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By

Gomes L.H.
Duarte K.M.R.
Lira S.P.

Research Article

Increase on ethanol production by blocking the ADH2 gene expression in GFP3-transformed *Saccharomyces cerevisiae*

Gomes L.H. [1], Duarte K.M.R.[2*], Lira S.P.[1]

[1,3]Universidade de São Paulo – ESALQ – Depto. Exact Sciences – Chemistry- Lab of Natural Products . Av. Pádua Dias 11, 13418-900 Piracicaba, SP, Brazil
[2*]Institute of Animal Science and Pastures – CPDGRA R. Heitor Penteado, 56, 13460-000, Nova Odessa, SP, Brazil

[2*]Corresponding Author´s Email: keila@iz.sp.gov.br

ABSTRACT

***Saccharomyces cerevisiae* was transformed with plasmid pYGFP3, spelling the GFP3 gene from *Aequorea victoria* whose expression is modulated by the enzyme promoter ADH2 yeast gene. The plasmid pYGFP3 (GFP3, TRP1$^+$, AMP) was re-constructed properly and used to transform the strain X2904-3C (met$^-$,ura$^-$ trp1$^-$). The transformed yeast strain X2904-3C-GFP3 produced 10.49 % (w/v) ethanol and ADH activity of 5.94 EUA.ml^{-1}, compared to the non-transformed yeast X2904-3C, with 9.49 % (w/v) of ethanol and ADH activity of 9.41 EUA.ml^{-1}, after 16 hours of fermentation. These differences can be explained by the ADH2 activity blocked in the transformed yeast, converting less ethanol to ATP at the end of fermentation process.**

Keywords: Alcohol deshidrogenase- 2; fermentation; green fluorescent protein; *Saccharomyces cerevisiae.*

INTRODUCTION

For industrial ethanol production some methods can be used to detect spoilage yeasts to avoid production losses (Gomes et al., 2000a). To monitoring the sugar cane fermentation season with massive fed batches or continuous processes demands continuous information about purity and predominant yeast inoculum. Genetic markers are useful for such purpose and the use of the green fluorescent protein gene would be adequate if they do not interfere with efficiency and productivity of the process. Of course the addition of genetic material can be deterrent and care must be taken in this regard.

When a marker gene is introduced in an organism it can be activated or inactivated and pathways can be manipulated aiming different objectives following transformation (Hinnen et al., 1978; Zhou et al., 2012). The expression of foreign genes in Saccharomyces cerevisiae is commonly regulated by native yeast promoters. Inducible promoters are employed to control the time and level of gene expression (Lee and DaSilva, 2005). Some very effective inducible promoters are copper metallothionein (CUP1), galactokinase (GAL1) (Romanos et al., 1992) and alcohol dehidrogenase II (ADHII) (Price et al., 1990). The ADH2 gene (alcohol dehydrogenase 2) from *Saccharomyces cerevisiae* is an example of catabolic repression in presence of low sugar content in the medium (Gancedo, 1998). The form of catabolic repression triggers transcription only when glucose concentrations are low. Upon this mechanism it allowed the ethanol produced to revert to energy for maintenance cell growth. Such catabolic repression is not desirable in industry and this end up of sugar conversion to ethanol must be followed through chemical analysis. The use of a marker, such as GFP modulated by the strong promoter of ADH2, helps to identify when the sugar contents are very low with no need to perform time consuming and labor for such.

MATERIAL AND METHODS

Strains

Two strains, wild type X20904-3C (met$^-$,ura$^-$ trp1$^-$) and the transformed yeast X2904-3C-GFP3 (met$^-$,ura$^-$) (Gomes et al., 2000) were grown in YEPD medium (2% glucose) at constant temperature of 30 ^{0}C, 200 rpm for 16 hours to obtain mass for the fermentation assay and isolation of GFP proteins during three times: 0; 12 and 14 hours.

Fermentation assay

In a 100 mL flask 3g of fresh yeast cells were added in 27 mL of YEPD (22 % glucose) at constant temperature of 30 ^{0}C for 16 hours. Samples were collected each two hours from both strains.

Electrophorese – PAGE 10%

This method was carried out using Davis 10% Polyacrilamide Gel and the samples were obtained according to Gomes et al. (2000b). After running gel, samples were observed under U.V. light to visualize the GFP fluorescence expression before silver stained.

Ethanol and reducing sugar

The ethanol production was evaluated by five fermentations and three repetitions assay. The reducing sugar analysis was performed according to Somogyi and Nelson method and the alcohol yield was measured by gas chromatograph (CG-37D) column CG-Bore-530 (Somogyi, 1952).

Protein concentration and enzymatic activity test

Protein concentration of each sample was assessed by Lowry technique. ADH activity was assayed spectrophotometrically by a modification of the Bergmeyer method (Bergmeyer *et al*., 1974). The reaction mixture contained 650 □L of 0.1 M Tris/HCl buffer (pH 8.8), 200 □L of 30 mM NAD+, 100 □L of absolute ethanol. The protein amount was standardized as 200mg.mL-1. The enzyme reaction was carried out at 30°C in a 1 cm cuvettes, and the reaction was followed by measuring the increase in absorbance at 340 nm. One unit of enzyme activity (EAU) was defined as the amount of enzyme catalyzing the production of 1 mmol of NADH per minute under these conditions (Blandino et al., 1997).

RESULTS AND DISCUSSION

Table 1: Results of ethanol % (w/v); ADH activity (EUA . mL^{-1}) and sugar content, measured by TRS, along the 16 hours of fermentation for both strains – non transformed and GFP transformed yeast.

	Ethanol %(w/v)		ADH activity EUA.mL^{-1}		Sugar %(w/v)	
HOURS	X2904-GFP3	X2904-3C	X2904-GFP3	X2904-3C	X2904-GFP3	X2904-3C
0	0,00 ± 0,00	0,00 ± 0,00	11,29 ± 0,21	16,59 ± 0,64	22,20 ± 0,20	22,20 ± 0,20
2	1,78 ± 0,10	1,74 ± 0,25	10,29 ± 0,15	15,38 ± 0,23	18,60 ± 0,17	17,90 ± 0,30
4	3,48 ± 0,10	3,68 ± 0,31	12,60 ± 0,14	12,29 ± 0,22	14,50 ± 0,25	15,60 ± 0,26
6	5,44 ± 0,18	5,01 ± 0,06	12,44 ± 0,09	11,67 ± 0,10	12,30 ± 0,30	11,30 ± 0,36
8	6,04 ± 0,09	6,15 ± 0,13	10,91 ± 0,16	12,29 ± 0,06	9,60 ± 0,10	9,80 ± 0,26
10	7,24 ± 0,15	7,92 ± 0,19	11,67 ± 0,10	11,47 ± 0,08	6,60 ± 0,20	7,20 ± 0,26
12	8,94 ± 0,10	8,42 ± 0,17	10,39 ± 0,18	12,37 ± 0,18	4,70 ± 0,20	4,30 ± 0,17
14	9,85 ± 0,04	9,16 ± 0,12	10,18 ± 0,09	11,76 ± 0,13	2,20 ± 0,10	2,30 ± 0,10
16	10,49 ± 0,04	9,29 ± 0,33	5,94 ± 0,22	9,61 ± 0,13	1,70 ± 0,10	1,80 ± 0,10

According to table 1, we can observe that the transformed strain X2904-3C-GFP3 and the non-transformed strain starting fermentation with the same media, with average of 22,20% initial TRS (total reducing sugar), presenting at the end of fermentation process, within 16 hours , 10.49% of ethanol (w/v), which means 92.5 % of theoretical fermentation yield, with DAH activity of 5.94 EUA.mL-1 and final TRS of 1.70 % while the non transformed strain presented 9.29 % of ethanol, which means 81.93% from theoretical fermentation yield, ADH activity of 9.61 EUA.mL^{-1} and TRS of 1.80%. At the end of fermentation (16 hours) the transformed strain showed an average of 11.45% more alcohol than non-transformed strain and 38.19% less ADH enzyme activity, even the TRS at the end was the same for both strains, what leads to the explanation based upon ADH II blocking activity, which inhibits the consumption of alcohol by the yeast at the end of sugar source in the fermentation process (Gancedo, 1998).

Low sugar content allied to high alcohol production, on non transformed strains leads to the consumption of some alcohol as carbon source (Beier and Young, 1985). Since the GFP expression, on transformed yeast strain is modulated by the ADH II enzyme promoter, on Figure 1, we can see the fluorescence expressed by the

GFP gene during fermentation, appearing after 14 hours, when the sugar content is already low (less than 2.5 %) fact that can also be observed on table 1.

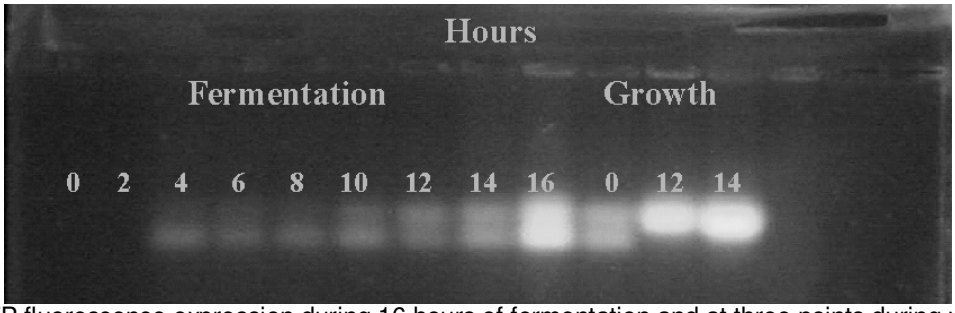

Figure 1: GFP fluorescence expression during 16 hours of fermentation and at three points during yeast growth, showing higher expression as the time comes close to the end of fermentation process (low sugar content).

CONCLUSIONS

Such research confirmed the utility of the ADHII for industrial applications (Lee and DaSilva, 2005) and opens up a wide study area in alcohol fermentation, using models as GFP as markers to block other genes of interest, aiming higher ethanol production. For industrial alcohol production, improvement of fermentation performance can be activated by overexpression of genes which controls stress compounds such as trehalose accumulation and ergosterol contents, as done by Yu et al. (2012) or can be achieved by strains transformation changing the adh expression in the yeast cell (Zhou et al., 2012), aiming not only ethanol increase but also fine sugars production such as xylitol. The knowledge of gene pathways in a process like alcohol fermentation is crucial to the Brazilian and to world sustainable economy, in which alcohol plays a big role in fuel industry.

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