GLUCOSE FERMENTATION UNDER CARBON DIOXIDE PRESSURE

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The effect of CO_2 pressure on commercial *S.cerevisiae* cells viability and on the rate of ethanol production in yeast, has been studied in batch cultures under 1,10 and 18 bar of CO_2 pressures. The results show that the effect of CO_2 pressure on ethanol productivity strongly depends on the temperature, glucose and starting biomass concentration and pressure itself. Under atmospheric CO_2 pressure condition, fermentation was completed after 62 hours with a 10% (w w^{-1}) of ethanol in the fermentation broth. In the same conditions, using up to 10 bar of CO_2 pressure, ethanol productivity was about 8 % (w w^{-1}). No productions were observed at a CO_2 pressure of 18 bar, probably due to a complete failure of enzymatic activity. The fermentation was obtained starting from a high concentration of biomass, however no cellular growth was observed during the process, when 10 and 18 bar of CO_2 pressure were used. Inositol addition, in the millimolar range concentration, greatly influenced the viability of yeast cells, allowing the production of ethanol more rapidly in the first 24 hours of fermentation. Nevertheless after 62 hours, the final ethanol concentration was lower than the ethanol obtained in the unsupplemented media. Finally, we demonstrated the feasibility of using a new pressurized reactor system, developed in our laboratory, for fermentation under higher carbon dioxide pressure.

INTRODUCTION

Biofuels represent a very good alternative to fossil fuels in the short and middle-term, reducing the dependence on oil and supporting the development of depressed rural areas. The most likely alternative to the fuels currently used are renewable sources such as bioethanol and biodiesel, but their production is more expensive than petroleum refining, so that the biofuels industry is not fully developed yet, especially in the less industrialized countries. Both biodiesel and bioethanol pollute the environment less than the fossil fuels and in addition they come from renewable sources i.e. annual cultures with a short vital cycle. The limitations of bioethanol industry are represented by variable production and high distribution costs [1]. The traditional distillation, commonly used to purify ethanol, is a very expensive technique. In addition, the ethanol-water mixture forms an azeotrope, which further increases the cost of purification. In order to reduce production costs, ethanol extraction by supercritical carbon dioxide could be used [2]. To reduce the need of energy due to the supercritical carbon dioxide extraction process, the development of a fermentation reactor that works under high CO_2 pressure could be helpful. Since CO_2 is more soluble in organic solvents than in water, ethanol may be extracted maintaining it at a low concentration in solution. In this way the product inhibition effect can be reduced, making also more realistic a continuous fermentation process, where the substrate is continuously added and the product removed. In addition, the loss of supercritical solvent would not be critical, because this gas is produced from the fermentation process itself. The bacteriostatic action of the CO_2 and the growth and metabolism of different microorganisms including *S.cerevisiae* have been extensively described [3,4,5,6,7]. Increasing CO_2 pressure resulted in a decreasing cell growth rate and cell yield [8]. Cell growth stopped at 280 kPa of CO_2 metabolically

produced during ethanol fermentation [9]. Fermentation up to 2,5 bar was associated with a more rapid cell death [10]. On the other hand fermentation capability of the cells was stimulated by the exposure to increased CO_2 partial pressure up to 0,48 bar [11,12]. It is inhibited at 1,5 bar and remains constant till 3,5 bar [10], therefore increasing pressure decreases the fermentation rate. The effect of pressure on cell viability strongly depends on the nature of the gas used for pressurization [13,14]; 6 bar of CO_2 pressure caused cell inactivation (confirmed by reduction of budded cells over time) and a significant decrease of fermentation rate. Some authors have studied the effect of hyperbaric CO_2 near supercritical conditions (70 bar) with the purpose of applying supercritical ethanol extraction from broth fermentation using unusually high pressure value in traditional bioreactor operation [14]. Generally, the increase of carbon dioxide pressure has a more detrimental effect on yeast growth compared with yeast fermentation. In this way, the problem of arrested growth, typical of an high pressure fermentation process, could be possibly overcome. using a starting high biomass concentration. Two interesting studies about physiological and genome-wide transcriptional response of *S.cerevisiae* to high carbon dioxide pressure and different pH values, were reported, but a more detailed analysis under high CO_2 is required before true signature transcripts for CO_2 stress can be identified [15,16].

In this work a set of different conditions of fermentation, as temperature, pressure, starting biomass and glucose concentration, has been studied in terms of final ethanol concentration by using a commercial yeast strain. In order to investigate the viability of yeast cells, we used inositol in a supplemented media to oppose carbon dioxide uptake into the plasma membrane. Inositol is the substrate for the synthesis of phosphatidilinositol, a phospholipid that allows tightening of the plasma membrane and increasing viability at higher ethanol concentration [17]. Finally, the possibility to use a new pressurized reactor system, developed in our laboratory, for the fermentation under high carbon dioxide pressure was described.

MATERIALS AND METHODS

Yeast strain and growth medium

An industrial strain of *S.cerevisiae* (supplied from Fermentis division of Lesaffre) was used for its highly efficient ethanol production capabilities; it was preserved at -80°C in 50% (v/v) glycerol. From this stock cultures, YPD agar plates (*Difco* 20 g L^{-1} agar, 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) were inoculated and colonies from these plates were grown aerobically at 28°C, 200 rpm in a medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose for 15 h; centrifuged and resuspended in 100 mL of a fresh medium with the same composition to obtain an optical density of 0,25 at 595 nm using the spectrophotometer *Gene Quant Pro*. Inocula were grown for 6 h at 28°C, 200 rpm in an orbital incubator in a 250 mL shaker flask to obtain about 200×10^8 cell per mL used as inocula for batch experiments under carbon dioxide pressure.

Cells preparation for batch fermentation

Yeast cells for fermentation monitoring were harvested in 2 mL eppendorf tubes (1,5 mL of culture for each tube) at the end of the exponential phase (6 h at 28°C, 200 rpm in YPD with a 0,25 optical density inocula) and washed twice in milliQ water. Then they were resuspended in 1,5 mL of a fresh medium with different composition depending on the type of the experiment.

Batch fermentation

Experiments were carried out in 2-mL stainless steel reactor (for a total of six reactors) at a different temperature, pressure, medium composition and starting biomass with a stirring rate of 400 rpm. The reactors were connected to a 55-60 bar pressurized bottle containing pure carbon dioxide. The operating pressure was set by manipulation of the pressure of the inlet gas. The line was provided with a reduction pressure valve to maintaining the pressure at a desired, constant value. Each reactor was equipped with a on-off valve for independent pressurization or depressurization. All the reactors were in a water bath suitable mixed, whose temperature was maintained at a constant value by thermostatic

bath (a mixture of water and glycol); the mixing of the samples were assured by magnetic stirrer coupled with an external agitator under the water bath. All the reactors had the same pressure and temperature.

Analytical methods

Samples for analysis of high pressure fermentation were extracted from the 2-mL reactors at the end of each experiment. The viability was determined by counting the number of colony forming unit (CFU) obtained after duplicate plating suitably diluted culture samples on a medium composed of 10 g L^{-1} yeast extract, 20 g L^{-1} peptone. They were directly plated on YPD-agar after the release of pressure, colonies were counted after incubation at 30°C for 24 h. Residual glucose concentration was estimated by spectrophotometry with an enzymatic method using Glucose Oxidase and Horseradish Peroxidase (*SigmaAldrich GO assay kit*). Ethanol concentration was measured by injection of 1 µL of the filtered solution (0,22 µm filters) into a Shimadzu GC-14A Gas Chromatography using TCD detector with the following conditions: Porapack QS column, 1,7 m x 5 mm x 2,6 mm; injector temperature 200°C; TCD temperature 170°C, resistors current 150 mA, oven temperature 150°C; carrier gas was helium with a flow rate of 20 mL min^{-1}.

RESULTS

Effect of glucose concentration

Three sets of batch fermentations were carried out in the presence of glucose concentration 100, 200 and 300 g L^{-1} in two different carbon dioxide pressure condition: 1 bar and 10 bar. The fermentation conditions were 62 h, 32°C with a $70x10^8$ cell per mL starting biomass concentration. Under atmospheric CO_2 pressure only 100 and 200 g L^{-1} of glucose completely fermented, while in 300 g L^{-1} samples, the sugar was incompletely fermented by *S.cerevisiae* and the final ethanol concentration did not exceed 10% (w w^{-1}). The data (Figure 1) clearly show that when pressure raised from 1 to 10 bar, ethanol concentration at the end of the reaction was reduced, pointing out the fact that high glucose concentration (300 g L^{-1}) slowed down the fermentation rate and inhibited ethanol productivity at 10 bar. On the other end, 200 g L^{-1} allowed is reached 7% (w w^{-1}) ethanol at 10 bar.

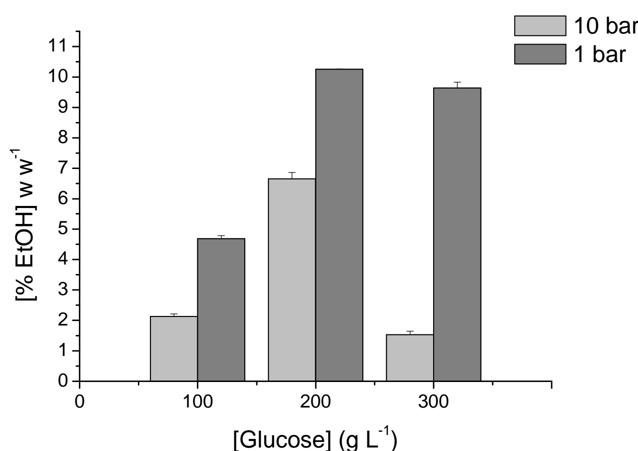

Figure 1: Ethanol concentration under different starting glucose concentration and CO_2 pressures

Effect of temperature and biomass concentration

To assess the combined effects of fermentation temperature and starting biomass on ethanol productivity (g L^{-1} per cell), media with six different starting biomass concentration (from 40 to $1400x10^8$ cell per mL) were fermented at the temperature ranging between 30 and 38 °C. The fermentation conditions were 24 h, 200 g L^{-1}, 10 bar CO_2.

Figure 2 summarizes the results. We found that increasing temperature from 30 to 36 °C increases the specific ethanol productivity. At a higher temperature, under the same starting biomass conditions, the final ethanol concentration is reduced. An increase of initial biomass concentration was always related to a decrease of specific ethanol productivity. We optimized the starting biomass concentration at about 200x10^8 cell per mL, which correspond to the late exponential phase in the yeast growth.

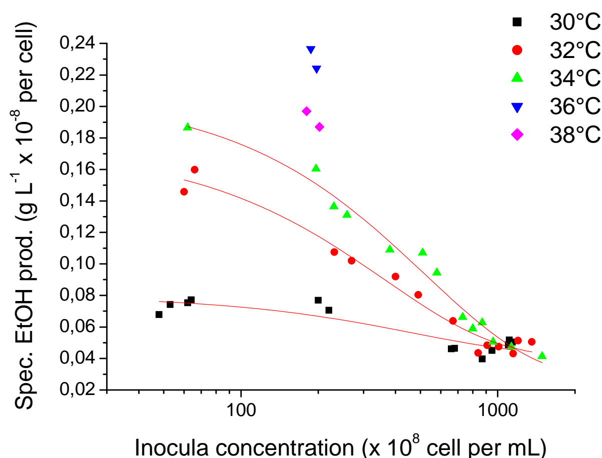

Figure 2: Specific ethanol productivity under different temperature and initial biomass concentration at 10 bar CO_2 pressure

Effect of pressure

The effect of pressure up to 10 bar of CO_2 at 200 g L^{-1} glucose, 36°C, 24 h has been examined. We studied alcoholic fermentation under 18 bar but no ethanol concentration at the end of the fermentation processes was observed in this case. This result is probably due to the complete inactivation of the enzyme Piruvate Decarboxilase, that is completely inhibited at pressure near 13 bar of CO_2 [7].

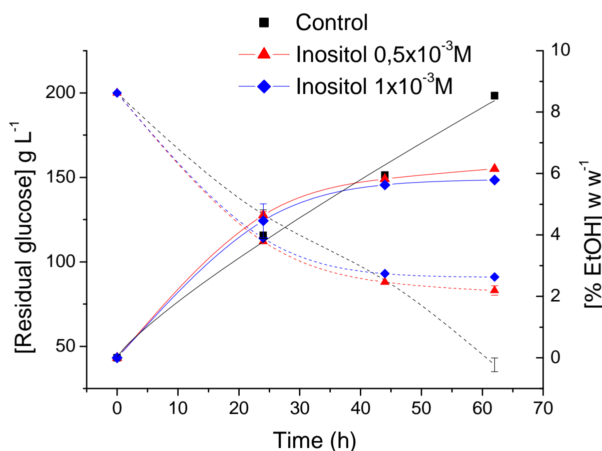

Figure 3: Fermentation kinetics under 10 bar CO_2 pressure with different millimolar inositol concentration, dash lines represent residual glucose concentration, solid line ethanol concentration

Effect of inositol concentration on ethanol productivity and cell viability

In order to assess the effect of inositol on the fermentation rate we carried out a set of experiments in the presence of 0,5 and 1 mmol L^{-1} of inositol. The conditions of fermentation were 10/18 bar CO_2, 200 g L^{-1} glucose, 36°C, 62 h. Inositol addition greatly influenced the viability of yeast cells, allowing

the production of ethanol more rapidly in the first 24 hours of fermentation; however, after 62 hours the ethanol concentration was lower than the unsupplemented media (Figure 3). The symmetric trend of the curves allows us to conclude that glucose consumption was almost entirely utilized for ethanol production. At the end of fermentation the ethanol concentration in the unsupplemented media was about 8,5% (w w^{-1}), 2% more if compared to supplemented media. The results shown in Figure 4 indicate that in the presence of inositol cells dead less rapidly and maintain a good viability during the latest step of fermentation. Figure 4 also shows that CO_2 pressure promote yeasts dead and works together with ethanol in reducing yeasts viability.

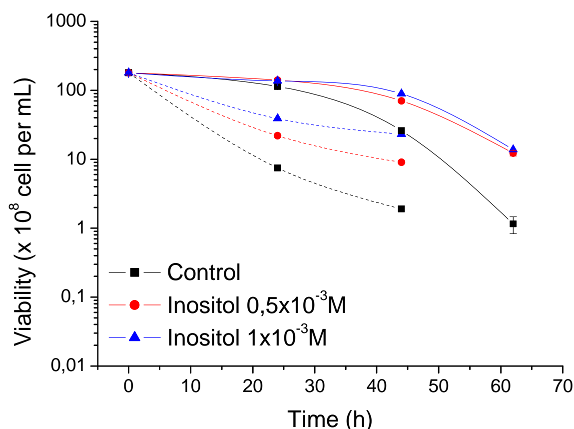

Figure 4: Viability at 10 and 18 bar of CO_2 in the presence of different millimolar inositol concentration, dash lines represent 18 bar of CO_2 pressure, solid line 10 bar of CO_2 of pressure

Pilot plant experiment

The lab-scale results received confirmation by running an experiment in a pilot plant to demonstrate the feasibility of the fermentation under carbon dioxide pressure at a larger scale. The works volume of this reactor was 0,5 L. We tested the fermentative capacity of the *S.cerevisiae* by monitoring fermentation for 45 h with the same conditions used for multibatch plant, i.e. 10 bar CO_2, 200 g L^{-1}, 36°C, 400 rpm, about 200×10^8 cell per mL and no inositol supplement. As reported in Figure 5 we found that fermentation rate is higher in the pilot reactor than in multibatch plant, probably due to a better mixing in the first one.

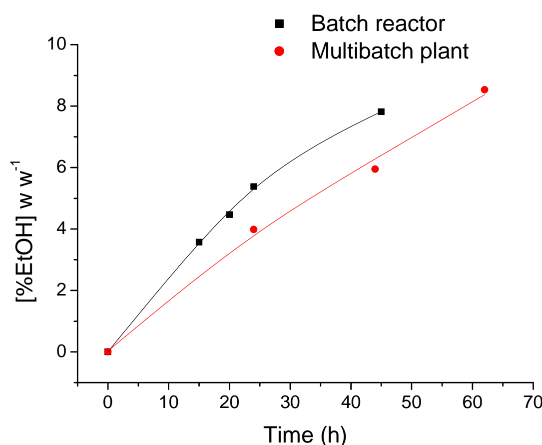

Figure 5: Fermentation comparison between batch reactor and multibatch plant under CO_2 pressure

CONCLUSION

In this work we have studied glucose fermentation under high carbon dioxide pressure using a commercial *S.cerevisiae* strain. We demonstrated that producing ethanol by fermentation under 10 bar of pressure could be possible whereas no ethanol production was obtained at 18 bar of CO_2 pressure. Fermentation conditions were optimized by changing temperature, glucose and biomass concentration obtaining an ethanol concentration of about 8,5% (w w^{-1}) instead of 10% (w w^{-1}) obtained in the atmospheric pressure conditions. The feasibility of using a new pressurized pilot reactor to ferment under high carbon dioxide pressure was verified. Finally, we found that the use of inositol in the supplemented media can improve yeasts viability mostly during the latest stage of fermentation, but increase in ethanol concentration were observed till 24 h of fermentation only.

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