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''ON THE FERMENTATIVE BEHAVIOR OF AUXOTROPHIC STRAINS OF SACCHAROMYCES CEREVISIAE''

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ABSTRACT

Background

The selection of latest yeast strains may lead to improvements in bioethanol production. Here, we've studied the fermentative capacity of various auxotrophic mutants of baker's yeast, which are routinely used as hosts for the assembly of heterologous proteins. it's recently been found that these strains exhibit physiological alterations and peculiar sensitivities concerning the parental prototrophic strains from which they derive. during this work the performance of auxotrophic S. cerevisiae CEN.PK strains were compared to the corresponding prototrophic strain, to S. cerevisiae T5bV, a strain isolated from grape must, and to a different auxotrophic strain, S. cerevisiae BY4741.

Results

The results indicate that the fermentative capacity of strains grown in 2% glucose was similar all told the strains tested. However, in 15% initial glucose, the auxotrophic strains exhibited a quite doubled ethanol yield on biomass (10 g g- 1dw) compared to the prototrophic strains (less than 5 g g- 1dw). Other tests have also evidenced that in medium depletion conditions, ethanol production continues after growth arrest.

Conclusions

The results highlight the capacity of auxotrophic yeast strains to provide ethanol per unit, during a higher amount concerning the prototrophic ones. This ends up in potential applications for auxotrophic strains of S. cerevisiae within the production of ethanol in both homogeneous and heterogeneous phases (immobilized systems). the upper ethanol yield on biomass would be advantageous in immobilized cell systems, as reduced yeast biomass could greatly reduce the mass transfer limitations through the

immobilization matrix.

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Keywords

Auxotrophic yeast.PK strainsEthanol yieldsFermentative capacityFermentative metabolism

1. Introduction

The last years are characterized by an unprecedented interest within the study of techniques for the assembly of bioethanol by fermentation to convert biomass into liquid fuel, as some way to interchange or supplement fossil fuels [1,2]. during this context, the research addressed to characterize new yeast strains able to produce ethanol in peculiar cultivation conditions represents a legitimate contribution during this field [3].

Auxotrophic Saccharomyces cerevisiae mutants have played a crucial role within the development of yeast classical genetic techniques, yeast biology, and genetic and metabolic engineering [4,5]. one amongst the foremost important uses of auxotrophic yeast strains is within the field of heterologous protein production using auxotrophy together with the selective medium as some way to confirm the steadiness of the plasmid and also the expression of the recombinant protein [6,7,8]. However, there haven't been, to our knowledge, previous studies focused on ethanol production by auxotrophic yeasts strains, probably because, initially sight, they constitute unlikely candidates for this purpose.

The present work was aimed toward investigating the potential of some auxotrophic S. cerevisiae strains to provide ethanol and comparing their performances to prototrophic strain.

The idea of studying ethanol production by auxotrophic yeast strains stemmed from the behavior of auxotrophic strains in aerated fed-batch reactor belonging to the CEN.PK

family of the yeast S. cerevisiae as observed by Landi et al. [9]. This work highlighted that the performance of auxotrophic yeasts, in terms of biomass yield and volumetric productivity, relied on both the sort and therefore the number of auxotrophies. within the latter case, the performance decreased with increasing the amount of auxotrophies [9]. Moreover, it absolutely was also observed that the upper the amount of auxotrophies the sooner was the transition from fully respiratory to the respiro-fermentative metabolism, notwithstanding the severe growth-limiting conditions applied over the whole time course of the fed-batch run, specifically designed to avoid over-flow metabolism [10].

In the present study, the ethanol production capacity of three strains belonging to the CEN.PK family [11] of S. cerevisiae, the prototrophic CEN.PK113-7D and two auxotrophic strains, CEN.PK113-5D and CEN.PK2-1C bearing one and 4 auxotrophies, respectively, are investigated. The fermentative capacity of the CEN.PK strains have also been compared to it of another auxotrophic strain, S. cerevisiae BY4741, commonly employed in the laboratory [12,13], bearing four auxotrophies, and to a yeast strain isolated from grape must, S. cerevisiae T5bV. The fermentative capacity of those strains was studied allowing them to proliferate in an exceedingly rich-complex medium supported YEP (Yeast Extract and Peptone), suited to push fermentation [14], at two different glucose concentrations, 2% and 15% (w/v). for 2 of the strains, CEN.PK113-7D and CEN.PK2-1C, the test made with 15% (w/v) initial glucose concentration, was prolonged by adding a high concentration glucose solution to revive glucose to fifteen after the initial glucose present within the medium had been completely depleted and monitoring the power of the strains to provide ethanol in these conditions.

The results highlighted that, in both cases, ethanol was still produced even after yeast growth had significantly dropped, ethanol yield relative to yeast biomass being always higher within the auxotrophic strain than within the prototrophic one. These results show ethanol as a not strictly growth-linked product.

2. Materials and methods

2.1. S. cerevisiae strains

All the strains utilized in this work are S. cerevisiae strains. Three of them belong to the CEN.PK family, CEN.PK 113-7D (MATa URA3 HIS3, LEU2 TRP1 MAL2-8c SUC2), CEN.PK113-5D (MATa ura3-52 HIS3, LEU2 TRP1 MAL2-8c SUC2) and CEN.PK2-1C (MATa ura3-52 his3-Δ1 leu2-3,112 trp1-289, MAL2-8c SUC2). They were kindly provided by Prof. D. Porro (University Milano-Bicocca, Italy) aside from CEN.PK2-1C which was purchased from the EUROSCARF collection (www.uni-frankfurt.de/fb15/mikro/euroscarf).

S. cerevisiae BY4741 (MATa, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1) was kindly provided by Prof. Jesus Zueco (Universitat de València-Spain). S. cerevisiae T5bV was isolated in our Laboratory from grape must, as a yeast strain ready to grow at high levels (over 12%) of ethanol concentration.

2.2. Shake-flask culture

Growth in shake-flask cultures was performed in 500 mL Erlenmeyer flasks containing 100 mL of rich-complex medium supported YEP, having the subsequent composition (w/v): 1% yeast extract, 2% peptone (Becton, Dickinson & Co.) to which 2% or 15% α-d-glucose (Sigma Aldrich) was added. These culture media are mentioned within the text as YEPD2 and YEPD15 where the latter are often also defined as exhausted when at the top of fermentation it's added with an amount of glucose like to revive the initial 15% w/v glucose concentration. The fermentation test was prepared in duplicate for every strain considered. In each test, the quantity of inoculum, coming from an exponential pre-culture, was evaluated to allow an initial optical density (O.D.590) of 0.1.

2.3. Biomass determination

Biomass resolve by a calibration curve relating optical density (O.D.590) to cell density (evaluated as dry weight). Yeast dry weight was obtained after washing broth culture

samples twice and achieving a continuing weight at 105°C. This procedure provided the subsequent correlation factors, 2.30, 2.45, and 1.90 (O.D.590 per g L- 1), for S. cerevisiae CEN.PK strains, S. cerevisiae BY4741 and S. cerevisiae T5bV, respectively.

2.4. Analysis

Samples were quickly withdrawn from shake-flasks, filtered on 0.45 μm GF/A filters (Millipore, Bedford, MA USA) and also the filtrates analyzed to see residual glucose and ethanol concentrations. Residual glucose (g L- 1) within the medium decided by enzymatic d-Glucose assay (GOPOD — Megazyme International, Ireland Ltd). Ethanol production was evaluated with the enzymatic kit also from Megazyme. All samples were analyzed in triplicate showing a regular deviation always not up to 5%.

3. Results

3.1. Screening of fermentative capacity of the S. cerevisiae strains

To test the capacity of ethanol production, all strains were allowed to grow in shake-flasks containing a rich-complex medium with two different initial glucose concentrations, YEPD2 and YEPD15, as described in Section 2.1. The fermentative capacity was evaluated by determining ethanol concentration in correspondence with the depletion of glucose within the medium.

Fig. 1 shows that every one the strains allowed to ferment in YEPD2 exhibited an analogous ethanol production of about 5–6 g L- 1. Similarly, no significant differences might be observed when cells were allowed to grow at a significantly higher glucose concentration (YEPD15), except within the case of S. cerevisiae T5bV, which produced the bottom amount of ethanol (Fig. 1). More interesting results were obtained when ethanol yield was evaluated as yield relative to both glucose consumption (YE/G) and biomass production (YE/X), rather than considering the ultimate ethanol concentration only (Table 1). Indeed, when YEPD15 was used as a fermentation medium, a rise in YE/G was always noticeable concerning YEPD2. This phenomenon was evident especially within the case of the CEN.PK strains and also the auxotrophic S. cerevisiae BY4741 strain,

whereas only a small increase in YE/G, were observed within the case of S. cerevisiae T5bV (Table 1).

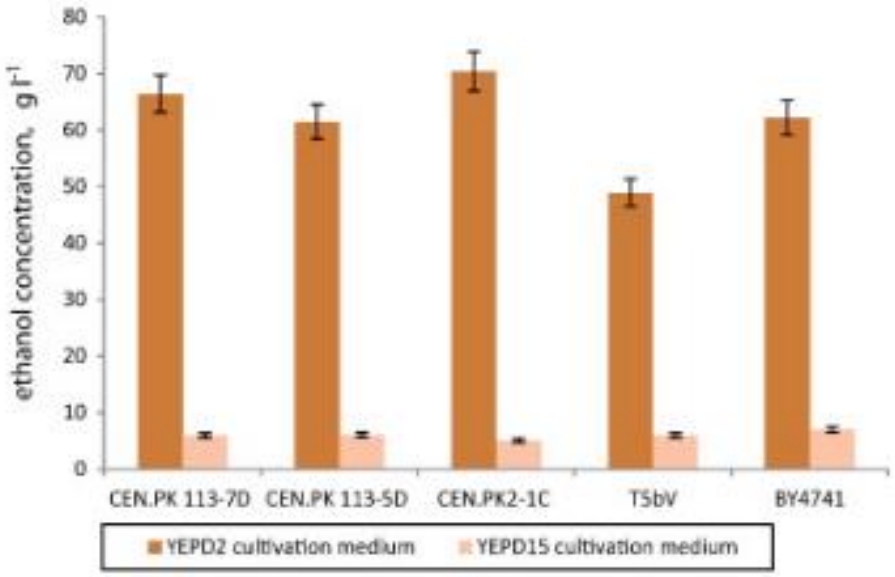

Fig. 1. Fermentative capacity of *S. cerevisiae* strains under investigation: YEPD2 cultivation medium and YEPD15 cultivation medium.

Table 1. Parameters considered studying the fermentative capacity of *S. cerevisiae* strains.

Strain	Glucose[a] ($g\ l^{-1}$)	Biomass[b] ($g\ l^{-1}$)	$Y_{X/G}$ ($g_{dw}\ g^{-1}$)	$Y_{E/G}$ ($g\ g^{-1}$)	$Y_{E/X}$ ($g\ g^{-1}_{dw}$)
T5bV	20	2.40	0.120	0.300	2.50
	150	13.6	0.0907	0.326	3.60
CEN.PK113-7D	20	2.17	0.108	0.300	2.76
	150	13.6	0.0907	0.443	4.87
CEN.PK113-5D	20	2.17	0.108	0.306	2.81
	150	6.01	0.0401	0.410	10.2
CEN.PK2-1C	20	2.33	0.116	0.253	2.17
	150	6.06	0.0404	0.469	11.6
BY4741	20	2.54	0.127	0.350	2.76
	150	5.84	0.0389	0.415	10.6

a Initial glucose concentration.

b Biomass concentration obtained after glucose depletion. The results are the means of three different experiments and that standard deviation was smaller than 10%.

The increase in ethanol yield relative to glucose consumption (YE/G), observed when YEPD15 was used, was parallel to the rise in ethanol yield relative to biomass produced (YE/X, Table 1) which was particularly noticeable within the case of the auxotrophic strains. Indeed, the worth of YE/X in YEPD15 for the auxotrophic strains CEN.PK113-5D and CEN.PK2-1C was quite twice that of the prototrophic CEN.PK113-7D strain. High YE/X value, similar for the 2 auxotrophic CEN.PK strains tested, was also a characteristic of the opposite auxotrophic strain examined, S. cerevisiae BY4741.

3.2. Effect of glucose addition on the fermentative capacity of prototrophic and auxotrophic S. cerevisiae strains

To assay the capacity of the yeast strains to still produce ethanol over time, the test with YEPD15 was prolonged for both the auxotrophic CEN.PK2-1C and therefore the prototrophic CEN.PK113-7D strains, by restoring the initial 15% w/v glucose concentration when the carbon source was exhausted. During this experiment, cell density (Fig. 2a), residual glucose (Fig. 2b), and ethanol (Fig. 2c) concentrations before and after refreshing the medium with new glucose, were monitored.

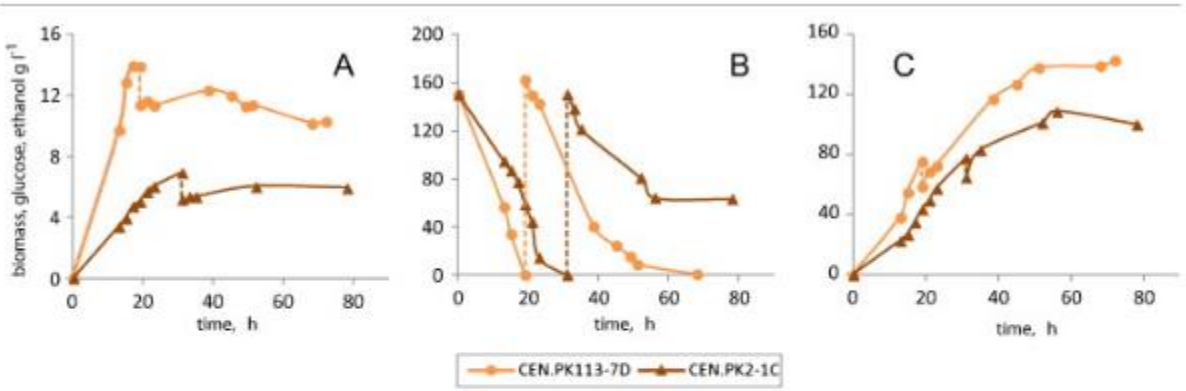

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Fig. 2. Effect of glucose addition on the fermentative capacity of *S. cerevisiae* CEN.PK strains: CEN.PK113-7D (circles) and CEN.PK2-1C strains (triangles). Cell density, (A), residual glucose, (B), and ethanol concentration, (C) *vs* time. Points of discontinuity marked with dotted line represent the time at which glucose was replenished after its depletion.

During the primary fermentation innovate YEPD15, the prototrophic S. cerevisiae CEN.PK113-7D grew more vigorously compared to the auxotrophic CEN.PK2-1C (Fig. 2a). However, the quantity of ethanol produced was comparable for both strains after 20 h (Fig. 2c), when the prototrophic strain had depleted all the glucose within the medium. At this moment, approximately one-third of the initial glucose concentration was still available to be used within the case of the auxotrophic strain, allowing it to grow for an additional 10 hands to provide a rather higher ethanol concentration, from the identical amount of glucose, than the prototrophic strain.

In the second fermentation phase, after the restoration of the initial 15% w/v glucose within the exhausted medium, both strains were capable to consume glucose (Fig. 2b) without a net increase in biomass (Fig. 2a). Indeed, the values of ethanol yield on glucose (YE/G) evaluated within the second fermentation phase were very near the theoretical ones [18], 0.51 g ethanol g- 1 glucose for both strains. Glucose was depleted only within the case of the prototrophic strain (Fig. 2b) whereas the auxotrophic strain consumed only 60% of the added glucose (Fig. 2b). In these conditions, the ethanol

concentration profile of CEN.PK113-7D prototrophic strain diverged from that of the CEN.PK2-1C auxotrophic strain (Fig. 2c), because of the upper biomass produced and therefore the capacity of the prototrophic strain to completely deplete the carbon source.

To better compare the fermentative capacity of the strains examined, ethanol yield relative to biomass produced was plotted against the clock (Fig. 3). This highlighted that yield increased until glucose within the medium was consumed (Fig. 2b) and confirmed the upper YE/X value of CEN.PK2-1C concerning the prototrophic strain. Finally, when further addition of glucose was made, no glucose uptake was observed by any of the strains thanks to a significantly high loss of viability (data not shown) presumably caused by both the high ethanol concentration achieved and nutrient depletion.

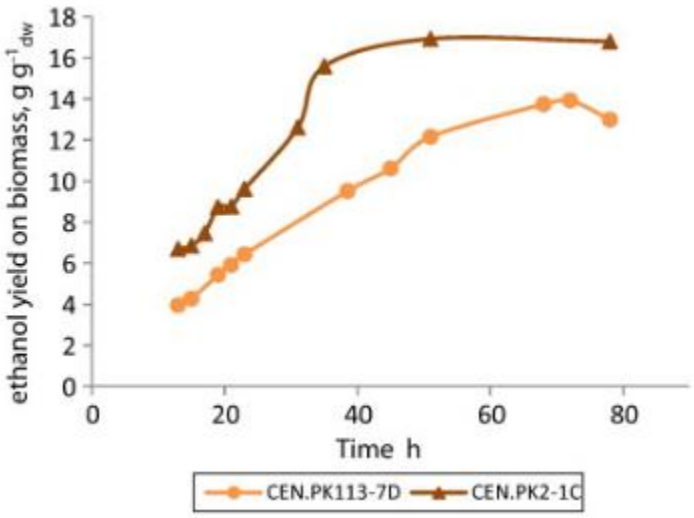

Fig. 3. Ethanol yield on biomass vs time: CEN.PK113-7D and CEN.PK2-1C. Experiments were performed in duplicate with a standard deviation smaller than 15%.

4. Discussion and conclusions

In this work, we've got studied ethanol production in auxotrophic strains of S. cerevisiae. Experiments were performed at two different levels of the initial carbon source, glucose at 2% and 15% (w/v). A 2% (w/v) glucose concentration is usually employed in all sorts of studies in shake flasks for the expansion of S. cerevisiae [19], while the very best value was suitably chosen to attain a significantly higher ethanol concentration

[20]. for 2 of the strains examined, CEN.PK2-1C and CEN.PK113-7, the test in YEPD15 was prolonged by adding a volume of a more concentrated glucose solution to the depleted medium, to revive the initial 15% concentration, allowing during this way a second fermentation.

The results obtained show that the correlation between growth and ethanol production strongly depends on both, yeast strain and environmental conditions. During cultivation in YEPD2, ethanol production proceeded for sure from a growth-linked metabolite (data aren't shown), and also the amount of ethanol produced per cell biomass, was within the range reported within the literature, that is, about 3 g g- 1dw [21]. This occurred irrespective of whether or not the strains were auxotrophic.

When YEPD2 was replaced by YEPD15, yeast growth was related to vigorous ethanol production and a rise in ethanol yield relative to both, glucose consumed (YE/G) and biomass produced (YE/X), the latter ranging between 5 and 10 g g- 1dw, with the very best YE/X values being achieved by the auxotrophic strains, independent of the quantity of the auxotrophies.

Moreover, an extra increase in YE/X, with time was found when yeast went on growing after the addition of glucose to the exhausted medium to revive the initial glucose concentration. This behavior suggests that ethanol production isn't always growth-associated which it should be strongly full of another factor like cell age, the ratio between the most energy and carbon source (glucose), and other nutrients or the requirement to divert the next amount of energy towards maintenance rather than growth [9].

In the light of the results obtained, this work is considered because the start line for a scientific investigation on yeast strains able to ferment at a awfully low rate of growth. Indeed, these strains can be exploited within the field of ethanol production by immobilized yeast cells [22,23,24] with countless advantages deriving from the chance to regu-

late the thickness of biofilm and to cut back mass transfer limitations [25] and cell leakage through the immobilized system. this can be also, to our knowledge, the primary study on the fermentative behavior of auxotrophic strains of S. cerevisiae, strains that are routinely employed in a good range of applications, including the expression of recombinant proteins.

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