Biomass and Bioenergy 81 (2015) 234–241

Contents lists available at ScienceDirect

Biomass and Bioenergy

journal homepage: http://www.elsevier.com/locate/biombioe

Research paper

Covalent immobilization of enzymes and yeast: Towards a continuous simultaneous saccharification and fermentation process for cellulosic ethanol

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ARTICLE INFO

Article history:
Received 18 August 2014
Received in revised form
19 May 2015
Accepted 9 July 2015
Available online xxx

Keywords:
Cellulosic ethanol
Covalent immobilization
Plasma treatment
Continuous saccharification and fermentation

ABSTRACT

The immobilization of enzymes and yeast cells is a key factor for establishing a continuous process of cellulosic ethanol production, which can combine the benefits of a separated hydrolysis and fermentation process and a simultaneous saccharification and fermentation process. This paper investigates the use of cellulase enzyme and yeast cell immobilization under a flow regime of ethanol production from soluble substrates such as cellobiose and carboxymethyl cellulose. The immobilization was achieved by incubating enzymes and yeast cells on polystyrene surfaces which had been treated by nitrogen ion implantation. The saccharification by immobilized enzymes and the fermentation by immobilized yeast cells were conducted in two separate vessels connected by a pump. During the experiments, glucose concentrations were always maintained at low levels which potentially reduce product inhibition effects on the enzymes. Covalent immobilization of enzymes and yeast cells on the plasma treated polymer reduces loss by shear flow induced detachment. The potential for continuous flow production of ethanol and the influence of daughter yeast cells in the circulating flow on the immobilized enzyme activity are discussed.

1. Introduction

In ethanol production from cellulosic biomass based on enzymatic catalysis, there are three common designs of processes: consolidated bioprocessing (CBP), separated hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). CBP is a process in which enzyme production, cellulose hydrolysis and fermentation are all accomplished by microorganisms [15]. Apart from having fermentation capability, the microorganisms selected for CBP must be capable of expressing enzymes for hydrolysing cellulose (i.e. cellulases) as well as enzymes for converting glucose into ethanol. Microorganisms that are efficient in carrying out all of these processes are rare in Nature and must be created by genetic engineering [11]. SHF is a simple process in which the hydrolysis step occurs before fermentation. The separation of these two steps allows the optimum operation of each step to be achieved [20]. However, the separation of these two steps also causes the accumulation of hydrolysis products in the hydrolysis vessel which inhibit enzyme activity, consequently reducing the reaction rate over time [1].

To overcome the disadvantages of both of these processes, Takagi et al. [19] proposed a SSF process in 1977 and since then, it has been widely used for studying cellulosic ethanol production. In the SSF process of Takagi et al., enzyme hydrolysis and fermentation occur at the same time and in the same vessel. The simultaneous saccharification and fermentation enhances the hydrolysis efficiency by reducing the accumulation of hydrolysis products that would otherwise impede the activity of the key enzymes. Ghosh et al. [7] reported an increase in hydrolysis rate by 13–30% by using SSF compared to a normal saccharification process that allows glucose to accumulate. Ohgren et al. [14] also reported a 13% higher yield of ethanol from the SSF process compared to SHF process. However, some disadvantages of the SSF process have been reported in the literature. Ooshima et al. [16] found that when the ethanol concentration exceeded 0.2 M, it disturbed the adsorption of exoglucanase on cellulose and depressed the saccharification rate. In addition, the presence of cellulase enzyme mixtures in the same vessel significantly influences yeast growth [17]. Since the hydrolysis and fermentation steps have so far been carried out in

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http://dx.doi.org/10.1016/j.biombioe.2015.07.009
0961-9534/

the same vessel, the process is often operated in the temperature range between 30 and 40 °C due to the restricted thermostability of yeast. Removing this constraint by separating the yeast from the enzyme processes in separate vessels would enable the use thermophilic enzymes to achieve higher activities by allowing operation at higher temperatures. Here we report on the operation of such a process in which the yeast and enzyme reaction steps are carried out in separated zones, while allowing a continuous flow process to be operated.

The proposed separated continuous simultaneous saccharification and fermentation (continuous SSF) process is shown in Fig. 1. In this process, cellulosic biomass is continuously pre-treated and pumped to the hydrolysis vessel where a mixture of cellulases converts it into fermentable sugars. The fermentation substrates are pumped continuously to the fermentation vessel where the sugars are fermented to produce ethanol. Ethanol is continuously separated from the bulk by an ethanol selective membrane or continuous distillation. The remaining liquids, consisting of water and ethanol with incompletely reacted substrates, are recycled to the hydrolysis tank, and combined with newly pre-treated biomass, for further hydrolysis. The use of a circulating flow of the liquid medium allows the separation of saccharification and fermentation into two vessels while the concentrations of sugars and ethanol can be maintained stable over time, hence reducing the end product inhibition and enabling the optimum temperature to be used for each step. The activities of the enzymes used in hydrolysis and the yeast for ethanologenisis are thereby prolonged. Such a continuous SSF process would not only reduce the cost of enzymes and ethanologens but would also give higher yield as the intermediate products in the bulk are recycled. A continuous process based on immobilised agents would also require less reaction volume than a batch process, and would reduce the labour cost, minimise unproductive down time and reduce the production of waste water [4,8].

The most important requirements for a continuous SSF are the maintenance of a stable population of active enzymes and ethanologen organisms inside the reactors. Under continuous flow, enzymes and yeast cells would normally be washed away quickly, reducing the activity of the reaction vessels over time. Therefore, a key prerequisite for a continuous SSF is strong immobilization of both enzymes and yeast.

The immobilization of cellulose enzymes (enzyme celB and β-glucosidase) and yeast cells on plasma activated polymers has been studied in our previous works [13,21,22]. The polymer surfaces were activated using a plasma immersion ion implantation (PIII) technique to create radicals that remain embedded in the ion implanted surface layer for a long period after treatment. Through reactions with these highly reactive radicals, the PIII treated polymer is able to form covalent bonds with side chain groups of biomolecules, providing strong surface immobilisation while preserving biomolecule activity [2,3]. In this paper, we demonstrate the usefulness of this type of immobilization for the implementation of circulating flow continuous SSF. Two separate experiments were conducted with two soluble celluloses. In the first experiment, we demonstrate that immobilized β-glucosidase and yeast cells can be used to convert cellobiose into ethanol. In the second experiment, we use two immobilized enzymes (celB and β-glucosidase) working together to hydrolyse carboxymethyl cellulose and use immobilized yeast cells to convert fermentable sugars into ethanol. Corresponding saccharification experiments were carried out at the same time with the circulating SSF to estimate the effectiveness of the enzyme hydrolysis.

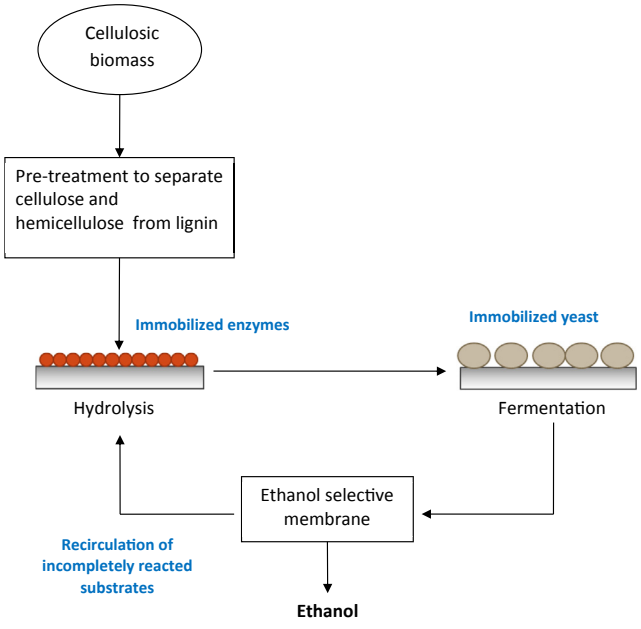

Fig. 1. A schematic diagram depicting the separated continuous simultaneous saccharification and fermentation process. The enzymatic hydrolysis and yeast fermentation steps are carried out in separate reaction vessels.

2. Materials and methods

2.1. Immobilization of enzymes and yeast cells

Polystyrene films (0.19 mm thick from Goodfellow Cambridge Ltd) were treated by plasma immersion ion implantation (PIII) and used as supports for the immobilization of enzyme and yeast. The PIII treatment was conducted in a vacuum chamber with plasma, generated in nitrogen (2×10^{-3} torr) by inductively coupled radio frequency (13.56 MHz) power. Polystyrene film (8×8 cm^2) was mounted on a conductive sample holder that was connected to a high voltage power supply. A bias of 20 kV was applied in pulses of 20 μs duration at a frequency of 50 Hz for 400 s (providing an ion implantation fluence of 5×10^{15} ions/cm^2) to accelerate ions from the plasma towards the polymer. This treatment process and its effects on polymers have been reported in literature [10].

Enzyme immobilization: thermophilic β-glucosidase and celB enzymes were cloned, expressed and purified as described in previous works [9,22]. CelB is a thermophilic endo/exo glucanase [18] which can hydrolyse cellulose to produce cellobiose. PIII treated samples were incubated with an enzyme solution in a falcon tube or petri dish while being rocked over night at room temperature. Note that these two enzymes were separately immobilized onto separate PIII treated polystyrene sheets and their quantities were modulated by varying the areas of the polymer supports used. The incubation solution containing each enzyme was prepared with an enzyme concentration of 95–100 μg/ml in appropriate buffers. We used acetate buffer 10 mM at pH 5.0 for β-glucosidase immobilisation and acetate buffer 10 mM at pH 5.5 for celB immobilisation. After incubation, samples were transferred to new containers with fresh buffer solutions and washed three times on a rocker for 20 min each to remove any unbound enzyme molecules. During incubation and washing, sample surfaces were always kept wet to guard against drying of the enzymes.

Yeast immobilization: rehydrated *Saccharomyces cerevisiae* (YSC2, Sigma Aldrich) were prepared and suspended in phosphate buffer saline [21] to obtain a cell density of approximately $5–6 \times 10^7$ cells/ml. Each side of the PIII treated samples was incubated with yeast suspension in a petri dish for 30 min. After