CHEMICAL ENGINEERING TRANSACTIONS
VOL. 38, 2014
A publication of AIDIC
The Italian Association of Chemical Engineering
www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Taj Keshavarz

ISBN 978-88-95608-29-7; **ISSN** 2283-9216
DOI: 10.3303/CET1438078

Auxotrophic *Saccharomyces Cerevisiae* CEN.PK Strains as New Performers in Ethanol Production

Lucia Paciello, Palma Parascandola, Carmine Landi*

Industrial Engineering Department, University of Salerno, via Giovanni Paolo II 132, 84084 Fisciano, Salerno, Italy
clandi@unisa.it

In this work, the capacity to produce ethanol of auxotrophic strains belonging to the CEN.PK family of the yeast *Saccharomyces cerevisiae* has been investigated.
Shake flask experiments were set up to study the ethanol production by the auxotrophic *S. cerevisiae* CEN.PK strains in terms of biomass and ethanol yield, the latter was evaluated on both glucose and biomass. The test was carried out in a rich-complex medium with 2 and 15 % w/v initial glucose concentration. The results obtained, compared with those of the prototrophic CEN.PK strain, indicated that the fermentative capacity of the auxotrophic strain in 2 % glucose was comparable at all to the prototrophic one, whereas using 15 % initial glucose, the auxotrophic CEN.PK strain exhibited a more than doubled ethanol yield on biomass.
The fermentative capacity of both the prototrophic and the auxotrophic strain was also tested after having refreshed the exhausted medium with glucose to restore the initial 15 % concentration. In these conditions, the amount of ethanol produced by the auxotrophic strain per mass unit, resulted to be double with respect to the prototrophic one.
In the light of these results, yeast culture based on auxotrophic CEN.PK, strains could be taken into consideration as culture capable to produce ethanol with a low amount of microbial cells and so potentially suitable to be used for ethanol production by immobilized system.

1. Introduction

Nowadays is of considerable interest the study of techniques for the production of ethanol by fermentation in order to convert biomass into liquid fuel, as a way to replace or supplement other type of fuels, especially fossil (McKendry, 2002; Caspeta et al., 2013).
To this purpose, the investigation addressed to search new yeast strains capable to produce ethanol in peculiar cultivation conditions, could represent a valid contribution for the research in this field (Nielsen et al., 2013).
To our knowledge, a study focused on ethanol production by auxotrophic yeasts strains has not yet been made. Among the auxotrophic yeast mutants, those obtained by the yeast *Saccharomyces cerevisiae* are of great importance. Indeed, they have played an important role in the development of yeast classical genetic techniques, yeast molecular biology, genetic and metabolic engineering (Sherman et al., 1986; Sherman, 1991).
The present work is aimed at investigating the capability of some auxotrophic *S. cerevisiae* strains to produce ethanol and comparing their performances to the prototrophic strain.
The idea of studying ethanol production by auxotrophic yeast strains stems from the behaviour in aerated fed-batch reactor of auxotrophic strains belonging to the CEN.PK family of the yeast *S. cerevisiae* observed by Landi et al.(2011). Particularly, it was observed that, notwithstanding severe growth limiting conditions were applied to avoid over-flow metabolism (Enfors, 2001), auxotrophic yeasts had a strong tendency to produce ethanol with respect to the prototrophic parental strain. In addition, it was observed that the greater the number of autotrophies the faster was the metabolic shift from respiratory to fermentative metabolism with ethanol production (Landi et al., 2011).

Please cite this article as: Paciello L., Parascandola P., Landi C., 2014, Auxotrophic saccharomyces cerevisiae cen.pk strains as new performers in ethanol production, Chemical Engineering Transactions, 38, 463-468 DOI: 10.3303/CET1438078

In the present study, two strains belonging to the CEN.PK family (van Dijken et al., 2000), the prototrophic CEN.PK113-7D and two auxotrophic strains CEN.PK113-5D and CEN.PK2-1C bearing one and four auxotrophies, respectively, have been taken into consideration to investigate the differences in their capability to produce ethanol.
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 (Hahn-Hagerdal et al., 2005) at two different initial glucose concentration, namely 2 and 15 % (w/v). The test made at 15 % (w/v) glucose concentration has been prolonged, for the two strains, CEN.PK113-7D and CEN.PK2-1C, after glucose depletion in the medium, by renewing only glucose concentration and by monitoring the ability to produce ethanol.

2. Materials and methods

2.1 *Saccharomyces cerevisiae* CEN.PK strains

The microorganisms used in this work are three yeasts strains belonging to the CEN.PK family of *Saccharomyces cerevisiae* namely the prototrophic CEN.PK 113-7D (MATa URA3 HIS3, LEU2 TRP1 MAL2-8c SUC2) and the auxotrophs 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 purchased at EUROSCARF collection (www.uni-frankfurt.de/fb15/mikro/euroscarf).

2.2 Shake-flask culture

Growth in shake-flask cultures was performed at 30 °C in 500 ml Erlenmeyer flasks containing 100 ml of rich-complex medium based on YEP (Yeast Extract and Peptone), having the following composition (w/v): 1 % yeast extract, 2 % peptone (BectonDickinson & Co.) added with 2 or 15 % α-D-glucose (Sigma Aldrich). These culture media are mentioned in the text as YEPD2 and YEPD15. When glucose in YEPD15 was completely depleted, the fermentation test was prolonged by refreshing the exhausted medium with new glucose in order to achieve again the 15 % w/v initial glucose concentration. The fermentation test was prepared in duplicate for each strain considered and the shake flask inoculum came from an exponential pre-culture in an amount such as to give the initial optical density at 590 nm ($O.D._{590}$) of 0.1.

2.3 Analysis

Samples were quickly withdrawn from shake-flasks, filtered on 0.45 µm GF/A filters (Millipore, Bedford, MA USA) and the filtrates analysed 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 from Megazyme. All the samples were analysed in triplicate showing a standard deviation always lower than 5 %.
Biomass dry weight for yields calculation was determined by a calibration curve relating $O.D._{590}$ to cell density.

3. Results

3.1 Screening of *S. cerevisiae* CEN.PK strains for their fermentative capacity

To test fermentative capacity, three strains belonging to the CEN.PK family of the yeast *S. cerevisiae* namely a prototrophic strain CEN.PK 113-7D and two auxotrophic, CEN.PK 113-5D and CEN.PK2-1C bearing one and four auxotrophies, respectively (for details see Materials and Methods) were taken into consideration. These strains were allowed to grow in 500 mL shake-flasks containing 100 ml YEPD2 or YEPD15, as described in section 2.2. The fermentative capacity has been evaluated by determining ethanol concentration in the medium when glucose was completely exhausted. As far as the test at low glucose concentration, in YEPD2, the time of glucose depletion was quite similar for all the strains tested and it resulted to be 9-10 h (data not shown). Contrarily, with high glucose concentration of YEPD15, glucose depletion time differed significantly between the prototrophic strain and the auxotrophic ones, being 19 h for CEN.PK 113-7D and 30-31 h for CEN.PK 113-5D and CEN.PK2-1C.
Figure1, shows that, all the strains in YEPD2, exhibited a similar final ethanol production of about 5-6 g L^{-1}. Also in the presence of a significantly higher glucose concentration as in the case of YEPD15, no significant difference in final ethanol was observed between the prototrophic and the auxotrophic strains even if final ethanol achieved was obviously higher than that produced in YEPD2.

A more fruitful comparison between prototrophic and auxotrophic strains was accomplished when, besides ethanol amount obtained, ethanol yields (Table 1) based on biomass unit ($Y_{E/X}$) or glucose consumed ($Y_{E/G}$)

were considered. Indeed, a significant increase in $Y_{E/G}$ was always noticeable in YEPD15 with respect to YEPD2.

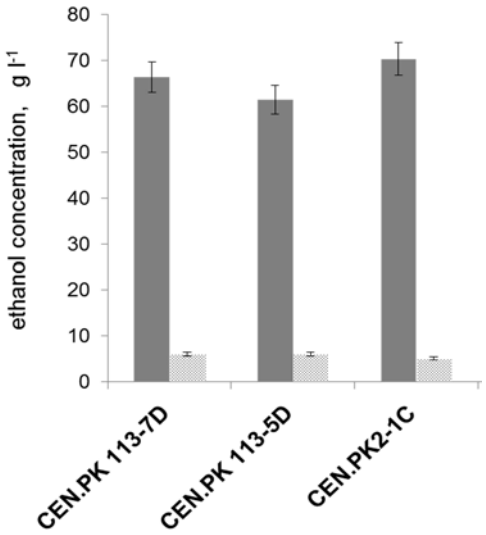

Figure 1: Fermentative capacity of S. cerevisiae CEN.PK strains under investigation: YEPD2 cultivation medium (light grey), YEPD15 cultivation medium (dark grey). Experiment were performed in duplicate and S.D. was always lower than 7 %.

The increase in ethanol yield on glucose ($Y_{E/G}$), observed in YEPD15, for all the strains considered, joined with the increase of ethanol yield per biomass unit ($Y_{E/X}$, see Table 1) which was particularly relevant in the case of the auxotrophic strains. Indeed the $Y_{E/X}$ values in YEPD15 for both the auxotrophic strains CEN.PK113-5D and CEN.PK2-1C, were more than double if compared to the prototrophic CEN.PK113-7D (Table 1).

Table 1: Ethanol yield on glucose ($Y_{E/G}$) and ethanol yield on biomass ($Y_{E/X}$). The parameters were obtained after glucose depletion for 20 and 150 g L^{-1} initial glucose. S.D. was always lower than 5%.

	glucose **g L^{-1}**	biomass **g L^{-1}**	$Y_{E/G}$ **g g^{-1}**	$Y_{E/X}$ **g g^{-1}** (d.w.)
CEN.PK113-7D	20	2.17	0.300	2.76
	150	13.6	0.443	4.87
CEN.PK113-5D	20	2.17	0.306	2.81
	150	6.01	0.410	10.2
CEN.PK2-1C	20	2.33	0.253	2.17
	150	6.06	0.469	11.6

3.2 Fermentative capacity of CEN.PK strains in exhausted medium

In order to assay the capability of the auxotrophic yeast strains to produce ethanol over time, the fermentation test in YEPD15 (section 3.1), has been prolonged for the CEN.PK2-1C strain having four auxotrophies, by restoring the 15 % glucose in the exhausted medium and compared with the prototrophic CEN.PK113-7D strain assayed in the same condition. During this experiment, ethanol production was monitored over time (Figure 2A) since the beginning of the first fermentation test in fresh YEPD15.

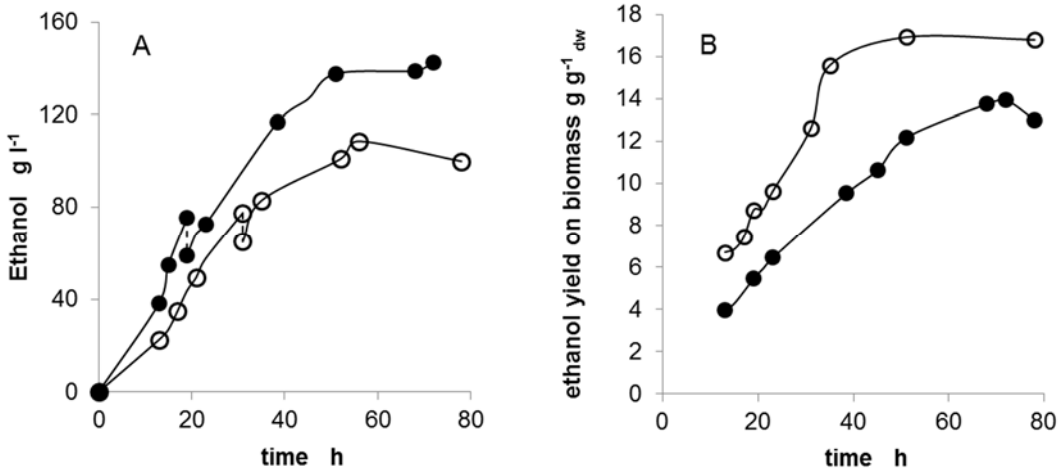

Figure 2: Effect of glucose addition on the fermentative capacity of S. cerevisiae CEN.PK113-7D (full circles) and CEN.PK2-1C (empty circles): ethanol concentration (A) and ethanol yield on biomass (B). Points of discontinuity marked with dotted line represent the time at which glucose was replenished after its depletion.

It is worth noting that during fermentation test, in both fresh and exhausted YEPD15, the prototrophic *S. cerevisiae* CEN.PK113-7D, grew faster and produced always more ethanol in comparison with the auxotrophic CEN.PK2-1C (Figure 2A). The better performance exhibited by CEN.PK113-7D corresponded to faster glucose consumption. As a consequence, refreshing of the exhausted medium in the case of the prototrophic strain, was anticipated (after 19 h incubation) with respect to the auxotrophic one (after 31 h incubation) as shown in Figure 2A by dot lines which represent the link between two points that are ethanol concentration achieved before and after medium refresh, the latter resulting always lower due to dilution effect.

To better compare the fermentative capacity of the strains examined, ethanol yield on biomass ($Y_{E/X}$) has been plotted against the time (Figure 2B) highlighting that $Y_{E/X}$ increased continuously for both CEN.PK113-7D and CEN.PK2-1C and achieved a plateau value when the strains were not able to produce ethanol anymore due to the higher concentration of this by-product (more than 100 g L^{-1}) (Walker, 1998) as shown in Figure 2A. It is interesting to observe that the capability of CEN.PK 2-1C to produce ethanol per mass unit was always higher than that of CEN.PK 113-7D (Figure 2B).

The results showed that, notwithstanding the prototrophic strain grew faster, the auxotrophic one had the ability to produce higher amount of ethanol per mass unit without a net increase in biomass.

After the last glucose addition, further medium refreshing attempt did not produce any change in the brothculture probably due to a, significantly cell viability loss (data not shown) caused by the high ethanol concentration achieved and nutrient depletion.

4. Conclusions

To study aspects of ethanol production related to both prototrophic and auxotrophic yeast strains, experiments were performed at two different concentration of the initial carbon source, namely 2 and 15 % (w/v) of glucose. 2 % (w/v) glucose concentration is commonly used in shake flasks for cultivation study with *S. cerevisiae* (Saghbini et al., 2001) whereas YEPD15 broth formulation was suitably chosen with the aim to achieve a significant ethanol concentration (Alfenore et al., 2002).

For CEN.PK2-1C and CEN.PK113-7D, the test in YEPD15 was prolonged by adding to the depleted medium new glucose to test both their fermentative capacity and their robustness. This is the reason why a four auxotrophies-bearing strain, CEN.PK2-1C was chosen as a representative auxotrophic strain having been resulted as the highest potential producer strain (Landi et al., 2011).

During cultivation in YEPD2, as expected, ethanol resulted to be growth associated (data not shown) and its amount produced per cell mass, was in the range reported in the literature, 3 g $g^{-1}_{d.w.}$ (Valadi et al., 1998) as well as biomass and ethanol yields on glucose (Lievense and Lim, 1982; Enfors, 2001). This occurred regardless of whether the strains were auxotrophic or not.

When YEPD2 was replaced by YEPD15, an increase in ethanol yield on both glucose ($Y_{E/G}$) and biomass ($Y_{E/X}$) was observed, the latter ranging between 5-10 g $g^{-1}_{d.w.}$. The highest $Y_{E/X}$ values were achieved with the auxotrophic strains independently of the number of the auxotrophies.
Moreover a further increase in $Y_{E/X}$, with time, has been found, when yeast went on growing after addition of glucose to the exhausted medium to restore the initial glucose concentration. This behaviour highlighted that ethanol produced per mass unit can be strongly affected by cell age, ethanol concentration in the culture medium and/or the ratio between the main energy and carbon source (glucose) and other nutrients that is by non-balanced medium where, presumably, yeast cell are forced to divert an higher amount of glucose towards maintenance. This behaviour was more pronounced in the case of auxotrophic strains as already shown in the literature (Paciello et al., 2009; Paciello et al., 2010; Landi et al., 2011).
The present work represents the starting point to initiate an investigation on yeast physiology to have a deeper insight on yeast catabolism under different environmental conditions. This study should be aimed at isolating new auxotrophic strains capable to ferment with very low biomass yield. Such type of yeast strains could be exploited in the field of ethanol production with immobilized cells (Najafpour et al., 2004; de Alteriis et al., 1992; Parascandola et al., 1992) with countless advantages such as diminishing of cell leakage, control of biofilm thickness and reduction of mass transport limitation phenomena (Qureshi et al., 2005).

Acknowledgement

This work was supported by the University of Salerno funds (FARB 2012) to Palma Parascandola in the framework of the research project “La Lipasi A da Bacillus subtilis espressa nel lievito *Saccaromyces cerevisiae*: realizzazione e modellazione del bioprocesso con studio di fattibilità”.

References

Alfenore S., Molina-Jouve C., Guillouet S. E., Uribelarrea J. L., Goma G., Benbadis L., 2002, Improving ethanol production and viability of *Saccharomyces cerevisiae* by a vitamin feeding strategy during fed-batch process, Applied Microbiology and Biotechnology, 60, 67-72.

Caspeta L., Buijs N. A. A., Nielsen J., 2013, The role of biofuels in the future energy supply, Energy Environmental Science, 6, 1077-1082.

de Alteriis E., Parascandola P., Scardi V., 1992, Ethanolic fermentation by yeast cells immobilized in polyhaldehyde-hardened gelatin beads. Journal of Fermentation and Bioengineering, 73, 73-75.

Enfors S.O., 2001, Baker’s yeast. In: Ratledge C. and Kristiansen B. Editors, Basic Biotechnology, Cambridge University Press.

Hahn-Hägerdal B., Karhumaa K., Larsson C. U., Gorwa-Grauslund M., Görgens J., van Zyl W. H., 2005, Role of cultivation media in the development of yeast strains for large scale industrial use. Microbial Cell Factories, 4, 31, doi:10.1186/1475-2859-4-31.

Landi C., Paciello L., de Alteriis E., Brambilla L., Parascandola P., 2001, Effect of auxotrophies on yeast performance in aerated fed-batch reactor. Biochemical Biophysical Research Communication, 414(3), 604–611, 10.1016/j.bbrc.2011.09.129.

Lievense J.C., Lim H.C., 1982, The growth and dynamics of *Saccharomyces cerevisiae*, Annu. Rep. Ferm. Process, 5, 211-262.

McKendry P., 2002, Energy production from biomass (part 1): overview of biomass, Bioresource Technology, 83, 37-46.

Najafpour G., Younesi H., Syahidah K., Ismail K., 2004, Ethanol fermentation in an immobilized cell reactor using *Saccharomyces cerevisiae*, Bioresource Technology, 92, 251–260.

Nielsen J., Larsson C., van Maris A., Pronk J., 2013, Metabolic engineering of yeast for production of fuels and chemicals, Current Opinion in Biotechnology, 24, 1-7.

Paciello L., Romano V., de Alteriis E., Zueco J., Parascandola P., 2009, Performance of the auxotrophic *Saccharomyces cerevisiae* BY4741 as host for human Interleukin-1β production in an aerated fed-batch reactor, *Microbial Cell Factories,* 8, 70, doi:10.1186/1475-2859-8-70.

Paciello L., Andrès I., Zueco J., Bianchi M.M., de Alteriis E., Parascandola P., 2010, Expression of human interleukin-1b in Saccharomyces cerevisiae using PIR4 as fusion partner and production in aerated fed-batch reactor, Ann. Microbiol, 60, 719–728.

Parascandola P., de Alteriis E., Farris G. A., Budroni M., Scardi V., 1992, Behaviour of grape must fermentation by *Saccharomyces cerevisiae* cells immobilized within insolubilized gelatin, Journal of Fermentation and Bioengineering, 74, 123-125.

Qureshi N., Annous B. A., Ezeji T. C., Karcher P., Maddox I. S., 2005, Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. Microbial Cell Factories, 4, 24, doi:10.1186/1475-2859-4-24.

Saghbini M., Hoekstra D., Gautsch D., 2001, Media Formulations for various two-hybrid systems, in: MacDonald, P.N., (Ed.), Methods in Molecular Biology, Vol. 177, Two-Hybrid Systems: Methods and Protocols, Humana Press Inc., Totowa, NJ

Sherman F., Fink G. R., Hicks J. B., 1986, Laboratory course manual for methods in yeast genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y..Sherman F., 1991, Getting started with yeast, Methods in Enzymology, 194, 3-21.

Valadi H., Larsson C., Gustafsson L., 1998, Improved ethanol production by glycerol-3-phosphate dehydrogenase mutants of *Saccharomyces cerevisiae*. Applied Microbiology and Biotechnology, 50, 434-439.

van Dijken J. P., Bauer J., Brambilla L., Duboc P., Francois J. M., Gancedo C., Giuseppin M. L. F., Heijnen J. J., Hoare M., Lange H. C., Madden E. A., Niederberger P., Nielsen J., Parrou J. L., Petit T., Porro D., Reuss M., van Riel N., Rizzi M., Steensma H. Y., Verrips C. T., Vindeløv J., Pronk J. T., 2000, An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. Enzyme Microbial Technology, 26, 706-714.

Walker G.M.,1998, Yeast physiology and biotechnology, John Wiley &Sons.