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Short communication

On the fermentative behavior of auxotrophic strains of *Saccharomyces cerevisiae*

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ABSTRACT

Background: The selection of new yeast strains could lead to improvements in bioethanol production. Here, we have studied the fermentative capacity of different auxotrophic mutants of *Saccharomyces cerevisiae*, which are routinely used as hosts for the production of heterologous proteins. It has recently been found that these strains exhibit physiological alterations and peculiar sensitivities with respect to the parental prototrophic strains from which they derive. In this work the performance of auxotrophic *S. cerevisiae* CEN.PK strains was compared to the corresponding prototrophic strain, to *S. cerevisiae* T5bV, a strain isolated from grape must and to another auxotrophic strain, *S. cerevisiae* BY4741.
Results: The results indicate that the fermentative capacity of strains grown in 2% glucose was similar in all the strains tested. However, in 15% initial glucose, the auxotrophic strains exhibited a more than doubled ethanol yield on biomass (10 g g$^{-1}_{dw}$) compared to the prototrophic strains (less than 5 g g$^{-1}_{dw}$). 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 produce ethanol per mass unit, in a higher amount with respect to the prototrophic ones. This leads to potential applications for auxotrophic strains of *S. cerevisiae* in the production of ethanol in both homogeneous and heterogeneous phases (immobilized systems). The higher ethanol yield on biomass would be advantageous in immobilized cell systems, as a reduced yeast biomass could greatly reduce the mass transfer limitations through the immobilization matrix.

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1. Introduction

The last years have been characterized by an unprecedented interest in the study of techniques for the production of bioethanol by fermentation in order to convert biomass into liquid fuel, as a way to replace or supplement fossil fuels [1,2]. In this context, research addressed to characterize new yeast strains able to produce ethanol in peculiar cultivation conditions represents a valid contribution in this field [3].

Auxotrophic *Saccharomyces cerevisiae* mutants have played an important role in the development of yeast classical genetic techniques, yeast molecular biology and genetic and metabolic engineering [4,5]. One of the most important uses of auxotrophic yeast strains is in the field of heterologous protein production using the auxotrophy in combination with selective medium as a way to ensure stability of the plasmid and the expression of the recombinant protein [6,7,8]. However, there have not been, to our knowledge, previous studies focused on ethanol production by auxotrophic yeasts strains, probably because, at first sight, they constitute unlikely candidates for this purpose.

The present work was aimed at investigating the capability of some auxotrophic *S. cerevisiae* strains to produce 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, depended on both the type and the number of auxotrophies. In the latter case, the performance decreased with increasing the number of auxotrophies [9]. Moreover, it was also observed that the higher the number of auxotrophies the earlier was the transition from fully respiratory to the respiro-fermentative metabolism, notwithstanding the severe growth limiting conditions applied over the entire time course of the fed-batch run, specifically designed to avoid over-flow metabolism [10].
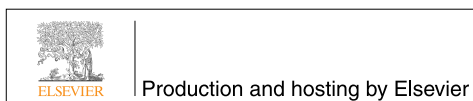
In the present study, the ethanol production capacity of three strains belonging to the CEN.PK family [11] of *S. cerevisiae*, the prototrophic CEN.PK113-7D and two auxotrophic strains, CEN.PK113-5D and CEN.PK2-1C bearing one and four auxotrophies, respectively, has been investigated. The fermentative capacity of the CEN.PK strains has also been compared to that of another auxotrophic strain, *S. cerevisiae* BY4741, commonly used in the laboratory [12,13], bearing four auxotrophies, and to a yeast strain isolated from grape must, *S. cerevisiae* T5bV. The fermentative capacity of these strains, was studied allowing them to proliferate in a rich-complex medium based on YEP (Yeast Extract and Peptone), suited to promote fermentation [14], at two different glucose concentrations, 2% and 15% (w/v). For two 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 restore glucose to 15% after the initial glucose present in the medium had been completely depleted, and monitoring the ability of the strains to produce 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 in the auxotrophic strain than in 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 used 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 MAL2-8^c SUC2), CEN.PK113-5D (MATa ura3-52 HIS3, LEU2 TRP1 MAL2-8^c SUC2) and CEN.PK2-1C (MATa ura3-52 his3-Δ1 leu2-3,112 trp1-289, MAL2-8^c SUC2). They were kindly provided by Prof. D. Porro (University Milano-Bicocca, Italy) except for 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 able to grow at high levels (over 12%) of ethanol concentration.

The strains of CEN.PK family are isogenic laboratory strains [11] obtained from Dr P. Kotter, Frankfurt, Germany [15]. CEN.PK2-1C is the strain used in the yeast gene functional analysis project made in collaboration with 15 lab that worked on the deletion of the genes and on the functional analysis of the deletion strains [16]. The other laboratory strain, BY4741, is based on the well-known *S. cerevisiae* S288c in which, four commonly used selectable marker genes have been deleted [13]. *S. cerevisiae* S288c although not closely related to the CEN.PK strains, has many features in common with them [17]. The T5bV is a fresh isolate that has not been characterized apart from it being able to grow at high 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 based on YEP, having the following 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 in the text as YEPD2 and YEPD15 where the latter can be also defined as exhausted when at the end of fermentation it is added with an amount of glucose such as to restore the initial 15% w/v glucose concentration. The fermentation test was prepared in duplicate for each strain considered. In each test, the amount of inoculum, coming from an exponential pre-culture, was evaluated so as to give an initial optical density (O.D.$_{590}$) of 0.1.

2.3. Biomass determination

Biomass was determined 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 constant weight at 105°C. This procedure provided the following 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 the filtrates analyzed to determine residual glucose and ethanol concentrations. Residual glucose (g L^{-1}) in the medium was determined 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 standard deviation always lower than 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 determining ethanol concentration in correspondence with the depletion of glucose in the medium.

Fig. 1 shows that all the strains allowed to ferment in YEPD2, exhibited a similar ethanol production of about 5–6 g L^{-1}. Similarly, no significant differences could be observed when cells were allowed to grow at a significantly higher glucose concentration (YEPD15), except in the case of *S. cerevisiae* T5bV, which produced the lowest amount of ethanol (Fig. 1). More interesting results were obtained when ethanol yield was evaluated as yield relative to both glucose consumption ($Y_{E/G}$) and biomass production ($Y_{E/X}$), instead of considering the final ethanol concentration only (Table 1). Indeed, when YEPD15 was used as fermentation medium, an increase in $Y_{E/G}$ was always noticeable with respect to YEPD2. This phenomenon was evident especially in the case of the CEN.PK strains and the auxotrophic *S. cerevisiae* BY4741 strain, whereas only a slightly increase in $Y_{E/G}$ was observed in the case of *S. cerevisiae* T5bV (Table 1).

The increase in ethanol yield relative to glucose consumption ($Y_{E/G}$), observed when YEPD15 was used, was parallel to the increase in ethanol yield relative to biomass produced ($Y_{E/X}$, Table 1) which was
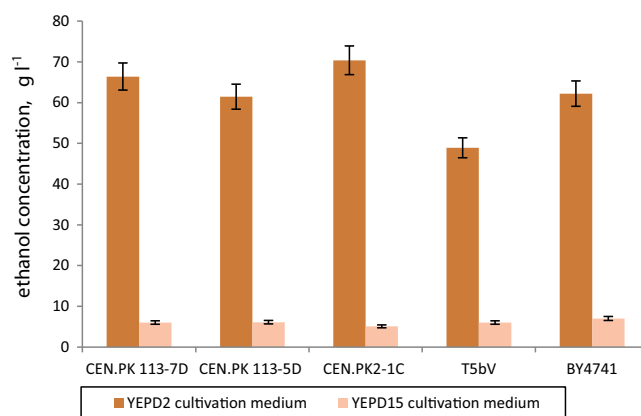

Fig. 1. Fermentative capacity of *S. cerevisiae* strains under investigation: YEPD2 cultivation medium and YEPD15 cultivation medium.

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%.

particularly noticeable in the case of the auxotrophic strains. Indeed, the value of $Y_{E/X}$ in YEPD15 for the auxotrophic strains CEN.PK113-5D and CEN.PK2-1C, was more than twice than that of the prototrophic CEN.PK113-7D strain. High $Y_{E/X}$ value, similar for the two auxotrophic CEN.PK strains tested, was also a characteristic of the other auxotrophic strain examined, S. cerevisiae BY4741.

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

In order to assay the capacity of the yeast strains to continue to produce ethanol over time, the test with YEPD15 was prolonged for both the auxotrophic CEN.PK2-1C and 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.

During the first fermentation phase in YEPD15, the prototrophic S. cerevisiae CEN.PK113-7D grew more vigorously compared to the auxotrophic CEN.PK2-1C (Fig. 2a). However, the amount of ethanol produced was comparable for both strains after 20 h (Fig. 2c), when the prototrophic strain had depleted all the glucose in the medium. At this moment, approximately one third of the initial glucose concentration was still available for use in the case of the auxotrophic strain, allowing it to grow for a further 10 h and to produce a slightly higher ethanol concentration, from the same amount of glucose, than the prototrophic strain.

In the second fermentation phase, after restoration of the initial 15% w/v glucose in 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

($Y_{E/G}$) evaluated in the second fermentation phase were very close to the theoretical ones [18], 0.51 g ethanol g^{-1} glucose for both strains. Glucose was depleted only in 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), due to the higher biomass produced and 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 time (Fig. 3). This highlighted that yield increased until glucose in the medium was consumed (Fig. 2b) and confirmed the higher $Y_{E/X}$ value of CEN.PK2-1C with respect to the prototrophic strain. Finally, when a further addition of glucose was made, no glucose uptake was observed by any of the strains due to a significantly high loss of viability (data not shown) presumably caused by both the high ethanol concentration achieved and nutrient depletion.

4. Discussion and conclusions

In this work we have 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 commonly used in all types of studies in shake flasks for the growth of S. cerevisiae [19], while the highest value was suitably chosen with the aim to achieve a significantly higher ethanol concentration [20]. For two 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 restore the initial 15% concentration, allowing in 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 as expected from a growth-linked metabolite (data not shown), and the amount of ethanol produced per cell biomass, was in the range reported in the literature, that is, about 3 g g^{-1}$_{dw}$ [21]. This occurred regardless of whether or not the strains were auxotrophic.

When YEPD2 was replaced by YEPD15, yeast growth was associated to a vigorous ethanol production and an increase in ethanol yield relative to both, glucose consumed ($Y_{E/G}$) and biomass produced ($Y_{E/X}$), the latter ranging between 5 and 10 g g^{-1}$_{dw}$, with the highest $Y_{E/X}$ values being achieved by the auxotrophic strains, independent of the number of the auxotrophies.

Moreover, a further increase in $Y_{E/X}$, with time was found when yeast went on growing after addition of glucose to the exhausted
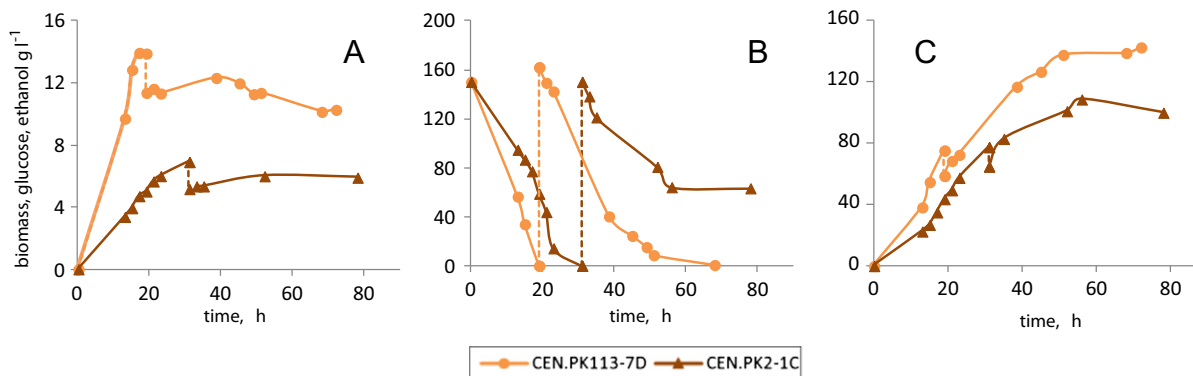

Fig. 2. Effect of glucose addition on the fermentative capacity of S. cerevisiae CEN.PK strains: CEN.PK113-7D (circles) and CEN.PK2-1C strains (triangles). Cell density, (A), residual glucose, (B), and ethanol concentration, (C) vs time. Points of discontinuity marked with dotted line represent the time at which glucose was replenished after its depletion.
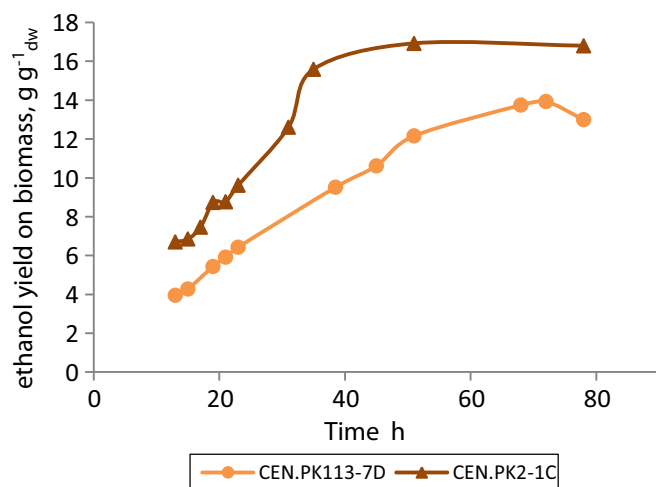
Fig. 3. Ethanol yield on biomass vs time: CEN.PK113-7D and CEN.PK2-1C. Experiments were performed in duplicate with a standard deviation smaller than 15%.

medium to restore the initial glucose concentration. This behavior suggests that ethanol production is not always growth-associated, and that it may be strongly affected by other factor such as cell age, the ratio between the main energy and carbon source (glucose) and other nutrients or the need to divert a higher amount of chemical energy towards maintenance instead of growth [9].

In the light of the results obtained, the present work can be considered as the starting point for a systematic investigation on yeast strains able to ferment at very low growth-rate. Indeed, these strains could be exploited in the field of ethanol production by immobilized yeast cells [22,23,24] with countless advantages deriving from the possibility to control the thickness of biofilm and to reduce mass transfer limitations [25] and cell leakage through the immobilized system. This is also, to our knowledge, the first study on the fermentative behavior of auxotrophic strains of *S. cerevisiae*, strains that are routinely used in a wide range of applications, including the expression of recombinant proteins.

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Author contributions

Proposed the theoretical frame: CL; Conceived and designed the experiments: LP, CL; Wrote the paper: LP, JZ, CL; Analyzed the data: LP, CL.

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