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Growth kinetics and physiological behavior of co-cultures of *Saccharomyces cerevisiae* and *Kluyveromyces lactis*, fermenting carob sugars extracted with whey

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ABSTRACT

Alcoholic fermentation of carob waste sugars (sucrose, glucose and fructose) extracted with cheese whey, by co-cultures of *Saccharomyces cerevisiae* and *Kluyveromyces lactis* has been analyzed. Growth and fermentation of *S. cerevisiae* in the carob-whey medium showed an inhibition of about 30% in comparison with water-extracted carob. The inhibition of *K. lactis* on carob-whey was greater (70%) when compared with the whey medium alone, due to osmolarity problems. Oxygen availability was a very important factor for *K. lactis*, influencing its fermentation performance. When *K. lactis* was grown alone on carob-whey medium, lactose was always consumed first, and glucose and fructose were consumed afterwards, only at high aeration conditions. In co-culture with *S. cerevisiae*, *K. lactis* was completely inhibited and, at low aeration, died after 3 days; at high aeration this culture could survive but growth and lactose fermentation were only recovered after *S. cerevisiae* became stationary. To overcome the osmolarity and *K. lactis*' oxygen problems, the medium had to be diluted and a sequential fermentative process was designed in a STR–3l reactor. *K. lactis* was inoculated first and, with low aeration (0.13 vvm), consumed all the lactose in 48 h. Then *S. cerevisiae* was inoculated, consuming the total of the carob sugars, and producing ethanol in a fed-batch regime. The established co-culture with *K. lactis* increased *S. cerevisiae* ethanol tolerance. This fermentation process produced ethanol with good efficiency (80 g/l final concentration and a conversion factor of 0.4 g ethanol/g sugar), eliminating all the sugars of the mixed waste. These efficient fermentative results pointed to a new joint treatment of agro-industrial wastes which may be implemented successfully, with economic and environmental sustainability for a bioethanol industrial proposal.

1. Introduction

Agro-industries produce a great amount of carbon-rich wastes, whose industrial abatement are mandatory and could be the source of new added-value products. On the other hand, water is a scarce resource and its use in biotechnological processes should be optimized. The production of second generation bioethanol has been a subject of great interest since is recognized as the main replacement for the fossil fuel based energy. Agro industrial wastes have the potential to be one of the major feedstock for 2nd generation ethanol production [1]. From previous works [2,3] it has been demonstrated that carob waste sugars, extracted with water, can be fermented efficiently by *S. cerevisiae*. A multi-wastes approach would enable the simultaneous management of several agro-industrial wastes, providing the high amounts of sugar and water needed. However, this approach also brings new complexity to the fermentation processes.

In this work a mixture of two wastes has been tested - goat cheese whey and carob kibbles. The whey is an effluent of dairy industries produced in large amounts all around the world. It has been proposed, long ago, to be used for several purposes in industry, including the production of 2nd generation bioethanol [4]. However, its direct use in biofuel production is hindered by the limited concentration of its fermentable sugar, lactose, which is about 5%

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(w/v) [5,1]. The carob pod is a fruit produced in Mediterranean countries. A solid waste named carob kibbles that has high content of soluble sugars (40–60%w/v), is produced from the fruit after the carob seeds are removed for the high-added value compounds extraction [6]. We have shown previously that the sugars from the carob pod can be easily extracted with water using very low energy input and are efficiently transformed into ethanol by *Saccharomyces cerevisiae* [2]. Moreover the problems and bottlenecks of the process, when using high carob sugar concentrations, have been identified and solved by the design of a fed-batch process [2,3]. However, the need for large quantities of water in the sugars extraction from the solid waste is an economic and environmental problem.

Our purpose with this work was to evaluate the possibility of the joint treatment of both wastes, obtaining alcohol as a final product. This mixed substrate process would have the potential benefit of increasing the fermentable sugar concentration (and the consequent final ethanol concentration), performing the treatment of both residues and avoiding water waste.

Some difficulties in the process could be envisaged from the beginning. The first one is that *S. cerevisiae*, that can ferment efficiently and completely the carob sugars (sucrose, fructose and glucose) if optimal conditions are provided [2], cannot ferment lactose, the main sugar of whey. To solve this problem, a mixed population approach should be taken and a lactose-fermenting yeast species, such as *Kluyveromyces lactis* or *Kluyveromyces marxianus,* widely used in whey fermentation [5,7,8] should be included in the process. However, previous studies with co-cultures of other strains of *K. lactis* and *S. cerevisiae* than those selected for this work, had shown that the latter inhibited almost completely the growth of *K. lactis* during the first days of fermentation and, after 8 days, all the *K. lactis* population was dead [9]. Several factors have been referred to justify this early death of the non-*Saccharomyces* species (NS) in wineries, such as the high levels of ethanol and organic acids, low pH values, depletion of certain nutrients, as well as other yeast–yeast interactions (*e.g.* killer toxins) [10,11]. The limited oxygen availability is another factor mentioned. It has been reported that NS species as *T. delbrueckii* and *K. thermotolerans* die at low oxygen availability conditions in co-cultures with *S. cerevisiae* and this could be also the case with *K. lactis* [12]. Gonzalez-Siso considered *K. lactis* as a respirofermentative yeast with a high, unlimited respiratory capacity, that uses the pentose phosphate pathway preferentially for the first part of glycolysis [8]. For this reason and, to regenerate the NADP formed in that pathway, the need for oxygen is quite high [8]. So, a careful control of aeration seems to be very important for ethanol production by *K. lactis* as it should be high enough to permit growth but not too high to be able to promote alcoholic fermentation and also to avoid the consumption of the ethanol produced [5]. With this physiological information, it seemed that there were several fundamental physiological problems that had to be solved preceding the implementation of a successful process. The purpose of this research was to identify all the problems involved and the physiological mechanisms underlying them, to end up with a technological proposal which solved or avoided those problems and that could improve the economic and environmental sustainability of the process.

2. Materials and methods

2.1. Yeast strains, maintenance and inocula

Two yeast strains were selected: *Saccharomyces cerevisiae* F13A [2,3] and *Kluyveromyces lactis* CBS 2360. Stock cultures was maintained in 20% glycerol at −80 °C. To prepare inocula the strains were cultured on solid YEPD medium (20 g/l peptone, 10 g/l yeast extract, 20 g/l glucose and 15 g/l agar) and incubated at 30 °C. Pre-inocula were prepared in YEPD broth inoculated with one isolated colony. Inocula was incubated in an orbital shaker (IKA KS4000i, Portugal) at 150 rpm and 30 °C, until the cultures reached the late exponential phase. Fermentations started with an initial cell concentration of about 1×10^7 cells/ml.

2.2. Extraction of the sugars from the carob powder with aqueous whey

The extraction procedure to obtain soluble sugars from the carob waste was adapted from the previously published method with distilled water of Manso and collaborators [13].

The carob kibbles provided by the industry were dried, ground and suspended in cheese whey at different solid/liquid ratios. These mixtures were homogenized at 150 rpm, for 2 h. In this stage the carob pod extract was centrifuged at 7500*g* and 4 °C for 30 min (Beckman J2-MC Centrifuge with a JA14 rotor) to remove solid fraction and to clarify the mixture. It was stored at −20 °C.

Soluble sugars analyses were performed by high-performance liquid chromatography (HPLC), as it is described below at "Analytical methods".

2.3. Culture media

Three media with different carbon sources were assayed. The whey medium was composed by liquid whey obtained as a waste of a dairy industry with about 60 g/l of lactose. The carob-water medium was obtained extracting the carob sugars with distilled water [2]. The total sugar concentration of this medium changed and it is indicated in the corresponding experiments. The carob-whey medium was made of carob powder extracted with whey, as described above, with a total concentration of sugars of about 140 g/l, 60 g/l corresponding to lactose from the whey and 80 g/l to sucrose, glucose and fructose from the carob extract. These media were all enriched with 5 g/l peptone and 3 g/l yeast extract [2].

2.4. Fermentation conditions

2.4.1. Batch experiments in shake flasks

Batch fermentations were carried out in 250 ml shake flasks, filled with 150 ml of culture. They were incubated in an orbital shaker at 30 °C and 150 rpm for 96 h. Optical density, biomass dry weight, sugar consumption and ethanol production were measured in the broth as described in "Analytical methods". All the tests were carried out in triplicate and the results were the mean of six values (three replicates of the process and two replicates of the analysis).

2.4.2. Batch experiment in aerated STR

The effect of aeration was studied in a 3-l STR (ADI 1010/1025 Applikon, Holland). Aeration was provided by a Rushton-type turbine operated at 250 rpm and an L-shaped sparger, (0.13 or 0.50 vvm, as indicated). Incubation temperature was 30 °C. The monitoring of the fermentation was carried out with the BioXpert program, version 2.1. The measurement of temperature, pH and dissolved oxygen was made with specific probes and the values were constantly registered. Samples were taken at regular intervals to determine the optical density, biomass dry weight, cell viability, sugar consumption and ethanol production, as described in "Analytical methods".

The culture medium used was the carob-whey mixture of 40%(w/v) with a total sugar concentration of 140 g/l added with antifoam B (Sigma A-5757). The inocula had a cell concentration of about 1×10^7 cell/ml.