlo 110, NewBruswick Scientific, Edison, NJ); a water bath (Boekel Scientific ORS-200); and a micro centrifuge (Eppendorf Centrifuge 5415D).

2.2. Cultivation

The *A. foetidus* culture was maintained on potato dextrose agar (PDA). The shake flask medium used for seed culture preparation was a modified Mandels and Weber medium [33]. The medium composition was: 1.4 g/L $(NH_4)_2SO_4$, 2.0 g/L KH_2PO_4, 0.3 g/L $MgSO_4{\cdot}7H_2O$, 0.4 g/L $CaCl_2{\cdot}2H_2O$, 0.3 g/L urea, 1.0 g/L proteose peptone, 0.2 g/L Tween 80, 20 g/L soybean hull, 0.005 g/L $FeSO_4{\cdot}7H_2O$, 0.0016 g/L $MnSO_4{\cdot}H_2O$, 0.0014 g/L $ZnSO_4{\cdot}7H_2O$, and 0.002 g/L $CoCl_2{\cdot}2H_2O$. Three wire-loops of culture on PDA were added to 50 mL medium in a 250 mL shake flask. This seed culture was incubated under 250 rpm shaking for 2 days at room temperature with a cheesecloth covering.

The seed culture was used to inoculate the stirred tank fermentors at 10% (v/v). The fermentors were operated with 1–1.5 L working volume under controls of dO2, pH, agitation, temperature and foaming, the last by automatic addition of Trans-278 (Trans-Chemco, Inc., Bristol, WI). The production medium was same as the seed culture medium, unless specified otherwise. In all of the fermentation experiments, the dO2 was maintained at above 20% air saturation (i.e., ~1.6 mg/L) by automatic supplementation of pure oxygen to the influent air. The influent air stream was sterilized by being passed through a sterile 0.22-μm in-line disc filter. The agitation rate was maintained at 350 rpm. pH was controlled by automatic addition of 1.0 M NaOH and 1.0 M HCl. Daily samples were taken for enzyme analysis. Samples were centrifuged at 10,000 *g* for 10 min to remove the solid biomass, and the supernatants collected were stored at −20 °C prior to analysis.