Bioethanol production from oilseed rape straw hydrolysate by free and immobilised cells of *Saccharomyces cerevisiae*

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Abstract

Oilseed rape (OSR) straw can serve as a low-cost feedstock for bioethanol production. Glucose and other fermentable sugars were extracted from OSR straw using sulfuric acid pre-treatment and enzymatic hydrolysis. Batch fermentation of enzymatic hydrolysate with *Saccharomyces cerevisiae* immobilised in Lentikat® was found to be superior to free cells in terms of bioethanol yield. The maximum bioethanol concentration from free and immobilised cells was 6.73 and 9.45 $g.l^{-1}$, respectively, with corresponding yields of 0.41and 0.49 g bioethanol. g $glucose^{-1}$.

Keywords: bioethanol, dilute acid pre-treatment, immobilisation, oilseed rape straw.

1 Introduction

In 2007, consumption of liquid fuels in the transportation sector was 46 million barrels per day and is expected to increase by 67 million barrels per day by 2035 EIA [1]. In 2007, the use of liquid fuels was responsible for 38% of global greenhouse gas (GHG) emissions, providing a significant contribution to climate change EIA [1]. The replacement of gasoline (petrol) with bioethanol is encouraged globally as a mechanism to reduce exposure to volatility in the oil market, and minimise the extent to which road transport contributes to global warming. Bioethanol can be produced from two different types of feedstocks: first-generation feedstocks (maize, wheat and sugarcane) and second-generation feedstocks (lignocellulosic materials such as straws, forest residue or any agriculture waste) (Balat [2]). Commercial production of bioethanol from first-generation feedstocks is limited by land availability, and concerns regarding the

WIT Transactions on Ecology and the Environment, Vol 150, © 2011 WIT Press
www.witpress.com, ISSN 1743-3541 (on-line)
doi:10.2495/SDP110771

use of land for fuel as opposed to food production. Second-generation bioethanol production from lignocellulosic material is a complex process compared to first-generation feedstocks due to the presence of lignin and hemicellulose. Additional processing steps, referred to as pre-treatment and hydrolysis are essential for extracting sugar from lignocellulosic materials. The pre-treatment process is highly energy intensive and expensive (due to enzyme application during hydrolysis), which means the production of second-generation bioethanol is currently non-competitive to first-generation bioethanol (Lora *et al.* [3]). Consequently bioethanol produced from second-generation feedstocks is the focus of considerable research and development.

Global cultivation of OSR was 31 million ha in 2009. Assuming a straw yield of 1.5 – 3.0 tonnes per ha (Newman [4]), the amount of OSR straw produced in 2009 was between 46.5 and 93.0 million tonnes. Assuming a bioethanol yield of 270 l tonne^{-1} (Larson [5]) of straw (using existing technology) it is predicted that between 12.5 and 25.0 billion liters of bioethanol could have been produced from OSR straw. Currently OSR straw does not have an existing market and is normally ploughed back into field. Hence bioethanol production from OSR straw could add value to existing crops.

Dilute acid pre-treatment is one of the most commonly used pre-treatment techniques for altering the structure of lignocellulosic materials (Moiser *et al.* [6]). It mainly breaks the structure of hemicellulose and a small portion of lignin. Dilute acid pre-treatment also leads to the formation of fermentation inhibitors such as acetic acid, hydroxymethylfurfural (HMF) and furfural as a result of sugar degradation (Palmqvist and Hahn-Hagerdal [7]). Dilute acid pre-treatment has been widely studied for a range of feedstocks. Jeong *et al.* [8] optimised the dilute acid pre-treatment of OSR straw based on the extent to which hemicellulosic sugars (mainly xylose, mannose and galactose) were extracted. Under optimum pre-treatment conditions (1.76% H_2SO_4, 152.6^{o}C for 21 min) 85.5% of total sugars were recovered from OSR straw. The inhibitors present in the pre-treated hydrolysate were acetic acid (2.94 g l^{-1}), 5-hydroxymethylfurfural (0.04 g l^{-1}) and furfural (0.98 g l^{-1}). Subsequent enzymatic hydrolysis resulted in a digestibility of 95.4% after 72 h, compared to a digestibility of 27.1% for untreated OSR straw. Castro *et al.* [9] optimised the dilute acid pre-treatment of OSR straw using pre-treatment temperatures between 140 and 200^{o}C, pre-treatment times between 0 and 20 min and sulfuric acid concentrations between 0.5 and 2.0% (w/w). A mathematical model was used to predict the pre-treatment conditions that would result in a cellulose conversion efficiency of approximately 100%. The optimum conditions were predicted to be the application of temperature at 200^{o}C for 27 min at an acid concentration of 0.40%. Mathew *et al.* [10] studied the dilute acid pre-treatment of OSR straw based on the concentration of glucose recovered after enzymatic hydrolysis. Under optimum pre-treatment conditions (5% (w/w) biomass loading, 2.5% (w/w) acid concentration and 90 min pre-treatment time) 81% of glucan was converted into glucose after 72 h of enzymatic hydrolysis.

The production of bioethanol using immobilised cells has been well studied. However, previous research has focused on the use of either sugar cane or starch

hydrolysate as substrate. The advantages of cell immobilisation over free cell fermentation for bioethanol production include a higher volumetric productivity due to higher cell density, enhanced yield and cell viability for repeated cycles of fermentation (Swain *et al.* [11]). The research presented compares the bioethanol yield and volumetric productivity obtained from the batch fermentation of OSR straw hydrolysate using free and immobilised cells of *S. cerevisiae.*

2 Materials and methods

2.1 Microorganism and media

S. cerevisiae Type I was grown at 30°C and maintained on agar slants at 4°C as described by Liu *et al.* [12]. *S. cerevisiae* was cultivated in 150 ml conical flasks with 50 ml growth medium (Glucose, 5.0; yeast extract, 0.5; peptone, 0.5; K_2HPO_4, 0.1; $MgSO_4$. $7H_2O$, 0.1; expressed in g 100 ml^{-1}) and incubated at 30°C in a shaking incubator at 150 rpm. After overnight incubation (10^8 cells ml^{-1}) cells were harvested by centrifugation at 4000 rpm for 15 min and resuspended in 10 ml growth medium.

2.2 Sugar extraction from OSR straw

OSR straw was pre-treated using the optimum dilute sulfuric acid pre-treatment conditions determined previously (Mathew *et al.* [10]). Following pre-treatment, the slurry was filtered, washed with purite water and the liquid fraction collected. The solid fraction was used for enzymatic hydrolysis using cellulase from *Trichoderma ressei* ATCC 26921 (25 FPU g^{-1} biomass) and β-glucosidase from Aspergillus niger (70 CBU g^{-1} biomass) for 72 h, at 50°C and 5% biomass loading using sodium citrate buffer (pH 4.8). The hydrolysate was filtered using No.1 Whatman filter paper and liquid fraction was used for fermentation.

2.3 Immobilisation of yeast cells

Lentikat® was obtained from geniaLab (Germany) and prepared for immobilisation according to Bezbradica *et al.* [13] after melting at 90 ± 3°C. *S. cerevisiae* cells were immobilised into Lentikat® by mixing 10 ml of *S. cerevisiae* cell suspension with 40 ml of Lentikat® liquid. The mixture was extruded onto petri dishes through a syringe fitted with a needle (1.25 x 40 mm). The petri dishes were left to dry in a laminar flow cabinet under a downwards vertical airstream at room temperature for approximately 2 h. The Lentikat® discs were stabilised and re-swollen in 100 ml stabilising solution (geniaLab, Germany) for 2 h. Lentikat® immobilised *S. cerevisiae* were allowed to proliferate through overnight incubation in 100 ml growth medium according to Liu *et al.* [12]. Lentikat® was selected as an immobilisation support because of its mechanical strength.

2.4 Fermentation conditions

2.4.1 Bioethanol production from enzymatic hydrolysate using free and immobilised cells of *S. cerevisiae*

Batch fermentation was completed using free and Lentikat® immobilised cells of *S. cerevisiae* in 150 ml sterile conical flasks with 50 ml of fermentation medium (enzymatic hydrolysate with glucose concentration of 16 – 19 g l^{-1}) for 24 h at 30 ± 3°C and 150 rpm. The fermentation medium was composed of glucose, yeast extract 0.5 g 100 ml^{-1}; peptone, 0.5 g 100 ml^{-1}; K_2HPO_4, 0.1 g 100 ml^{-1} and $MgSO_4.7H_2O$, 0.1 g 100 ml^{-1}. The fermentation medium was inoculated (10% w/v) either with immobilised or free cells. Samples (1 ml) were withdrawn from the fermentation medium after 2, 4, 6, 10 and 24h, and analysed to determine the glucose and bioethanol concentration. Batch fermentations were completed in triplicate.

2.4.2 Bioethanol production by immobilised cells of *S. cerevisiae* using acid pre-treatment and enzymatic hydrolysate as substrate

Batch fermentation was completed using Lentikat® immobilised cells of *S. cerevisiae* as per the method presented in section 2.4.1, but the fermentation medium was composed of the liquid fraction collected immediately following pre-treatment and the enzymatic hydrolysate. The sugars present in the pre-treated hydrolysate were xylose, 7.46 g l^{-1}; glucose, 1.77 g l^{-1}; galactose, 1.35 g l^{-1}; arabinose, 0.85 g l^{-1}. The glucose concentration of the fermentation medium was adjusted to 23 g l^{-1} using pure glucose.

2.5 Analytical methods

The concentration of bioethanol and glucose present in the fermentation media was analysed using HPLC fitted with a refractive index detector. HPLC analysis was completed according to NREL [14] laboratory analytical procedure.

2.6 Statistical analysis

Statistical analysis was completed using Genstat 13th edition. The effect of immobilisation of *S. cerevisiae* in Lentikat® supports and free cells was analysed separately by using one-way analysis of variance (ANOVA).

3 Results

3.1 Bioethanol production from enzymatic hydrolysate using free and immobilised cells of *S. cerevisiae*

Batch fermentation was completed using glucose extracted from the enzymatic hydrolysis of OSR straw as carbon source. Batch fermentation was completed over a 24 h time period and employed either free or Lentikat® immobilised cells of *S. cerevisiae* (Figure. 1). The concentration of bioethanol produced after 24 h of fermentation was approximately 40% higher ($p = 0.021$) for immobilised cells

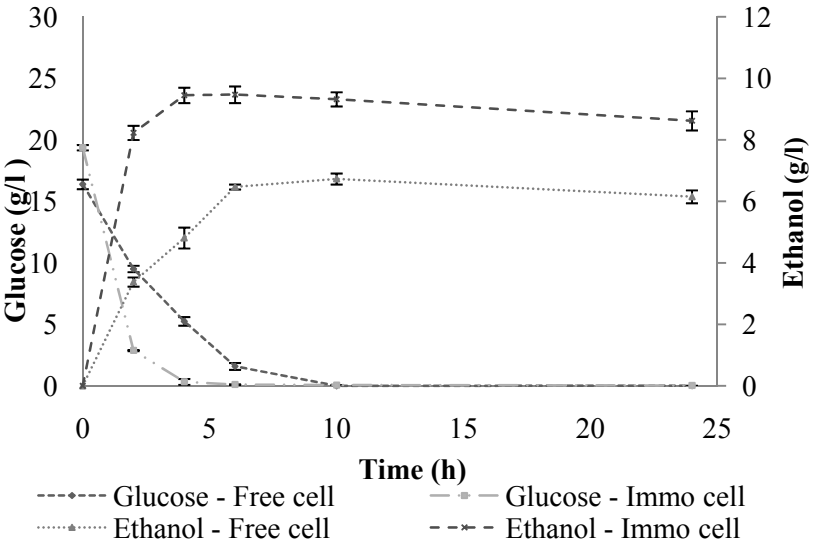

Figure 1: Glucose consumption and ethnaol production using free and immobilsed cells of *S. cerevisiae*.

(8.62 ± 0.31 g l^{-1}) compared to free cells (6.15 ± 0.21 g l^{-1}). This finding is supported by Swain *et al.* [11], who demonstrated a 6.7% increase in bioethanol yield when mahula flowers were fermented with cells immobilised in calcium alginate as opposed to free cells. Glucose was fully consumed within the 24 h fermentation time period; hence a lower bioethanol yield cannot be attributed to a reduced level of glucose uptake by free cells. The conversion of glucose to bioethanol was significantly faster with immobilised cells than with free cells. A maximum bioethanol concentration of 9.45 ± 0.25 g l^{-1} was achieved within 4 h of fermentation when immobilised cells were used. This represents a glucose conversion efficiency of approximately 98% compared to 67% glucose conversion by free cells in the same time period. The final biomass concentration recorded in Swain *et al.* [11] was higher in free cell fermentation, suggesting glucose may have been diverted from bioethanol production in order to support cell growth. It took approximately 24 h for free cells to consume 99% of the initial glucose present in the medium, demonstrating cell immobilisation resulted in significantly enhanced volumetric productivity. The volumetric productivity of bioethanol fermentation using immobilised *S. cerevisiae* cells was 4.12 g l^{-1} h^{-1} compared to 1.69 g l^{-1} h^{-1} for free cells after 2 h. The volumetric productivity reduced to 0.26 g l^{-1} h^{-1} and 0.36 g l^{-1} h^{-1} for free and immobilised *S. cerevisiae* after 24 h of batch fermentation due to a lower concentration of glucose present in the fermentation medium. At the initial stages of batch fermentation (until 4 h), the volumetric productivity of immobilised *S. cerevisiae* cells was found to be approximately two to three times higher than that of free cells. According to Nedovic and Willaert [25], immobilised cells have less

hydrodynamic and mechanical stress compared to cells in suspension, which enables the cells to utilise more cellular energy for product formation, resulting an improvement in volumetric productivity.

A decrease in bioethanol concentration was observed with free cells and immobilised cells between 6 and 24 h of fermentation. However statistical analysis suggested this was not a significant reduction ($p = 0.289$ for free cells and $p = 0.224$ for immobilised cells). The same trend was observed in a study conducted by Kuhad *et al.* [15], where the bioethanol concentration and bioethanol yield reduced between 16 and 24 h fermentation. The reduction in bioethanol concentration may be due to the oxidation of bioethanol to acetic acid (Christensen *et al.* [16]) or due to the formation of other by-products such as glycerol and butyric acid (as a result of contamination) (Cheng [17]). Another possible explanation is the consumption of accumulated bioethanol by *S. cerevisiae* that has adapted to simultaneously consume fermentable sugars and bioethanol (Ramon-Portugal *et al.* [18]). The maximum bioethanol yield obtained from free cells was 0.41 g bioethanol. g glucose^{-1} and that of immobilised cells was 0.49 g bioethanol. g glucose $^{-1}$. These yields are less than the stoichiometric bioethanol yield of 0.51 g bioethanol. g glucose^{-1}. The lower bioethanol yield observed may have been due to the use of glucose for cell growth during fermentation, essentially diverting substrate from bioethanol production.

The maximum theoretical bioethanol conversion (%) was found to be 80.51% after 10 h of fermentation with free cells and 95.66% after 4 h of fermentation with immobilised cells. Behera *et al.* [19] studied the production of bioethanol from mahula flowers using *S. cerevisiae* (strain CTCRI) cells either free in solution or immobilised in agar-agar or calcium alginate. Behera *et al.* [19] reported theoretical bioethanol conversions of 87%, 93% and 95% for free cell, cells immobilised in agar-agar and calcium alginate, respectively after 96 h of fermentation. Bioethanol conversion (%) with immobilised *S. cerevisiae* cells in the current study was found to be approximately equal to that of Behera *et al.* [19]. In contrast to this, Rakin et al. [20] compared bioethanol production from corn meal hydrolysate using *S. cerevisiae var. ellipsoideus* immobilised into calcium alginate and Lentikat®. *S. cerevisiae var. ellipsoideus* immobilised in calcium alginate resulted in a higher theoretical bioethanol yield of 111% compared to 77% for cell immobilised in Lentikat® discs. In comparison to free cells, immobilised cells were found to result in higher bioethanol concentrations and improved volumetric productivity of batch fermentation by increasing the local population density of cells (Yu *et al.* [21]; Najafpour *et al.* [22]).

3.2 Bioethanol production by immobilised cells of *S. cerevisiae* using acid pre-treatment and enzymatic hydrolysate as substrate

Batch fermentation was conducted over a 24 h time period using fermentable sugars extracted from pre-treatment and hydrolysis of OSR straw (i.e. acid pre-treatment and enzymatic hydrolysis hydrolysate were combined). The results are shown in figure 2. The bioethanol concentration obtained after 24 h of batch fermentation from acid and enzymatic hydrolysate fermentation medium was

8.66 ± 0.09 g l^{-1}. A maximum bioethanol concentration of 9.26 ± 0.04 g l^{-1} was obtained after 6 h of fermentation. Similarly to when enzymatic hydrolysate was used as sole substrate, there was a significant reduction ($p = 0.044$) in bioethanol concentration between 6 and 24 h of batch fermentation. There was no significant difference observed ($p = 0.873$) in the concentration of bioethanol produced when enzymatic hydrolysate only and acid pre-treatment and enzymatic hydrolysate were used as substrate, even though the initial concentration of fermentable sugars was higher in the latter.

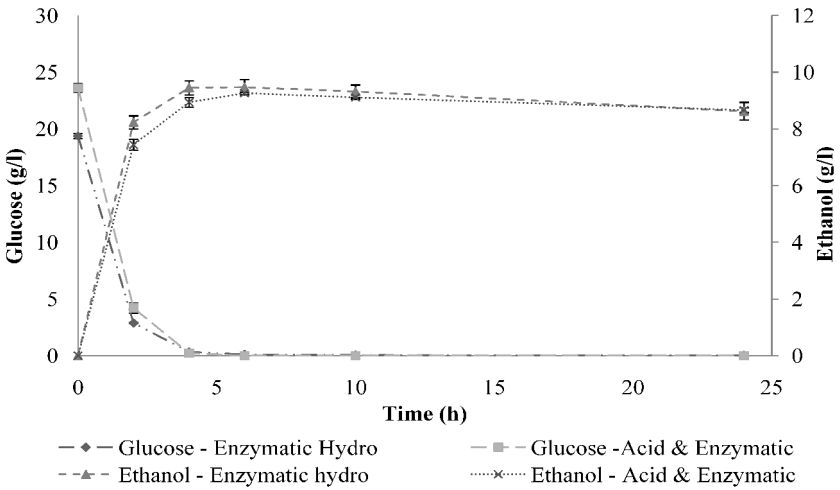

Figure 2: Glucose consumption and bioethanol production from enzymatic hydrolysate, acid and enzymatic hydrolysate using immobilised *S. cerevisiae* cells.

A bioethanol yield of 0.39 g bioethanol g $glucose^{-1}$ was achieved with immobilised cells using acid pre-treatment and enzymatic hydrolysate as substrate. A yield of 0.49 g bioethanol g $glucose^{-1}$ was obtained for immobilised cells when enzymatic hydrolysate was used as substrate. The lower bioethanol yield observed when acid and enzymatic hydrolysate was added to the fermentation medium may have been due to the presence of inhibitors such as acetic acid, hydroxymethylfurfural (HMF) and furfural that are produced during acid pre-treatment and which were present in the pre-treatment hydrolysate. These products can inhibit bioethanol production by reducing the activity of several enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase and pyruvate dehydrogenase (Modig *et al.* [23]).

The volumetric bioethanol productivity of immobilised cells when acid and enzymatic hydrolysate was added to the fermentation medium was 2.24 g l^{-1} h^{-1} after 4 h of batch fermentation. After 24 h of fermentation, the volumetric bioethanol productivity was reduced to 0.36 g l^{-1} h^{-1}. This is similar to the volumetric bioethanol productivity observed after 24 h, for free and immobilised

cells of *S. cerevisiae* using enzymatic hydrolysate (0.26 and 0.36 g l^{-1} h^{-1}, respectively). Behera *et al.* [19] reported volumetric bioethanol productivities of 0.258, 0.262 and 0.268 g l^{-1} h^{-1}, for cells free in solution, and immobilised in agar-agar and calcium alginate, respectively. The current study demonstrates higher bioethanol productivity in immobilised fermentation compared to Behera *et al.* [19] whereas the volumetric productivity of free cells was found to be similar to the study conducted by Behera *et al.* [19]. Petersson *et al.* [24] demonstrated a bioethanol productivity of 0.91 g $l^{-1}h^{-1}$ from simultaneous saccharification and fermentation of wet oxidised OSR straw. The ethanol productivity in the current study was found to be lower due to a lower concentration of glucose present in the fermentation medium initially.

From the current study, the maximum theoretical bioethanol yield from acid and enzymatic hydrolysate was approximately 76%, which is less than the maximum theoretical bioethanol yield observed from free cells in suspension using enzymatic hydrolysate as fermentation medium. Hence the current study concluded that fermentation using enzymatic hydrolysate would be better than combined use of pre-treatment and enzymatic hydrolysate for bioethanol production. Potentially the bioethanol yield obtained when pre-treatment hydrolysate is used as carbon source could be increased through the fermentation of pentose sugars.

4 Conclusion

Bioethanol production from OSR straw using dilute acid pre-treatment was studied using *S. cerevisiae* cells free in suspension and immobilised in Lentikat® discs. Batch fermentation with enzymatic hydrolysate demonstrated the immobilisation of *S. cerevisiae* cells in Lentikat® discs resulted in improved volumetric productivity and a higher concentration of bioethanol (9.47 ± 0.27 g l^{-1}) than when cells free in suspension were used (6.73 ± 0.18 g l^{-1}). Batch fermentation of pre-treatment and enzymatic hydrolysate using Lentikat® immobilised cells did not improve the concentration of bioethanol produced (9.26 ± 0.04 g l^{-1}).

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