Conversion of winery wastes into a sugar-rich hydrolysate for the biotechnological production of succinic acid

K. Filippi, M. Alexandri, H. Papapostolou, A. A Koutinas

Department of Food Scinece and Human Nutrition, Agricultural University of Athens, Iera Odos 75, 118 55, Athens, Greece

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Presenting author email: katiana.philippi@gmail.com

Introduction

The depletion of fossil resources in combination with environmental issues generated by their extensive utilisation, has arisen a global interest in finding alternative renewable feedstocks to replace the petroleum-based ones. The wine-producing industry is one of the most important industrial sectors associated with significant amounts of by-products such as pomace, stalks and wine lees. Grape pomaces and stalks are mainly composed of lignocellulose but grape pomace contains also significant amounts of free sugars. Owing to their composition, these by-product streams are potential renewable resources for the biotechnological production of platform chemicals such as succinic acid. Succinic acid is one of the top 12 building block chemicals serving as a precursor for the production of a wide spectrum of chemicals or as intermediate in pharmaceutical, food and cosmetic industry.

The present study demonstrates the development of a bioprocess based on the efficient bioconversion of grape pomace and stalks into succinic acid. The lignocellulosic part of the winery wastes was hydrolysed using thermochemical pretreatment methods and enzymatic hydrolysis. The ability of *Actinobacillus succinogenes* to effectively consume the carbohydrates generated from winery wastes after hydrolysis and free sugar extraction was subsequently evaluated in batch and fed-batch fermentations.

Experimental

Pomaces, skins, seeds and stalks generated from wine making of the Greek, red variety Agiorgitiko Nemeas were kindly provided by the Oenology laboratory of Agricultural University of Athens (Greece). Free sugars from pomaces, skins and seeds were extracted with deionized water at 45 ^{o}C for 2 h at a solid-to-liquid ratio of 1:10. Then solvent extraction of lipids was carried out utilising hexane at a solid-to-liquid ratio of 1:10 under agitation for 2 h. Subsequently, phenolic compounds were extracted using 70 % ethanol acidified with 0.5% HCl (0.1 M) in an ultrasonic waterbath. The remaining solids were mixed with grape stalks, in 1:1 ratio, and treated with sodium hydroxide (1% w/v) for 3 h at 100 ^{o}C for lignin removal. The hemicellulosic fraction was then hydrolysed with 3% v/v H_2SO_4 at 121 ^{o}C for 30 min. Enzymatic hydrolysis performed using three commercial enzyme preparations at different concentrations and combinations in order to optimize the process: cellulases from Aspergillus sp., β-glucosidase from almonds and Viscozyme L (Sigma-Aldrich). Specifically, the enzyme combinations were: 20 FPU/g cellulase and 80 U/g β-glucosidase (mix 1), 20 FPU/g cellulase and 40 U/g β-glucosidase (mix 2), 10 FPU/g cellulase 10 FPU/g Viscozyme L and 80 U/g β-glucosidase (mix 3), 10 FPU/g cellulase 10 FPU/g Viscozyme L and 40 U/g β-glucosidase (mix 4). All hydrolysis experiments were conducted at 50 ^{o}C in a solid to liquid ratio of 1:10 under mechanical stirring. The effect of the hydrolysis solution was also investigated, utilising 50 mM sodium citrate buffer solution, deionized water and the liquid obtained after the acidic hydrolysis of hemicellulose fraction. In all cases the pH of the hydrolysis was adjusted to 5.0. The addition of 8 mg per g substrate of bovine serum albumin (BSA) as surfactant was also tested (Yang and Wyman, 2006).

Finally, the hydrolysate produced after the enzymatic hydrolysis was combined with the free sugars fraction and used as carbon source for succinic acid production with the bacterial strain *A. succinogenes* 130Z. The fermentation broth was supplemented with 5 g/L yeast extract, 5 g/L $MgCO_3$ and minerals (Alexandri *et al.*, 2017). The production of succinic acid was carried out at 37 ^{o}C under agitation including continuous sparging of carbon dioxide (0.1 vvm) while the working volume was 500 mL. The feeding solution in all cases was concentrated free sugars from Agiorgitiko Nemeas pomaces.

Cellulose hemicellulose and lignin content of lignocellulosic biomass were determined according to a method proposed from National Renewable Energy Laboratory (NREL) (Sluiter *et al.*, 2008) sugars and organic acid were monitored using HPLC following the protocol presented by Alexandri *et al.* (2016).

Results and Discussion

Pomaces from Agiorgitiko Nemeas contained 2.55 g free sugars/ 100 g dry sample (consisted of glucose and fructose). After the treatment with sodium hydroxide 52 % lignin removal was achieved while the hemicellulose solubilisation was equal to 53%. Among the different enzymatic combinations evaluated, the addition of 20 FPU/g, 80 U/g β-glucosidase in 50 mM sodium citrate buffer resulted in the highest cellulose conversion yield (0.35 g/g). The addition of BSA in the mixture significantly enhanced the hydrolysis yield, reaching a value 0.48 g/g. Similar results were obtained when deionized water was utilized for the hydrolysis, indicating that citrated buffer could be omitted. The resulting hydrolysate presented a glucose concentration of 22.70 g/L.

Subsequently, fed batch fermentations were conducted with the produced hydrolysate as initial substrate for succinic acid production. Two feeding strategies were evaluated- addition with pulses and continuous feeding- in order to assess the effect on succinic acid concentration, yield and productivity. In both cases the final succinic acid concentration and yield were similar and equal to 37 g/L and 0.68 g/g, respectively. The productivity was significantly enhanced when continuous feeding was employed and it was equal to 0.71 g/(L·h), whereas the corresponding value for the experiment conducted using pulses was 0.48 g/(L·h). In both experiments, acetic acid was the main by-product (7.5-8.5 g/L) while formic acid formation was always lower (between 4-6 g/L). The production of acetic and formic acid was observed during the initial growth phase of the microorganism alongside with the production of succinic acid.

Conclusions

The results obtained in this work indicate that winery wastes (pomace and stalks) could be valorized via their efficient conversion into succinic acid production. The hydrolysis of the pretreated material could be successfully performed without the addition of buffer while the addition of BSA significantly improved the cellulose-to-glucose conversion yield. Finally, increased succinic acid productivities were achieved when a continuous supply strategy was applied.

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