

ETHANOL PRODUCTION FROM HIGH-GLUCOSE INDUSTRIAL SUBSTRATES USING ETHANOL-TOLERANT *SACCHAROMYCES CEREVISIAE* STRAINS

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ABSTRACT

Ethanol is well known as a toxic metabolite for yeast cells. Thus, strains that can grow well under high ethanol stress condition are highly desirable. This work aims to select and characterize *Saccharomyces cerevisiae* strains with improved ethanol tolerance. Moreover, it aims to evaluate the feasibility of industrial residues as fermentation media and to optimize the composition of such media.

The ethanol production and tolerance of the yeast strains have been evaluated, carrying out batch alcoholic fermentations with high-glucose YP medium. The most ethanol-tolerant strain was able to ferment 300 g/L glucose producing up to 17.4 % (v/v) of ethanol in trials carried out in anaerobic shake-flasks.

Aiming to develop a fermentation medium based in industrial substrates, corn steep liquor (CSL) has been tested as medium supplement, in order to replace nutrients that are needed to allow both cellular growth and fermentation. Supplementation of 300 g/L glucose medium with CSL concentrations around 90 - 110 g/L has resulted in fermentation performance similar to that observed in YP medium with the same glucose concentration, thus confirming the feasibility of CSL as peptone and yeast extract substitute.

Keywords: bioethanol, ethanol tolerance, ethanol titre, industrial media, corn steep liquor.

INTRODUCTION

Growing industrial development makes imperative to find environmentally sustainable energy sources. Biofuels have gained popularity in the last decades, especially due to the fossil fuels depletion and the increasing environmental concern. They have been claimed to be renewable and environmental friendly sources of energy, since they are produced from biomass resources and their production is based on CO₂-neutral concepts, thereby helping to minimize global warming.

Of the many fossil fuels' alternatives, bioethanol has been highly used and studied, since it can be added to fuels for combustion engines to levels of up to 20% without any modification to the engine [1]. In order to make ethanol production economically sustainable, a high productivity bioprocess is necessary. Ethanol productivity can be improved by using highly concentrated substrates. However, ethanol is well known as a toxic metabolite for yeast cells, which raises a serious problem. Thus, strains that can grow well under high ethanol stress condition are highly desirable.

Screening for strains or mutants with high ethanol tolerance has been often performed by viability observation on ethanol-containing agar plates [2] and by growth rates determination on ethanol-containing medium [3]. Although these and other similar techniques are easy and fast, they screen for the ability to grow under high ethanol stress but not for fermentation ability in these conditions. Thus, selection must be conducted under conditions similar to those faced by yeasts in real processes. In the present work strains were selected for ethanol tolerance by batch fermentations of high-glucose media, which causes osmotic (sugar) stress in the beginning and increasingly ethanol stress towards the end of the fermentation.

Ethanol fermentation productivity also depends on media composition. Specific nutrients, such as nitrogen and vitamins, are required for growth and fermentation. In laboratory scale, media are

often supplemented with peptone and yeast extract to supply those requirements. Nonetheless, such addition is not possible in industrial processes due to the high costs associated. Therefore, it is economically necessary to use inexpensive and readily available nutrient sources to supply all the nutritional requirements for growth and fermentation activity.

Corn steep liquor (CSL), a major by product of corn starch processing, is an inexpensive source of proteins, amino acids, minerals, vitamins, reducing sugars (such as dextrose), organic acids (in particular, lactic acid), enzymes, and elemental nutrients (such as nitrogen) [4, 5]. Its primary uses are as feed supplement for ruminants and nutrient source for poultry [4 - 6]. However, CSL has been reported in many works as a rich and effective nutrients supplement, for instance, for yeast extract replacement on ethanologic fermentation [6], as a cheap nitrogen source for recombinant yeast fermentation [7], as supplement on brewery's spent grain hydrolisates fermentation [8], for nutrients supply reducing the fungal inoculum amount required for biopulping [4] and as lactic acid source for enzyme stabilization [9].

In this work, CSL has been used as nutrients source in replacement of both peptone and yeast extract, being added to high glucose solutions in order to provide an inexpensive and highly fermentable industrial based medium.

MATERIALS AND METHODS

Yeast

Three *Saccharomyces cerevisiae* strains were initially tested for ethanol tolerance, from which CA 116 and RL 11 are industrial strains isolated from Brazilian "cachaça" fermentation and BY 4741 is a laboratorial strain. Further experiments were performed only with the industrial strain CA 116. Stock cultures were maintained on YPD [yeast extract 1% (w/v), bacto peptone 2% (w/v) and glucose 2% (w/v)] agar plates at 4 °C.

Inocula preparation

Prior to each fermentation yeast was first grown on agar plates in order to attain fresh cultures, and then inoculated and allowed to grow overnight on YPD liquid medium at 30 °C on an orbital shaker (150 rpm). The cells were collected by centrifugation at 4000 rpm for 10 min, washed and resuspended in supernatant to 25 mg fresh yeast/mL.

Fermentations

Yeast cells from cellular suspension described above were pitched at about 0.5 g/L into 100 mL culture medium in 250 mL Erlenmeyer flasks.

Two types of fermentation media were used: high-glucose YP with 250, 300 or 350 g/L glucose and 300 g/L glucose supplemented with the industrial substrate corn steep liquor (CSL) at concentrations ranging from 5 g/L to 150 g/L. Insolubles in CSL were removed by centrifugation. Glucose, CSL and peptone-yeast extract solutions were autoclaved separately and then add aseptically. CSL was kindly provided by a starch manufacturer (Copam, S. João da Talha, Portugal). Fermentations were carried out at 30 °C on an orbital shaker (150 rpm), either in shake-flasks fitted with cotton plugs or in anaerobic shake-flasks fitted with glycerol-filled fermentation locks. Samples were withdrawn during the fermentations in the shake-flasks fitted with cotton plugs, while in the shake-flasks with glycerol-locks, fermentation evolution was followed by mass loss, and samples were taken just at the start and end points.

Analytical methods

Biomass was estimated by optical density at 600 nm (OD₆₀₀) along fermentation and by cell dry weight determination at the end of the fermentation.

The concentrations of glucose and fermentation products (ethanol and glycerol) were determined by high performance liquid chromatography (HPLC). The HPLC column (Varian MetaCarb 87 H) was eluted at 60 °C with 0.005 M H₂SO₄ at a flow rate of 0.6 mL·min⁻¹. A refractive-index detector was used.

RESULTS AND DISCUSSION

Strains evaluation and selection

Yeast strains were firstly screened for growth ability under high ethanol stress by spotting on agar plates (data not shown). Ethanol tolerance was further assessed by fermentation ability evaluation under ethanol stress. Batch alcoholic fermentations of high-glucose YP media were carried out, being expected a high ethanol concentration towards the end of the process.

Table 1 presents the macrokinetic parameters obtained in the fermentations carried out with the 3 strains with different glucose concentrations.

Table 1. Macrokinetic characteristics of fermentations carried out by the 3 yeast strains in different glucose YP media [approximately 250 g/L (first column), 300 g/L (second to fourth columns) and 350 g/L (fifth and sixth column)]. Fermentations were performed in shake-flasks fitted with cotton plugs. Values are presented as mean \pm range of duplicate fermentations.

Real $C_{0, \text{glucose}}$ [g/L]	260.73 \pm 0.26	283.98 \pm 1.54	280.7 \pm 15.75	289.46 \pm 0.39	362.30 \pm 4.39	343.23 \pm 2.33
Strain	BY 4741	BY 4741	RL 11	CA 116	RL 11	CA 116
Fermentation time [h]	76	70	70	70	76	76
Maximum ethanol concentration [g/L]	110.18 \pm 1.07	97.39 \pm 1.19	116.00 \pm 0.70	119.57 \pm 1.52	121.12 \pm 0.48	123.01 \pm 1.66
Maximum ethanol titre [% v/v]	13.96 \pm 0.14	12.34 \pm 0.15	14.70 \pm 0.09	15.15 \pm 0.19	15.36 \pm 0.06	15.59 \pm 0.21
X_{max} [g/L]	4.97 \pm 0.01	4.92 \pm 0.05	6.23 \pm 0.04	5.92 \pm 0.06	4.38 \pm 0.00	5.33 \pm 0.08
Maximum ethanol yield, $Y_{P/S}$	0.42 \pm 0.00	0.42 \pm 0.00	0.42 \pm 0.02	0.41 \pm 0.01	0.42 \pm 0.01	0.43 \pm 0.02
Residual glucose [g/L]	0.39 \pm 0.33	27.73 \pm 6.47	0 \pm 0.00	0 \pm 0.00	53.27 \pm 1.55	50.41 \pm 1.19

X_{max} – Maximum biomass concentration (determined by OD₆₀₀) reached at 52.5 h with 300 g/L glucose and at 70 h for the other concentrations.

Firstly, fermentations in about 300 g/L glucose YP medium were carried out with the 3 strains, in duplicate. All the strains had in common the growth cessation at about 52.5 h and that they were able to continue fermenting after it (until 70 h of fermentation). This phase, in which growth and fermentation are uncoupled, has been observed before in fed-batch fermentations [10, 11]. During the whole fermentation course, glucose consumption and ethanol production were faster in the fermentations carried out by the industrial strains. Consequently, after 52.5 h fermentation, ethanol concentration was about 85 g/L for BY 4741 and 110 g/L for CA 116 and RL 11. After the ethanol peak at 70 h, strains further metabolize glucose, without further ethanol production. It is important to notice that the industrial strains were able to totally metabolize the glucose without increase of peptone and yeast extract concentrations, even with a 15-fold increase in glucose concentration (YPD is normally 20 g/L glucose, while we used 300 g/L). The laboratory strain was unable to ferment the glucose totally. Taken together, these observations indicate that both industrial strains are less sensitive to osmotic and ethanol stress, being able of higher productivities.

The results obtained prompted planning of further experiments at different glucose concentrations in order to estimate the limit glucose concentration of each strain in these conditions. The strain BY 4741 was tested in $C_0 = 250$ g/L and the other 2 strains in $C_0 = 350$ g/L.

The maximum ethanol concentration in the fermentations of 250 g/L and 350 g/L glucose was reached later than when fermenting 300 g/L ethanol – 76 h. In the 250 g/L glucose medium, the strain BY 4741 has almost totally consumed the glucose (0.39 ± 0.33 g/L residual glucose) and has produced approximately more 11.61 % ethanol than in the 300 g/L glucose fermentation. On the other hand, the strains CA 116 and RL 11 were not able to ferment completely the 350 g/L glucose, leaving a glucose residual of approximately 50 g/L. Nonetheless, the ethanol production was slightly higher than in the fermentations with 300 g/L initial glucose.

The ethanol yield on glucose was approximately 0.42 (82 % of the theoretical value) for all the strains and initial glucose concentrations. Thus, the higher ethanol production obtained by CA 116 and RL 11 seems to be due to a higher ethanol tolerance that has allowed them to further consume the glucose.

In order to maximise the efficiency of the process, it is desirable to achieve the highest concentration of ethanol and the lowest residual glucose concentration. Thus, from the results obtained, 300 g/L glucose seems to be the best initial glucose concentration in these conditions. The two industrial strains have shown similar behaviour, however CA 116 is flocculent. The strain CA 116 was therefore chosen for further work since flocculation is a useful trait for development of high cell density fermentation systems [12], which is one of the objectives of our future work.

Corn steep liquor as an effective medium supplement

The development of a fermentation medium based in industrial substrates is economically desirable. Corn steep liquor (CSL) is a by-product of corn starch processing and an inexpensive and nutrient rich source. Therefore, it has often been proposed as a media supplement.

Aiming to evaluate the feasibility of CSL as sole nutrient source to sustain high-performance fermentation, and to optimize the CSL concentration, fermentations of glucose supplemented with CSL in concentrations ranging from 5 g/L to 110 g/L were carried out.

As seen in Figure 1, although higher CSL concentrations in the medium led to enhanced ethanol production, it seems that, in these conditions, ethanol titres higher than 16 % v/v can not be achieved regardless of the CSL concentration.

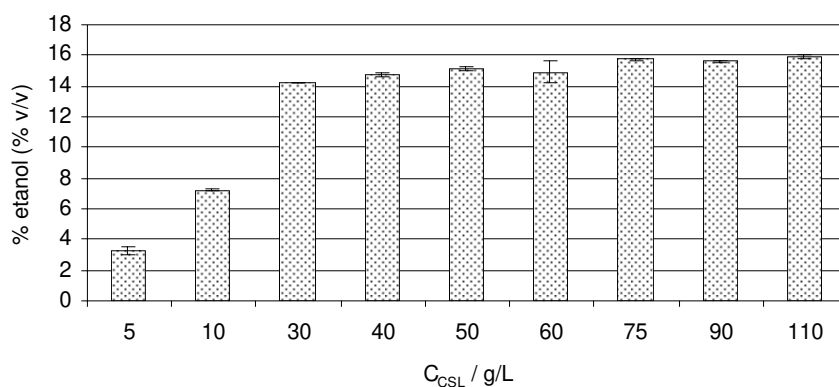


Figure 1. Maximum ethanol titres obtained by CA 116 in 300 g/L glucose medium supplemented with different CSL concentrations. Trials were performed in shake-flasks fitted with cotton plugs. Ethanol titres presented were obtained after 120 h of fermentation for CSL concentrations from 5 g/L to 50 g/L, after 72 h for 60 g/L and after 93 h for higher concentrations. Error bars represent range values of duplicate fermentations.

Results obtained were consistent with literature, showing that CSL is an alternative and effective nutrient source. High CSL concentrations (75 – 110 g/L) provided similar ethanol titre, ethanol yield and glucose utilization to those obtained with YP medium. However, fermentation in glucose-CSL media was rather slower. Values presented on Table 1 were reached in 70 h and 76 h, while the

concentrations referred above for glucose-CSL fermentations where reached just after 93 h of fermentation. Moreover, CSL concentration needed was much higher than yeast extract and peptone concentrations. At least 75 g/L CSL were necessary to obtain the same results as in 300 g/L glucose YP (20 g/L peptone and 10 g/L yeast extract) medium. Ethanologic fermentation by *Bacillus Stearotherophilus* T-13 in medium supplemented with the same amount (5 g/L) of CSL or yeast extract have resulted on similar glucose utilizations and maximum ethanol concentrations within a 10 h fermentation period [6]. However, that study was performed on 10 g/L glucose medium, while our results refer to the fermentation of 300 g/L glucose. Therefore, higher nutrient requirements and fermentation time might be explained by the greater stress induced on yeast by high osmotic pressure in the beginning and by high ethanol concentration at the end of fermentation.

Insolubles contained in CSL were removed by centrifugation prior to fermentation. Although we have not verified whether this removal affects fermentation performance, others have reported that it has enhanced fermentation dynamics, in terms of cell mass yield, fermentation time [13] and productivity [14].

Higher ethanol titres obtained in anaerobic shake-flasks

Fermentations of YP- and CSL-300 g/L glucose media have been performed in shake-flasks fitted with glycerol-filled fermentation locks. Such configuration allows release of carbon dioxide, but blocks air entrance. Fermentation evolution was followed by mass loss, and samples were taken just at start and end points. Therefore, not only eventual ethanol losses are minimized, but also fermentation is favoured over respiration due to lower oxygen availability. However, conditions are not strictly anaerobic, since the oxygen present in the beginning of the process is not removed.

Figure 2 presents the ethanol titres obtained in 300 g/L glucose fermentations supplemented with YP or CSL (90, 110 or 150 g/L).

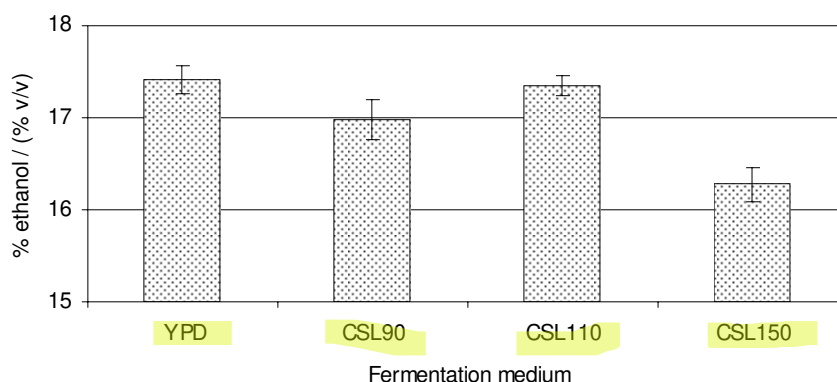


Figure 2. Maximum ethanol titres obtained at the end of fermentations (168 h) of 300 g/L glucose media supplemented with YP or CSL, carried out by CA 116 in anaerobic shake-flasks. Error bars represent range values of duplicate fermentations.

Two main observations can be made from the results of these fermentations. First, ethanol titre reached in YP-300 g/L glucose fermentation was higher in the anaerobic shake-flasks than those obtained previously, $17.42 \% \pm 0.15$ and $15.59 \% \pm 0.21$, respectively, corresponding to an increase of about 10.5 %. Secondly, as observed before, glucose supplemented with high CSL concentrations yielded ethanol titres similar to those of YP-300 g/L glucose medium. The highest value was attained for 110 g/L CSL ($17.35 \% \pm 0.11$).

The final biomass concentrations attained were similar in all the fermentations, ranging between 6 g/L and 8 g/L (determined by dry weight).

Recent studies have reported ethanol titres as high as 19 % v/v [10, 11]. However, these values have been achieved in fed batch processes with exponential vitamins feeding, besides other controls such as pH regulation and constant glucose concentration. In the current work,

fermentations were carried out in simple anaerobic shake-flasks without process control; thus, higher performance may be achieved by process improvement.

CONCLUSIONS

The industrial *Saccharomyces cerevisiae* strains evaluated are highly tolerant to ethanol stress, yielding ethanol titres up to 17.4 % (v/v), by fermentation of 300 g/L glucose. To growth and ferment, yeast requires nutrients that are usually supplied by peptone and yeast extract. For industrial processes a readily and inexpensive nutrient source is necessary. Corn steep liquor, a by-product of corn starch processing, has been shown to be an effective medium supplement, providing similar fermentation performance to that obtained with YP-300 g/L glucose. In order to design sustainable bioethanol production processes it is important to understand which factors contribute for a higher microbial ethanol tolerance, as well as to design fermentation media totally based in industrial by-products or other cheap resources.

REFERENCES

- [1] Minnesota Department of Agriculture (2007), "E20: The Feasibility of 20 Percent Ethanol Blends by Volume as a Motor Fuel". Available at <http://www.mda.state.mn.us/renewable/ethanol/default.htm>, 12 March 2008.
- [2] Carrasco, P; Querol, A and del Olmo, M (2001) "Analysis of the stress resistance of commercial wine yeast strains", Arch Microbiol, Vol. 175, pp 450.
- [3] Hirasawa, T; Yoshikawa, K; Nakakura, Y; Nagahisa, K; Furusawa, C; Katakura, Y; Shimizu, H and Shioya, S (2007) "Identification of target genes conferring ethanol stress tolerance to *Saccharomyces cerevisiae* based on DNA microarray data analysis", Journal of Biotechnology, Vol. 131, pp 34.
- [4] Akhtar, M; Lentz, JL; Blanchette, RA and Kirk, TK (1997) "Corn steep liquor lowers the amount of inoculum for biopulping", Tappi Journal, Vol. 80, pp 161.
- [5] Keller and Heckman LLP (2006) "Assessment plan for corn steep liquor (CAS #66071-94-1) in accordance with the USEPA high production volume chemical challenge program", Available at www.epa.gov/hpv/pubs/summaries/cornstlq/c16469.pdf, 10 March 2008.
- [6] Amartley, SA and Leungl, JPC (2000) "Corn steep liquor as a source of nutrients for ethanologic fermentation by *Bacillus Stearothermophilus* T-13", Bulletin of the Chemists and Technologists of Macedonia, Vol. 19, pp. 65.
- [7] Xiaoli, Z; Zhaojie, X; Binxia, Z; Zhihua, J (2002) "Efficient production of cloned α -amylase by culture of recombinant *Saccharomyces cerevisiae*", Chemical Journal on Internet, Vol. 4, pp 24.
- [8] Carvalheiro, F; Duarte, LC; Lopes, S; Parajó, JC; Pereira, H; Gírio, FM (2006) "Supplementation requirements of brewery's spent grain hydrolysate for biomass and xylitol production by *Debaryomyces hansenii* CCMI 941", J Ind Microbiol Biotechnol, Vol. 33, pp 646.
- [9] Marcussen, E and Henriksen, LR (2003) "Solid phytase composition stabilized with lactic acid provided by corn steep liquor", US Patent.
- [10] Alfenore, S; Molina-Jouve, C; Guillouet, SE; Uribe Larrea, J-L; Goma, G; Benbadis, L (2002) "Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process", Appl Microbiol Biotechnol, Vol. 60, pp 67.
- [11] Cot, M; Loret, MO; François, J and Benbadis, L (2007) "Physiological behaviour of *Saccharomyces cerevisiae* in aerated fed-batch fermentation for high level production of bioethanol", FEMS Yeast Res, Vol. 7, pp 22.
- [12] Domingues, L; Vicente, AA; Lima, N; Teixeira, JA (2000) "Applications of yeast flocculation in biotechnological processes", Biotechnol Bioprocess Eng, Vol. 5, pp 305.
- [13] Lawford, HG (1997) "Corn steep liquor as a cost-effective nutrition adjunct in high-performance *Zymomonas ethanol* fermentations", Applied Biochemistry and Biotechnology, Vol. 63-65, pp 287.
- [14] Lee, K (2005) "A media design program for lactic acid production coupled with extraction by electrodialysis", Bioresource Technology, Vol. 96, pp 1505.