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RESEARCH ARTICLE

THE PLACKETT-BURMAN MODEL - AN IMPROVED ALTERNATIVE TO INDENTIFY THE SIGNIFICANT FACTORS IMPLIED IN THE BIOCONVERSION OF THE COMPLEX CELLULOSIC WASTE TO ETHANOL

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Abstract

Ethanol generation from lignocellulose materials provides an alternative energy-production system. The objectives of this study were to identify the significant factors involved in the biotechnological process of waste cellulosic materials reusing bioconversion in the ethanol. The bioconversion process was conducted in two steps. The first one is the cellulose hydrolysis with the *Trichoderma reesei* commercial cellulase and the fermentation of reducing sugar with a *Saccharomyces cerevisiae* selected yeast strain with fermentative potential. By using the Plackett-Burman design of experiments model the optimized levels of significant factors for both biochemical steps were established. Based on results, the factors with positive effect are the solid/ liquid ratio, the pH, the enzyme concentration, the inoculum size, the fermentation temperature.

Keywords: bioethanol, complex cellulosic waste, enzymatic hydrolysis, fermentation, Plackett-Burman experimental design

Introduction

The utilization of fossil fuels in the form of oil, natural gas and coal, which modern society relies on for energy, contributes significantly to global warming (Rajesh *et al.* 2008). Production of ethanol (bioethanol) from biomass is one way to reduce both consumption of crude oil and environmental pollution (Balat, 2008). Bioconversion processes have been developed for the utilization of renewable resources to produce useful chemicals and feed stocks (Bahrim, 2004). Alternative fuels made from renewable resources, such as fuel ethanol, provide numerous benefits in terms of environmental protection, economic development, and national energy security. The production of fuel-ethanol from lignocellulose biomass is of growing interest around the world because it can provide a number of environmental advantages over conventional fossil fuels, most notably a reduction in greenhouse-gas emissions (Cuevas *et al.* 2010). Directive (2003/30/EC) was accepted that requests member states to establish legislation about utilization of fuels from renewable resources (Kosebent *et al.*, 2009). The importance of ethanol as a clean and safe transportation fuel has increased with the anticipated shortage of fossil fuel reserves and increased air pollution (Chen, 2007). Around 60% of the total ethanol is produced by fermentation (Kim *et al.* 2008). Ethanol can be derived from fermentation processes from any material that contains sugars or sugar precursors. Simultaneous hydrolysis and fermentation reduce the cost and increasing the yield (Lee, 2007). Many research and development efforts aimed at the commercial production of ethanol, by fermentation, from

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renewable resources such as crop residues and biomass waste, municipal solid wastes, municipal sludge, and dairy/cattle manures have increased (Kim *et al.* 2008; Olsson *et al.* 2005).
The conversion of biomass to ethanol generally includes four steps: pre-treatment, hydrolysis of polysaccharides and oligosaccharides into monomer sugars, fermentation of sugars to ethanol and, finally, ethanol concentration to absolute alcohol (to be used as motor fuel, ethanol must have a concentration of up to >99.8%) (Cuevas *et al.* 2010). The technologies consist of different pretreatment and enzymatic hydrolysis and fermentation orientations (Spatari *et al.* 2010). Lignocellulosic cell walls have a natural resistance, often called ''recalcitrance", to microbial and enzymatic deconstruction. Therefore, the biomass pretreatment is necessary to catalyze the hydrolysis of hemicellulose and make the cellulose fraction more accessible to enzymatic digestion prior to the ethanol fermentation (Scordia *et al.* 2010).

Extensive literature is available on different pre-treatment methods to enhance the susceptibility of lignocellulose materials to enzymatic hydrolysis. Diluted-acid pre-treatment, at temperatures of 120^0 C, is one of the most important procedures and has been reviewed for cellulosic materials, working with 2% sulfuric-acid. During this thermal process, the hemicellulose is depolymerized into a mixture of sugar oligomers and monomers, whereas less alteration is caused in lignin and cellulose. The removal of hemicellulose increases the porosity and therefore improves the enzymatic digestibility of cellulose. Simultaneous saccharification and fermentation processes combine enzymatic hydrolysis of cellulose with simultaneous fermentation of the D-glucose thus obtaining ethanol. The presence of yeast together with cellulase reduces the accumulation of D-glucose, thereby increasing the saccharification rate and ethanol yield. SSF also lowers capital costs and reduces the risk of microbial contamination (Cuevas *et al.* 2010).
In this study the factors affecting the biotechnological process of waste cellulosic materials reusing, were evaluated by the application of a two-level factorial Plackett-Burman design experiments model to get effect of various medium caracteristics on the substrate bioconversion rate and yield of ethanol.

Materials and methods

Waste cellulosic substrate and enzymatic hydrolysis conditions

Lignocellulosic materials used in this study are a complex waste cellulosic materials based on: office paper, newspaper and cardboard in ratio by 1:1:1 (w/w). The used materials were pretreated as follows: milled in a vibratory ball mill, autoclaving the waste cellulosic materials at 120^0 C, in wet atmosphere, with H_2SO_4, in a 2% (w/w) concentration (1/7 w/v ratio) for 24 hours, washed with distilled water until a neutral pH is reached and dried to a 80% dry mater. The enzymatic hydrolysis was carried out by using commercial cellulase, Onozuka R 10 (produced by *Trichoderma viride*), with 1 UE/mg enzymatic activity in 50 mM citrate buffer with pH= 4.8.
The hydrolysis assay procedure was performed in 700 ml flasks that contained: different concentrations of cellulosic material and cellulase and Tween 80 0.3%, suspended in acetate buffer, pH=4.8. The sample flasks were incubated in a lab shaker, at 45^0 C and 150 rpm for 24 hours. The enzymatic reaction was stopped by keeping the assays in boiled water for 10 minutes.

Yeast and substrate bioconversion into ethanol

The *Saccharomyces cerevisiae* yeast strain with fermentative potential was obtained from the Collection of microorganisms (coded MIUG) of the Bioaliment Platform and preserved and multiplied on malt extract agar medium. This served as the starter culture for the ethanol production. The optimized inoculum size was achieved by counting the yeast cells with a Thoma cytometer.
After the enzymatic hydrolysis, the flasks were cooled to 32^0 C, and supplied with: different concentrations of nutrient supplement and then inoculated with yeast suspension with different concentrations of cells/ml/g of substrate. The used

nutritive supplement contained: 7% N (nitrogen salt 3.8%, ammonia salt 3.2%), 4% P_2O_5 and 5% K_2O.
The flasks were sealed with a loop trap filled with concentrated H_2SO_4 and incubated at 32^0 C, for 144 hours. The CO_2 production was followed by the measuring of the weight loss indicative of the ethanol yield.
The yield of the ethanol**,** produced through fermentation**,** was measured with K-ETOH 03/06 ethanol kit produced by Megazyme International Ireland Ltd.

The Plackett-Burman experimental design
The Plackett-Burman experimental design, a fractional factorial design, was used in this study to demonstrate the importance of some factors implied in substrate hydrolysis and ethanol production. Eleven independent variables, in twelve combinations were organized according to the Plackett-Burman design matrix. For each variable, a high (+1) and low (-1) level was tested (Rajendran *et al.* 2007). The main effect of each variable was determined according to the following equation:

$$Exi = (\Sigma Mi+ - \Sigma Mi-) / N$$

where:
Exi is the variable main effect,
Mi+ and Mi- are the response percentage in trials, in which the independent variable (xi) was present in high and low concentrations, respectively,
N is the half number of trials.
Twelve experiments were generated for the 10 factors considered to affect the ethanol production. The factors tested were: substrate concentration, enzyme concentration, inoculum size, saccharification temperature, solid/liquid ratio, the pH, fermentation time, the nutritive supplement concentration, fermentation temperature, enzyme supplement, and the tested concentration range which is given in Table 1. The assays were performed in duplicate.

Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for the determination of variable significance (Al-Sarrani *et al.* 2006).

T a b l e 1. *Bioconversion factors at different levels used in Plackett-Burman experimental design*

Variables	Lower level (–1)	Higher level (+1)
A - substrate concentration, g	15	35
B - enzyme concentration, UE/g substrate	50	100
C - inoculum size, CFU*/g substrate	10^5	10^7
D - saccharification temperature, ^{0}C	40	55
E - solid/liquid ratio	1/10	1/20
F - the pH	4	5.5
H - fermentation time, h	72	168
I - the nutritive supplement concentration, %	0.05	0.8
J - fermentation temperature, ^{0}C	27	37
K - enzyme supplement, UE/g substrate	20	40

**CFU-Colony forming units*

Results and discussion
The ethanol produced by *Saccharomyces cerevisiae* was found to vary from 0.84 to 21.8 g /100 g dry weight cellulosic waste in the twelve experiments conducted (Table 2), which shows the strong influence of the medium components on the ethanol enzyme production.

Based on the statistical analysis of the experimental data it was found that the significant medium components were: the solid/ liquid ratio, the pH, the enzyme concentration, the inoculum size and the fermentation temperature (Figure 1).

T a b l e 2. Plackett-Burman experimental design for the evaluation of the 10 variables with coded values for ethanol production from complex cellulosic waste and the response of the design

Run	A	B	C	D	E	F	H	I	J	K	Ethanol, g /100 g dry weight cellulosic waste
1	-1	-1	1	-1	1	1	-1	1	1	1	0.84
2	1	-1	1	1	-1	1	1	1	-1	-1	10.5
3	-1	1	1	-1	1	1	1	-1	-1	-1	9.00
4	-1	1	1	1	-1	-1	-1	1	-1	1	5.25
5	1	-1	-1	-1	1	-1	1	1	-1	1	4.12
6	-1	1	-1	1	1	-1	1	1	1	-1	4.37
7	1	1	-1	-1	-1	1	-1	1	1	-1	9.00
8	1	1	1	-1	-1	-1	1	-1	1	1	9.00
9	1	-1	1	1	1	-1	-1	-1	1	-1	4.81
10	-1	-1	-1	1	-1	1	1	-1	1	1	9.18
11	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	4.12
12	1	1	-1	1	1	1	-1	-1	-1	1	21.8

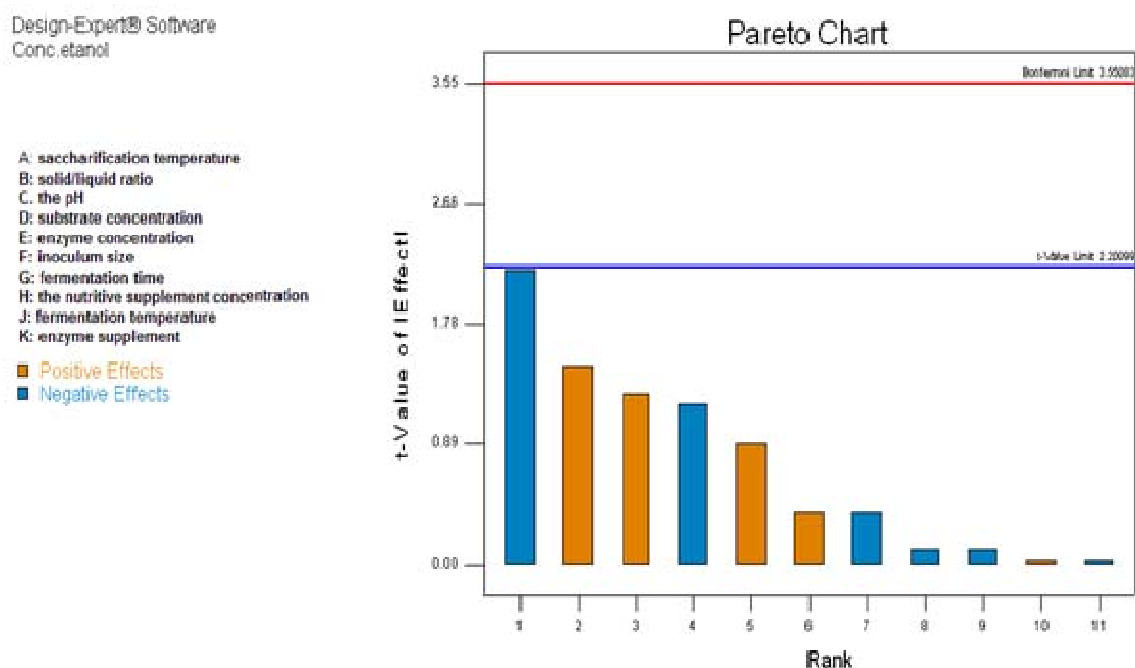

Figure 1. *The Pareto chart for the determination of the significant factors which influenced the ethanol bioproduction from cellulosic waste material*

Based on results can be concluded that the negative effects of the biotechnological process have the following factors:

– the saccharification temperatures adversely affect most biotech processes because the temperature increase leads to enzyme inactivation by the irreversible distortion of the protein (Segal, 2006)

– the substrate concentration can cause substrate inhibition,

– the pre-treatment of the cellulosic material is necessary to increase the reactivity of the cellulose from cellulosic material;
– the fermentation period has a reduced effect on the biotechnological process, as yeast consumes the sugar in the first 72 hours of fermentation;
– the nitrogen concentration has a reduced effect on the biotechnological process, by influencing the activity of the yeast, as a nutritive supplement to them;

Significant factors affecting the biotechnological process are:
– the solid/liquid ratio has a positive influence on the biotechnological process as the optimum water content is essential for the growth of microorganisms and thus producing ethanol. At a low level of water, the yeast cells are not able to grow normally so they cannot produce ethanol. The optimum water content can have a positive effect because the solved substances from the waste cellulosic material have a positive influence on the fermentation process by transporting the necessary nutrients for the development of yeast cells (Mohanty *et al.* 2009).
– the pH also has a strong positive effect on the biotechnological process because the pH affects both the cellulase activity and the yeast fermentation;
– the enzyme concentration has a strong positive effect on the process of biotechnology; to a low concentration of the enzyme the saccharification process is not complete because the substrate remains unchanged due to the enzyme depletion; a concentration too high of the enzyme causes a complete substrate saccharification but if it remained unused, the surplus increases the cost of the process, making it unprofitable;
– the inoculum size has a positive effect on the process, a very small inoculum concentration increases the fermentation time and a too high concentration can lead to losses in ethanol yield due to sugar consumption by the multiplying yeast cells;
– the fermentation temperature is one minor positive effect on the yeast fermentation process because the fermentative yeast is a mesofile.

Conclusion

Therefore, it is necessary to increase the cellulose content of the solids by means of other pre-treatments, and to ferment the pentoses in the hydrolysate, in order to make the process economically feasible. The Plackett-Burman experimental design, used in this work had demonstrated the relative importance of the following factors on the substrate enzymatic hydrolysis and the ethanol production: the solid/ liquid ratio, the pH, the enzyme concentration, the inoculum size, the fermentation temperature.

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