Enzymatic hydrolysis followed by alcoholic fermentation of prickly pears (*Opuntia ficus-indica* L.) cladodes and fruits using *Saccharomyces cerevisiae* E1A

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Abstract: The cultivation and production of biomass of prickly pear is of particular interest due to the fact that it can be grown in arid areas, producing higher amounts of biomass with lower inputs (water, fertilizers etc.) compared to other crops. The present study examines whether enzyme hydrolysis is capable to increase the amount of reducing sugars available to *S. cerevisiae* E1A strain to be used during alcoholic fermentation. Enzymatic hydrolysis was performed using the commercial enzymes Termamyl and Celluclast applied at various concentrations of dried cladodes and of dried fruit and added juice from discarded fresh prickly pears in order to enrich the quantity of sugars into the solution. The substrate used in the bioreactor experiment consisted of 100 g/L of dried cladodes with 40 g/L of dried fruit, which after the addition of the juice resulted in the synthesis of a liquid growth medium containing 48 g/L of sugars. The microorganism finally managed to produce a significant amount of endopolysaccharides inside the yeast biomass (22.12% w/w) while biomass (DCW) production was of 4.35 g/L. Ethanol at 21.54 g/L with a yield of ethanol produced per unit of sugar consumed $Y_{EtOH/S}$ was equal to 0.49 g EtOH / g of substrate.

Keywords: *Saccharomyces cerevisiae* strains, enzymatic hydrolysis, bioethanol, enzymes, prickly pear (*Opuntia ficus – indica* (L.)).

1. Introduction

After 1860 various agencies as well as companies began to use bioethanol as fuel. After the crises of the following years as well as the ban on drinking in the USA, the companies were turned to the use of fossil fuels such as petroleum (Datta et al., 2011). However, the excessive use of fossil fuels, especially in large urban areas, has led to an increase in pollution levels in recent decades, resulting in a huge increase in greenhouse gas levels in the atmosphere (Ballesteros et al., 2006; Sarkar et al., 2012). With the even increasing human population and industries as well in the near future it looks like global oil production will decrease. Hence, it will be replaced by the fuels derived from the processing of biomass and residues of the agricultural and industrial sectors (Campbell and Laherrere, 1998). In the EU the main sources for ethanol production are cereals and sugar beets (Demirbas, 2009), while in the US the vast majority of ethanol comes from hydrolyzed corn starch, while in Brazil ethanol mostly is produced from sugar cane (Sarris and Papanikolaou, 2016). Other raw materials that potentially could to be used for this purpose are fruits, oats, rice and lignocellulosic materials such as sawdust, agricultural waste, forestry waste and municipal or industrial waste which at the same time their utilization could address their disposal and treatment problems (Prasad et al., 2007). On the other hand, lignocellulosic biomass pretreatment is important concerning the potential use of this type of materials in the production of biofuels, as it dissolves the crystalline structure of cellulose and hemicellulose and releases fermentable sugars to achieve hydrolysis faster and with higher ethanol yields (Mosier et al., 2005). Proper pretreatment can prevent the formation of inhibitory by-products in subsequent hydrolysis and fermentation (Harun et al., 2011). It is known that ethanolic microorganisms can ferment and convert to bioethanol only monomeric and dimeric sugars (Khan et al., 2017), which proves the need for pretreatment and hydrolysis of biomass**.** In the present work, mechanical pretreatment was applied in combination with the use of enzymatic hydrolysis due to the fact that the latter is an environmentally friendly process. Moreover, neither high temperatures were developed nor harmful chemicals were used so as to increase the efficiency of the fermentation process. In Greece, the prickly pear plant firstly was appeared commercially in the 1950s, mainly on the islands and was used in the spare parts trade. In recent years, great efforts have been made to make the cultivation of prickly pear systematic because the shift to this cultivation would be a very important part of Greek agricultural production. The reason is that under the same irrigation conditions the productivity of prickly pears is higher than other plants while the production in biomass is more stable over time as it is less affected by the prevailing weather conditions. In addition, under favorable irrigation conditions prickly pear can produce up to 45-50 tons of dry biomass per hectare of land per year, which represents a very high yield compared to most other crops (Santos, 2016). Apart from these, the prickly pear plant can be used for the production of various products beyond the usual consumption of its

fresh fruits such as the production of oil from seeds, the use as animal feed (cladodes), the production of bioethanol after fermentation process, and the recovery of pigments used in the food industry (indixanthane and betanine).

The purpose of this study is to provide some information about the performance of a new strain of *S. cerevisiae* towards the production of bioethanol production. At the same time, we aim to contribute to the spread of a plant such as prickly pear (*Opuntia ficus-indica* L.) which is considered one of the alternative crops promoted in recent years in Greece.

2. Materials and Methods

The cladodes as well as the fruits used in the experiments came from the Mount Poikilo located in the area of Kamatero in Athens. Upon receipt and transport to the laboratory they were kept at room temperature. The portions used in the hydrolysates were chopped using a home blender and then placed in an oven at 100°C for about one day. After drying, they were put back in the blender to become a powder and then stored until use in a special food bag with zip.

2.1 Microorganisms

The microorganism used for the experimental procedure in the present study was the yeast *Saccharomyces cerevisiae* E1A strain (Photo 1), kindly provided by the Laboratory of Quality Control and Food Hygiene of the Food and Beverage Department of Agricultural University of Athens. In order to maintain the vitality of the microorganism at regular intervals it was renewed on YPD (Yeast extract, Peptone and Dextrose) substrate and maintained at 4° C until use.

2.2 Enzymatic Hydrolysis

Initially, tests were performed with the enzyme Termamyl (enzyme activity was 120 KNU/g) at various concentrations of dried cladode and fruit as well. Then the quantities were weighed, 100 mL of deionized water was added to each of the above quantities and the pH was adjusted with 5 N NaOH to 6. The appropriate dose of the enzyme was then added to each flask (20 μL). Then, it was transferred to a water bath at 85 ° C where it was left for one hour while, at regular intervals, the solution was stirred. At the end of the hour, they were placed in a boiling water bath for 10 minutes (in order to inactivate the enzyme) and then the flasks were left to be cooled, followed by vacuum filtration. The hydrolysate was stored in the freezer until use. A similar procedure was followed with Celluclast (enzyme activity was 1500 NCU/g) with the only difference that the temperature in the water bath was 50°C and the pH was adjusted to 5 using 3M HCl.

2.3 Alcoholic fermentation in the bioreactor

Strain E1A was selected to be cultured in a laboratory scale bioreactor L1523 Bioengineering, with a total volume of 8L with the ability to control temperature and agitation speed. The medium in which the fermentation took place consisted of: 1) 4 L of dried cladodes with dried fruit (in a ratio of 100 g/L of dried cladodes + 40 g/L of dried fruit) which was obtained after enzymatic hydrolysis (Photo 2) and from 2)1.5 L prickly pear juice obtained after grinding the whole fruit and filtering on a Buchner filter. The juice was made from discarded (viz. waste) fruits. The volume of the fermentation medium added to the bioreactor before inoculation was 5.5 L and it was inoculated with 300 mL of inoculum (5%) and HCl 3M (~ 200 mL). Prior to inoculation, the medium was sterilized after pH adjustment to the value of 5.0 (Photo 3). The fermentation temperature was 30±1 °C. The inoculum was made from YPD medium (yeast extract /peptone /dextrose at a 10 g/L of each). The agitation was adjusted to 150±10 rpm in the reactor while there was no air supply so trials were characterized as micaroaerobic /anaerobic.

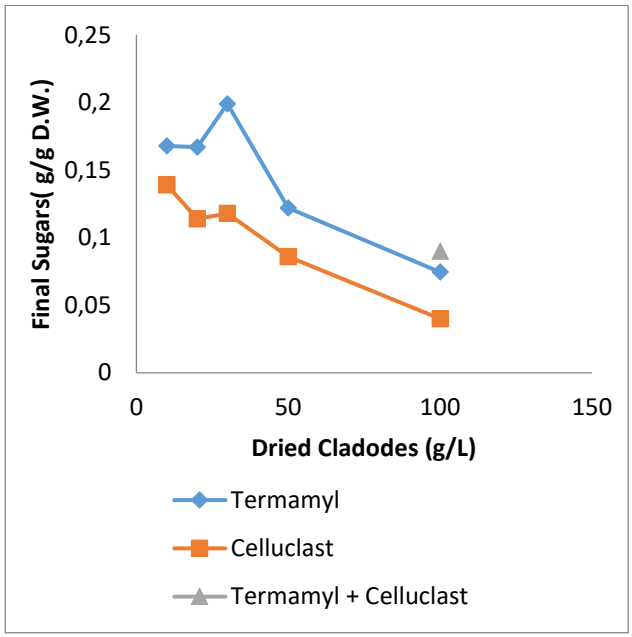
3. Results – Discussion

The procedure followed for sugar release in the prickly pear dried cladodes included 20 g/L of each sample dissolution in deionized water in separate 250 mL conical flasks and stirring them without heating by magnetic stirrers followed by filtering. Additionally, the sugars in the fruit juice came from the liquid that emerged after the process described above. The amount of initial sugars obtained from dried cladode, dried fruit and fruit juice is 1,93, 11,14 and 112,45 (g/L), correspondingly.

From the results of analyses done was noticed (Figure 1) that the higher the amount of substrate in which the specific enzyme acts, the lower the amount of sugar we receive (in both enzymes). It was observed that Termamyl has better efficiency in the extraction of sugars in contrast to Celluclast while the combination of the two enzymes did not increase the amount of sugars. It is possible that this decrease in the amount of sugars is due to the fact that the sugars that are available to the microorganism are destroyed during the inactivation of the enzymes at the end of the hydrolysis. It is showed (Figure 2) that the maximum amount of biomass is 4.35 g/L. With regard to endopolysaccharides, the microorganism seems to show stability in their values, while its maximum production is observed during inoculation (22.12%), which is particularly interesting as most of the time their accumulation occurs during the period of nitrogen lack from the substrate. The maximum value of ethanol is 21.54 g / L (Figure 3) with yield Y EtOH / S = 0.49 g EtOH / g substrate which is very close to the maximum theoretical yield (0.51 g EtOH / g substrate). The stability in the ethanol values may be due to the fact that the agitation rate in the reactor is quite small (150 rpm) and because we do not have external aeration for this so the phenomenon of ethanol re-consumption does not happened. Another reason for preserving the ethanol obtained may be that the microorganism uses most of the substrate sugars to produce its biomass and only a small part of it to produce ethanol, utilizing of course the proteins that are abundant in the substrate of fermentation. Finally, glycerol shows an upward course, reaching its

maximum amount in 28 hours, performing the amount of 2.02 g / L.

Figure 1: Reducing sugars variation after enzymatic hydrolysis at various concentrations of dried prickly pear cladodes (D.W.: Dry Weight)



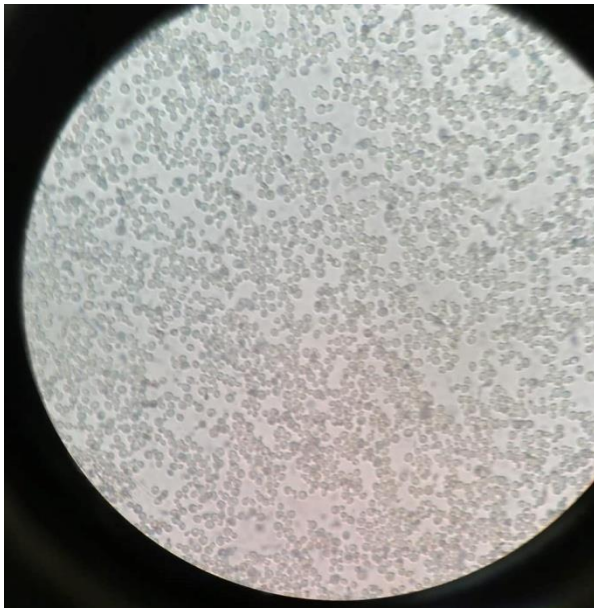


Photo 1: Preservation of the yeast strain *Saccharomyces cerevisiae* E1A in petri plates containing: 10 g/L Peptone, 10 g /L Yeast extract, 10 g/L Glucose and 20 g/L agar

Photo 2: The medium in which the enzymatic hydrolysis took place consisted of: 4 L of dried cladodes with dried fruit (in a ratio of 100 g / L of dried cladodes + 40 g / L of dried fruit)

Photo 3: Development of strain E1A of *S. cerevisiae* in the substrate from the parts of prickly pear in a laboratory scale bioreactor type L1523 Bioengineering, with a total capacity of 8L and with the ability to control: Ph(~5), temperature (30° C) and agitation speed (150 rpm)

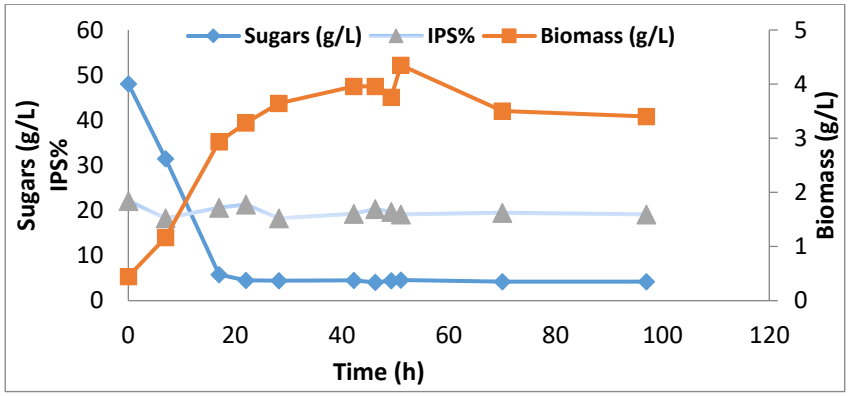


Figure 2: Kinetics of biomass, total sugars and endopolysaccharides during the development of strain E1A of *S. cerevisiae* in the substrate from the parts of prickly pear in a laboratory scale bioreactor type L1523 Bioengineering, with a total capacity of 8L and with the ability to control: Ph(~5), temperature (30° C) and agitation speed (150 rpm)

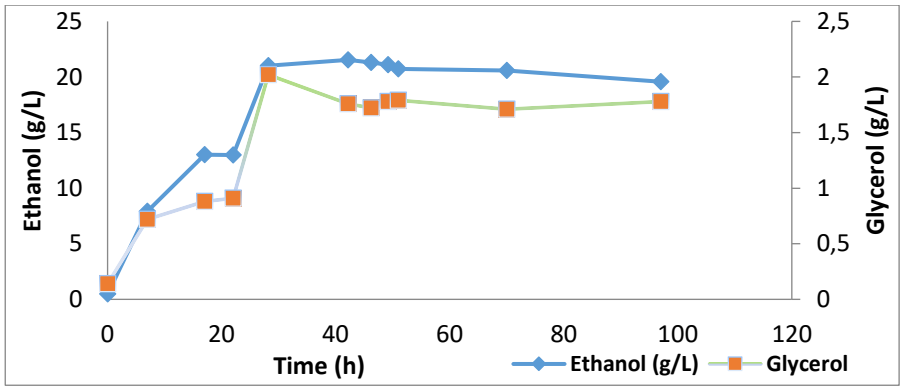


Figure 3: Kinetics of the ethanol produced as well as glycerol during the development of the E1A strain of *S. cerevisiae* on the substrate from the prickly pear parts in a laboratory scale bioreactor type L1523 Bioengineering with a total capacity of 8L and with the ability to control: Ph(~5), temperature (30° C) and agitation speed (150 rpm)

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