

Optimization of the enzymatic treatment of olive oil pomace for lignocellulosic ethanol production

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Measuring the activity of an enzyme is equivalent to describe its catalytic properties, that mainly they are influenced by the type of environment in which the enzyme is placed to operate. The objective of this work is to optimize the activity of the enzyme cellulase of *Trichoderma Reesei* to catalyze the reaction of saccharification of the contained cellulose on the Olive Pomace (final solid waste of the elaboration of the olives in the production process of olive oil).The saccharification reaction previews gradual deterioration of the cellulose in little parts until obtaining the monomer or dimers (cellobiose) of the glucose, final result of the reaction.

A design of experiments is used to determine the optimal enzyme dose for maximum ethanol production in a yeast fermentation process. Pomace pretreatment and quantity as well as enzyme concentration and hydrolysis temperature were varied selectively according to the design. In this way fewer experiments were needed to obtain the optimal formula for second generation ethanol production process.

1. Introduction

The enzyme in nature operates in a very defined environment or within that outside the cell, in fact it can be thought that exists a real optimization of the surrounding environment that functions as selector agent of the enzymatic abilities expressed from the organisms like result of millennia of evolution. Since the environment has fundamentally a role of selection towards a certain enzyme it can be thought that this environment can be modified, changing some of its parameters for being able to explore the true limits of the activity of the enzyme and not only those supplied from the conditions in nature. The main parameter of an enzyme is often the speed which it catalyzes the reaction for which it is destined, indicator in its turn of a combination of various conditions like the temperature, the pH, the structure of the substrate, the composition of medium and the presence of cofactors or inhibitors of the reaction, each one of which is specific for only one enzyme. Therefore an enzyme can be used considering the specific conditions of work in which it gives a higher reaction rate,

fortunately the specific parameters are not a single fix set of values but often they are intervals of values, whose largeness describes the plasticity of the certain enzyme, fundamental characteristic for the industrial application.

The more convenient technology for the production of bioethanol from olive pomace is the Simultaneous Fermentation and Saccharification (SSF), that it previews an enzymatic digestion of the lignocellulosic fraction contemporary to the fermentation of ethanol from microorganisms. The enzymatic digestion is operated from the cellulase enzyme, that belongs to the glicosidase family proteins, than hydrolyze the glycoside bound of cellulose fibers releasing in liquid means monomeric sugar compounds mainly glucose for the hexose and several pentose. In order to optimize the costs of the SSF process it is essential to study the catalytic activity of the enzyme in order to find the just concentration of active units for the substrate amount introduced expressed like FPU/g substrate. Enzyme dose is essential to optimize the production costs as the enzymatic step is often the part of the process that costs more. Moreover is should be consider that the enzymatic concentration influences on the growth of yeasts since its presence could have and inhibitor effect then turning out in a decrement of the amount of produced ethanol.

Previous works has been conducted by Ballesteros et al (2009). but for testing the cellulase activity crystalline and purified cellulose has been used. This is the ideal substrate of the cellulase. He found that 15 FPU/for 1 gram of substrate is the enzyme concentration that guarantees a good rate of saccharification and contextually it is not much toxic for cellular viability.

Nowadays is not present in literature data of the enzymatic activity of the cellulase and the just balance of active units (FPU) for gram of substrate on of olive pomace substrate. On the other hand, this characterization it is essential for the future activities of the development of a process SSF using pomace as a substrate. It must be optimized reducing the use of energy and enzymatic load. In this work the olive pomace has been hydrolysed to various concentrations (100, 150, 200, 250 g/l) with the enzyme cellulase to various units for gram of substrate (5, 15, 25 FPU/g substrate) in order to measure the amount of extractable and fermentable glucose. The olive pomace comes from two types of process for the extraction of the different oil of olive: a classic one with grinding followed from the squeezed without other treatment, and the second one in which the olive pomace after the classic treatment is chemically treated with hexane for an ulterior extraction of the not extracted lipid fraction to the previous step.

Theoretically from stoichiometric relations from 2 g/l of glucose, 1 g/l of Ethanol could be produced. So to reach high ethanol concentrations of 15 g/l of ethanol, at least 30 g/l of glucose should be released from the OP. From previous work (1) OP contains about 16% of glucose packed as cellulose. This means that at least 200 g/l of OP should be employed.

2. Description of the experiment

The objective of the work is to monitor the catalytic activity of the cellulase on the OP containing cellulose that results in an increase of fermentable sugars (glucose and cellobiose) in the solution. A screening of pomace type, concentration and enzyme dose is performed.

Since the specificity of the enzymatic reaction is high, it is possible to associate the increase of the concentration of sugars to the effective glucose increment in the solution. Josefsson colorimetric method for sugars determination was used that quantifies the sugar concentration in aqueous solutions. The saccharification process must be carried out in sterile conditions in order to avoid that the consequent glucose richness of the media favours the growth of polluting microorganisms that consume it.

In the present work two types of various OP have been used called Dry and Extr. Dry. Dry OP comes directly from the olive mill, while Extr Dry, comes from the hexane extraction of the Dry OP. Both samples have been used to concentrations of 10 g. and 15 g., while the active units of the cellulase has been chosen in 5, 15, and 25 FPU. A total of 16 experiments were performed. Enzyme dose and OP type and concentration are shown in Table 1. Each experiment was monitored hourly through time in order to determine the maximum enzyme activity.

Table 1. *Experiments performed.*

5 FPU per grams substrate							
Dry	10% w/v	15% w/v	20% w/v	25% w/v	Extracted	10% w/v	15% w/v
	10g OP	15g OP	20g OP	25g OP		10g OP	15g OP
	50 FPU	75 FPU	100 FPU	125 FPU		50 FPU	75 FPU
	100 ml 3D-w.	100 ml 3D-w.	100 ml 3D-w.	100 ml 3D-w.		100 ml 3D-w.	100 ml 3D-w.
15 FPU gram substrate							
Dry	10% w/v	15% w/v	20% w/v	25% w/v	Extracted	10% w/v	15% w/v
	10g OP	15g OP	20g OP	25g OP		10g OP	15g OP
	150 FPU	225 FPU	300 FPU	375 FPU		150 FPU	225 FPU
	100 ml 3D-w.	100 ml 3D-w.	100 ml 3D-w.	100 ml 3D-w.		100 ml 3D-w.	100 ml 3D-w.
25 FPU gram substrate							
Dry	10% w/v	15% w/v			Extracted	10% w/v	15% w/v
	10g OP	15g OP				10g OP	15g OP
	250 FPU	375 FPU				250 FPU	375 FPU
	100 ml 3D-w.	100 ml 3D-w.				100 ml 3D-w.	100 ml 3D-w.

2.1 Materials and methods

Treatment of OP: the OP called “Dry” wasn't particularly treated, instead as said before the “Extracted-Dry” comes from a hexane extraction process that removes the remaining olive oil. It is reasonable to think that the Extr.Dry pomace due to the hexane treatment has an expanded cellulose matrix with greater superficial area that facilitates the enzyme effect over all substrate. Before the tests, both OP have been

dried (for 4 hours at 130°C), milled and sifted (sieve of 1mm) then put in 100 ml 3D-water in a 250 ml Erlenmeyer flasks, then sterilized in autoclave at 121 °C for 20 minutes.

Saccharification: the working volume chosen is 100 ml with the appropriate mix of cellulase, OP and 3D-water as presented in table 1, in a 250 ml Erlenmeyer flask. After sterilization, then the first sample is captured (this is the sample at Time 0 of experiment) then the appropriate dose of enzyme is added. The flasks have been put in thermostatic bath at 47 °C continuously agitated at 150 RPM for 30 hours. Every hour a sample is taken till the seventh hour, then directly only one at the thirtieth hour.

Enzyme loading: the enzymes is loaded after the autoclave step, in particular when the temperature of medium down at 40-45°C.

Treatment of samples: every sample (at least 4 ml) is divided in two parts, one of 1 ml (that represents soluble sugars or the not soluble ones) and one of 3 ml. The latter is microfiltered with 0,2 um cellulase filter, to obtain soluble sugars. Then both samples are boiled at 100 °C for 4 minutes and stocked in freezer, to stop the enzymatic activity. All sampling was performed in aseptical conditions.

Total sugar analysis: all samples were analysed with Josefsson methodology to detect the sugar formed by enzymatic digestion. Every sample has been diluted in appropriate measure at least 100 fold until 300 fold for more concentrated samples like 200 g/L and 250 g/L, all measurements has been performed in triplicate.

3. Results

3.1. Dry Olive Pomace

The total sugar analysis gives a good linear result and suggest that a large part of cellulosic glucose is released in the first 1 or 2 hours for the 10 g of OP flasks, and in the first 2 or 3 hours for the 15 grams of OP flasks as shown on the figure 1 for Dry OP.

In the figure 1 are show the soluble sugars present in water, there are not distinctions between the glucose and the other sugars. If it is assumed that only the glucose is released, it can be possible to determine glucose fraction subtracting the initial soluble sugars content as shown in figure 2. The formation rate hour by hour from time 0 up to seven hours, and the standard deviations is shown.

In good agreement with the grams of OP added an increment of 50% of total sugars has been detected in comparison of the sample that containing increments of 50% of OP.

The best results is obtained with 250 g/l of OP that release medially 10 grams of glucose. All results can contain more glucose if it is consider that the initial rate of sugars have inside a certain percentage of glucose mixed with other sugars like pentose that are hydrolysed in autoclave (step one) and are not considered.

Generally there is not a big difference in the sugar contain of the sample with 5 FPU and 15 FPU loaded with same OP grams, in particular in the velocity of reaction.

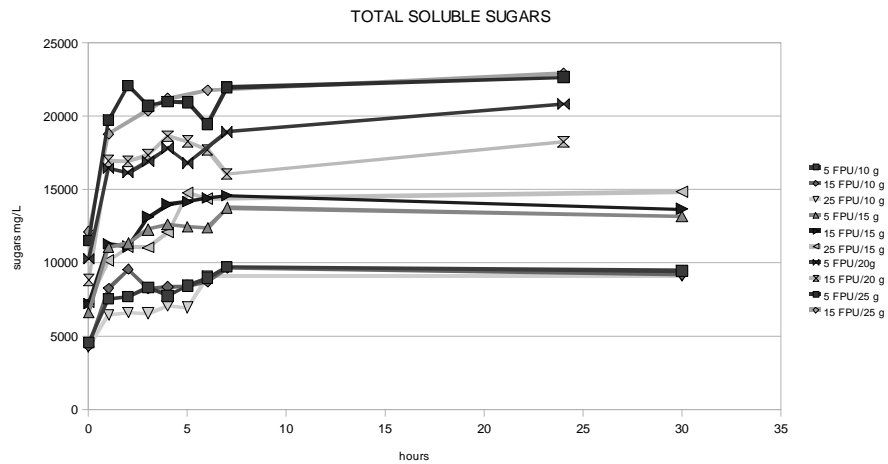


Figure 1: Total soluble sugars concentration through time.



Figure 2: Soluble glucose with standard deviation of concentration through the time

3.2. Extracted Dry Olive Pomace

The data are incomplete but the first results suggest that there are less sugars (c.a. 30 %) than the normal OP. The particulate of the sample used is finer than the grains of Dry OP, due to this reason the finer particulate could get through the 0.2 um filtered solution and therefore altered the measurement increasing the soluble sugars content. Results are presented in figure 3.

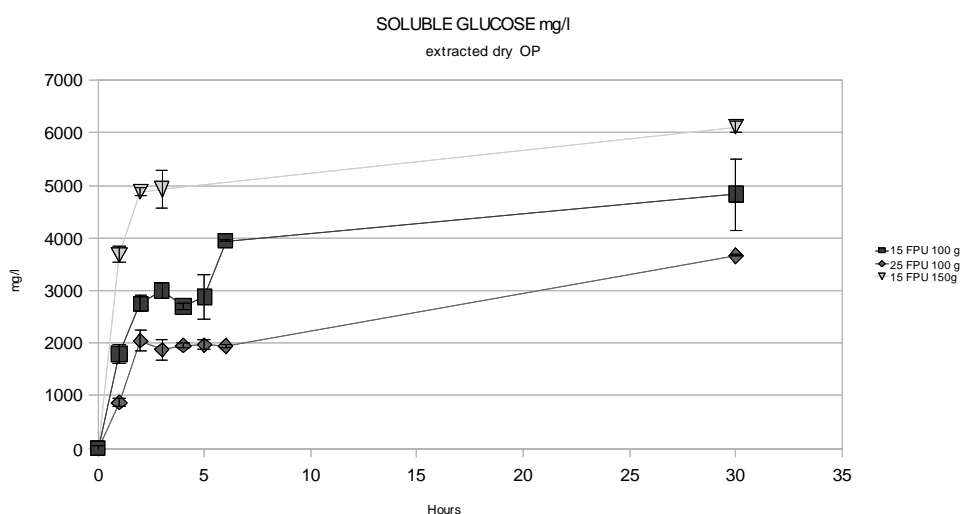


Figure 3: soluble glucose whit standard deviation of extracted OP.

4. Conclusion

A screening of the conditions that affect most the enzymatic activity were performed experimentally for the olive pomace. Two types of pomace were used in different concentrations and enzyme load. The best results are obtained whit 200 or 250 grams of dry olive pomace where are hydrolysed to obtain 8 and 10 grams of glucose respectively. These results can be improved if a pre-treatment of the fibres is employed. Generally the extracted OP give less glucose soluble rate respect the dry OP.

Acknowledgement

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 222331 –ETOILE

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