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ORIGINAL PAPER

# Overexpression of *ADH1* and *HXT1* genes in the yeast *Saccharomyces cerevisiae* improves the fermentative efficiency during tequila elaboration

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**Abstract** This work assessed the effect of the overexpression of *ADH1* and *HXT1* genes in the *Saccharomyces cerevisiae* AR5 strain during fermentation of *Agave tequilana* Weber blue variety must. Both genes were cloned individually and simultaneously into a yeast centromere plasmid. Two transformant strains overexpressing *ADH1* and *HXT1* individually and one strain overexpressing both genes were randomly selected and named A1, A3 and A5 respectively. Overexpression effect on growth and ethanol production of the A1, A3 and A5 strains was evaluated in fermentative conditions in *A. tequilana* Weber blue variety must and YPD medium. During growth in YPD and Agave media, all the recombinant strains showed lower cell mass formation than the wild type AR5 strain. Adh enzymatic activity in the recombinant strains A1 and A5 cultivated in *A. tequilana* and YPD medium was higher than in the wild type. The overexpression of both genes individually and simultaneously had no significant effect on ethanol formation; however, the fermentative efficiency of the A5 strain increased from 80.33% to 84.57% and 89.40% to 94.29% in YPD and Agave medium respectively.

**Keywords** *Saccharomyces cerevisiae* · *Agave tequilana* Weber blue variery · Fermentative efficiency · Alcohol dehydrogenase · Transporter hexose

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## Introduction

Tequila is a distilled beverage made from the fermented sugars of cooked *Agave tequilana* Weber blue variety. Tequila is classically associated with Mexico, particularly with Jalisco, a state located in the west of the country. The principal carbohydrate of Agave is insulin which can be hydrolyzed into fermentable sugars by heating it with steam and converting it to ethanol by means of yeast (Cedeño 1995). *Saccharomyces cerevisiae* is the principal yeast responsible for ethanol production due to its high fermentative capacity and ethanol tolerance. However, frequently slow and incomplete alcoholic fermentations are observed in the tequila industry probably caused by faulty hexose transport (Arrizon

and Gschaedler 2002). In *S. cerevisiae*, the hexose transporter family (Hxt) consists of more than 20 proteins comprising high and low affinity transporters (Bisson et al. 1993; Kruckeberg 1996). The transporter Hxt1p, one of the most important transporters in fermentative conditions, is strongly induced in high glucose concentration (>1% w/v o 56 mM) (Lewis and Bisson 1991; Özcan and Johnston 1995) and is expressed only during the growth phase (Luyten et al. 2002; Perez et al. 2005). Hexose transport through the plasmatic membrane in *S. cerevisiae* is known to be a control point in the metabolism of carbon compounds during fermentation (Elbing et al. 2004). Others authors have suggested hexokinase, phosphofructose kinase (PFK), or pyruvate kinase as control points (Galazzo and Bailey 1990; Cortassa and Aon 1994); however, the reduction of acetaldehyde to ethanol with the corresponding oxidation of NADH to NAD+, catalyzed for the enzyme Adh1p (Bennetzen and Hall 1982), could be besides of the sugar uptake, one of the critical steps during ethanol production. Overexpression of single (Schaaff et al. 1989) or simultaneous glycolytic enzymes (Hauf et al. 2000; Smits et al. 2000) has been used strategically to increase ethanol synthesis without successful results. Actually there are no studies describing the effect of simultaneous overexpression of *ADH1* and *HXT1* genes in *S. cerevisiae* strains isolated from the tequila industry. In this study *ADH1* and *HXT1* were placed in a yeast centromere plasmid and expressed in a *S. cerevisiae* tequila strain with the aim of improving fermentative efficiency during tequila production.

## Material and methods

### Strains and culture conditions

*Escherichia coli* DH5α [*sup*E44, Δ*lac*U169 (Φ80lacZDM15), *hsd*R17, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *rel*A1] was used for cloning purposes. It was grown and maintained according to standard protocols (Sambrook and Russell 2001). The *Saccharomyces cerevisiae* AR5 strain (Flores et al. 2005), was isolated from the tequila industry and conserved in our culture collection (Centro de Investigación y Asistencia en Tecnología y Desarrollo del Estado de Jalisco A. C.). Transformant strains A1, A3 and A5 were derived from the AR5 strain and were obtained in this work. Transformant strains and non-transformant AR5 were grown in aerobic conditions in a rotary shaker in YPD medium (2% bacto peptone, 1% yeast extract, 2% glucose [w/v]) or *A. tequilana* medium (*A. tequilana* Weber blue variety juice adjusted to a concentration of 20 g reducing sugar $l^{-1}$ and supplemented with 1.0 g $l^{-1}$ ammonium sulfate). Fermentative conditions were carried out without shaking in the same media except that the glucose and reducing sugar were adjusted to 115 g $l^{-1}$.

### Vector construction

Genomic DNA was isolated from the AR5 yeast cells according to standard protocols (Ausubel et al. 1994) and used for isolation of *ADH1*, *HXT7* and *HXT1* sequences. The yeast centromere plasmid pJRC34 was constructed by insertion of the 3.3-kb *Hin*dIII fragment, carrying the HygB phosphotransferase gene (Gritz and Davies 1983) transcriptionally fused to *U. maydis hsp70* gene promoter of plasmid pHL1 (Wang et al. 1988), into the *Hin*dIII site of yeast centromere plasmid pRS315 (Sikorski and Hieter 1989). The *ADH1* gene including its own promoter was recovered by PCR by using oligonucleotides olis5adh1sc and olias5adh1sc (Table 1), cloned in the TOPO pCR 2.1 vector (Invitrogen, Carlsbad, CA, USA) and sequenced. After that, the *ADH1* gene was subcloned in the *Bam*HI site into the plasmid pJRC34. This construction was named pJRC49. PCR was performed in a MyCycler thermo cycler (BioRad). The reaction consisted of: 12.5 µl of Kit PCR Master Mix (Roche), 500 ng of each primer, 100 ng of genomic DNA and 6.5 µl DNase water free. A pre-PCR step at 94°C for 1 min was applied. A total of 30 PCR cycles were run under the following conditions: Denaturation at 94°C for 10 s; annealing at 53°C for 1 min and extension at 68°C for 3 min 10 s. After the final cycle, the samples were incubated at 68°C for 5 min to complete the reaction.

A truncated 390-bp fragment of the *S. cerevisiae HXT7* promoter has been shown to cause up to 2.5-fold stronger expression than the full-length promoter of 1.2 kb (Hauf et al. 2000). This truncated promoter was used to overexpress the *HXT1* gene. The *HXT1*

**Table 1** Oligonucleotides used for overexpression constructs

| Oligonucleotide | Sequence |
|---|---|
| Olis5adh1sc | at**c ccg gg**a aga aga ggg ttg act aca tc |
| Olias5adh1sc | tt**c ccg gg**c atg cac gta tac act tga gta |
| Gen hxt1 | atg aat tca act ccc gat c |
| Oliassachxt1-2 | act gct t**ga gct c**tt atc aag caa gtg ttt ttc gtg |
| Olisspehxt7-3 | tag ga**a cta gt**t cgg gcc cct gc |
| Megaoligo hxt1 | gga gat att aga tcg gga gtt gaa ttc atg cgt ctt gtg aca ttt ttt g |
| Orf adh1 F2 | caa ggc tat ggg tta cag agt |
| Orf adh1 R1 | aga aca aca ctt ggc acc ag |
| Olis2hxt1 | gag gtc gcc ccc agt gaa atg aga gg |
| Orf hxt1 R1 | gag tca ctt aaa ccg aca gcc t |
| Olisact1 | tcg att tgg ccg gta gag att tga c |
| Oliasact1 | att gaa gaa gat tga gca gcg gtt tg |

gene ORF was fused to the truncated *HXT7* promoter that is constitutively active in ethanol as well as in glucose medium (Hauf et al. 2000). The *HXT1* ORF and the truncated *HXT7* promoter were amplified with PCR by using the oligomer pair gene hxt1 and oliassachxt1-2, olisspehxt7-3 and megaoligo hxt1, respectively (Table 1). The amplifying conditions were the same for both reactions: A pre-PCR step at 94°C for 3 min was applied. A total of 30 PCR cycles were run under the following conditions: Denaturation at 94°C for 1 min; annealing at 50°C for 2 min and extension at 72°C for 2 min 30 s. After the final cycle, the samples were incubated at 72°C for 10 min to complete the reaction. Both PCR products were used as a template in a second PCR reaction, and the oligomer pair used was olisspehxt7-3 and oliassachxt1-2 (Table 1). The PCR fusion conditions were 1 min at 94°C followed by 30 cycles of 10 s at 94°C, 1 min at 50°C and 2 min and 50 s at 68°C per cycle, and finally, 7 min at 72°C. The *HXT7* fusion promoter ORF *HXT1* of 2.621 kbp was cloned in the TOPO pCR 2.1 vector and verified by sequencing. The *HXT7* fusion promoter ORF *HXT1* was subcloned in the *Spe*I site of plasmid pJRC34. This construction was named pJRC52. Finally the *HXT7* fusion promoter ORF *HXT1* was cloned in the *Sac*I site of the plasmid pJRC49. This plasmid was named pJRC54 and contains a copy of the *ADH1* gene including its own promoter and a copy of the *HXT1* gene controlled by the truncated *HXT7* promoter.

## Yeast transformation

Transformation was performed by the electroporation procedure described by Becker and Guarente (1991). The transformants were selected on YPD medium plates with 150 μg $ml^{-1}$ of hygromycin. Three of the transformants were randomly selected for further analysis and were designated A1 carrying pJRC49 (*ADH1* gene), A3 carrying pJRC52 (*HXT1* gene) and A5 carrying pJRC54 (*ADH1* and *HXT1* genes).

## Semi-quantitative RT-PCR to evaluate ADH1 and HXT1 expression

Total RNA was prepared from yeast cell grown for 36 h in YPD. The RNA was quantified by spectrophotometry and its integrity verified on a 1.2% agarose gel. RT-PCR was performed as recommended in the retro tools SuperScriptTM III One-step RT-PCR System with Platinum® taq DNA Polymerase kit (Invitrogen, California, USA), with gene-specific primers for *ADH1* gene (orf adh1 F2 and orf adh1 R1), *HXT1* gene (olis2hxt1 and orf hxt1 R1) and actin gene (olisact1 and oliasact1). RT-PCR conditions for cDNA synthesis and PCR reaction were as follows: 1 cycle at 60°C for 30 min, followed immediately by one cycle at 94°C for 2 min, 30 cycles for 15 s at 94°C, 30 s at 55°C, 30 s at 72°C, and a final elongation step at 72°C for 5 min were performed. Aliquots of the PCR products were analyzed in 2% agarose gels, images obtained were digitalized and intensity of each band quantified by densitometry with the Quantity one program (Bio-Rad). The experiment was repeated on at least two independent occasions.

## Fermentation conditions

Culture starters of transformant strains and the non-transformant AR5 strain were developed in aerobic conditions growing all strains in 500 ml Erlenmeyer flasks containing 200 ml of YPD or *A. tequilana* medium with shaking for 18 h at 30°C.

Fermentation kinetics were carried out by inoculating $20 \times 10^6$ cells $ml^{-1}$ of each strain in five 125-ml flasks containing 100 ml of *A. tequilana* or YPD medium, both adjusted to 100 g reducing sugar $l^{-1}$

and 115 g glucose $l^{-1}$, respectively. After inoculation, the flasks were incubated at 33°C without shaking during 72 h. Samples were taken once from each flask every 12 h to determine cell growth, reducing sugar consumption, ethanol and glycerol production. Fermentation experiments were performed in duplicate. Alcohol efficiency was calculated from the ratio of the average ethanol produced at the end of fermentation and theoretical ethanol production from the biochemical conversion of the sugar consumed.

## Analytic methods

Ethanol determination was carried out by using an immobilized enzyme electrode method utilizing a Yellow Springs Instrument Company Model 2700 Industrial Analyzer (Yellow Springs Instruments Co., USA). Glucose was determined by using an enzymatic analyzer (Yellow Spring Instruments Co., USA). The reducing sugar concentration in the medium was determined by using 3,5-dinitrosalicylic acid reagent (Miller 1959). Biomass formation was measured as dry cell mass (Arrizon and Gschaedler 2002).

Glycerol determination was carried out by high performance liquid chromatography (HPLC) using a Varian Liquid Chromatograph (model 230, Varian, Mulgrade, Victoria). A HPX-87C column (BioRad) was used with water as mobile phase. The flow rate was of 0.5 ml $min^{-1}$. The column temperature was 85°C and the injection volume was 20 µl. Detection was performed by means of a differential refractive index detector (Varian model).

Alcohol dehydrogenase activity was assayed in the crude extracts according to the method proposed by Torres-Guzman et al. (1994) with some modifications. Briefly, yeast cells were washed and suspended in buffer TPI8.0 [50 mM Tris–HCl (pH 8.0) containing the protease inhibitors PMSF (10 µM), E64 (11.2 µM), leupeptin (2.5 µg $ml^{-1}$), pepstatin (1 µg $ml^{-1}$) and EDTA (5 mM)]. A volume of about 50 µl of the cells was mixed with an equal volume of glass beads (0.45–0.50 mm is diameter) and disrupted in an MSK cell homogenizer (Braun) for four 30-s periods, cooling with a stream of $CO_2$. The homogenate was centrifuged at 25,000*g* for 20 min. Supernatant (crude extract) was saved for the determination of enzyme activities. Protein concentration was estimated by the Bradford method (Bradford 1976) with bovine serum albumin as a reference. All enzyme assays, were carried out in a final volume of 1 ml. $NAD^+$-dependent Adh activity that was assayed in the oxidative direction according to Bergmeyer (1983). Assays were performed in reaction mixtures containing 50 mM Tris–HCl (pH 8.0), 1.9 mM $NAD^+$, cell-free extract (10–100 µg protein) and 0.8 M ethanol. The reaction was started by adding ethanol, and $NAD^+$ reduction was monitored by the increase in absorbance at 340 nm. One unit of enzymatic activity was defined as the amount of enzyme required to reduce 1 µmol of $NAD^+$ per min at 25°C. Specific Adh activity was expressed as units (U) per mg of protein.

### Miscellaneous

Sequencing was carried out at the Sequencing Facility (Unidad de Biología Molecular) at the Instituto de Fisiología Celular (UNAM, Mexico City), using an automated DNA sequencer (ABI PRISM; Model 310, version 3.4). The nucleotide sequence of the clone was generated on both strands by using universal primers and specific oligonucleotide primers based on *ADH1* and *HXT1* genomic sequences.

### Statistical analysis

The ANOVA and Least Statisitical Differences (LSD) tests were performed according to Montgomery (1991) using the Statgraphics software.

## Results

### Fermentative conditions

In order to assess the effect of individual or simultaneous overexpression of *ADH1* and *HXT1* genes on growth and ethanol production, the recombinant strains A1, A3 and A5 were cultivated in fermentative conditions in YPD and Agave medium. The growth of the recombinant strains was slightly decreased by the transformation effect (Fig. 1); the cell mass of recombinant strains A1,

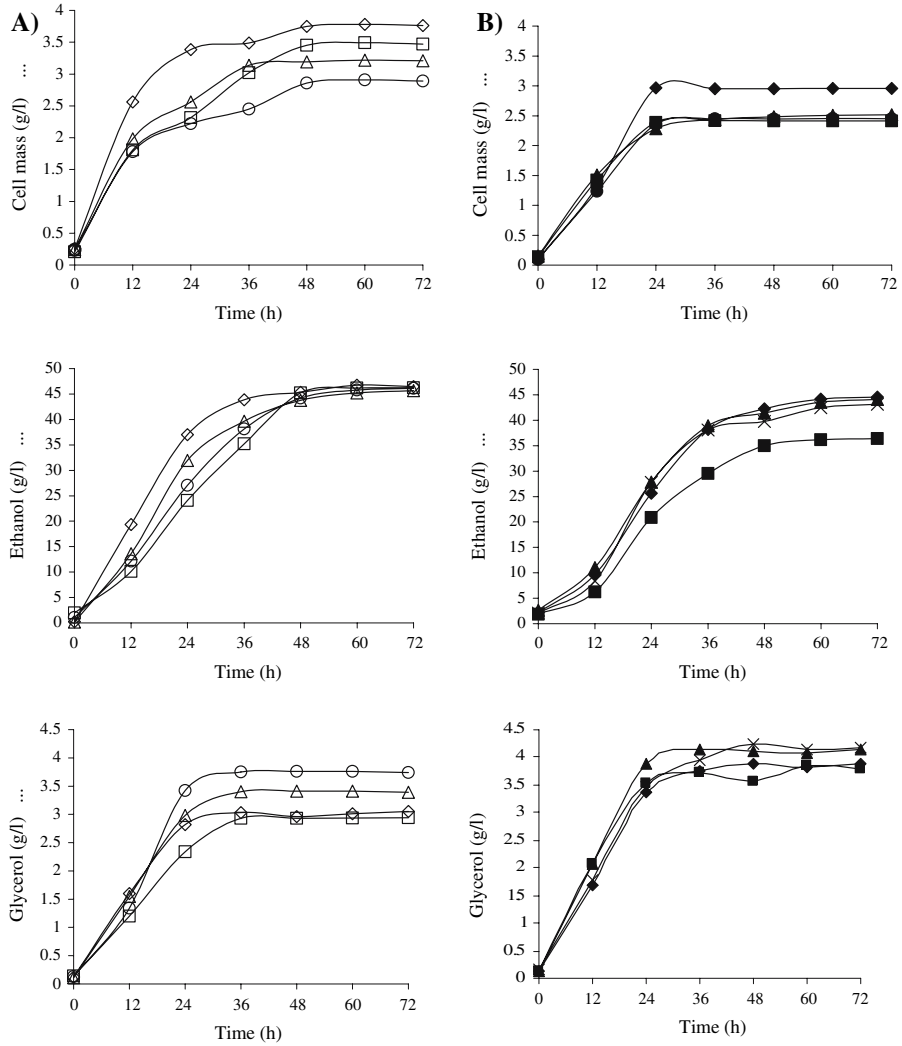


**Fig. 1** Determination of cell growth, ethanol and glycerol production, during fermentation of (**a**) YPD medium (open symbols) and (**b**) *A. tequilana* Weber blue variety (closed symbols): AR5 (◇, ◆), A1 (■, □), A3 (▲, Δ) and A5 (○, ●)

A3 and A5 on YPD medium was 7–24% lower that the growth obtained in the AR5 strain, whereas that the decrease of biomass formation on Agave medium ranging 15–19% (Table 2). When recombinant strains A3 and A5 containing the *HXT1* construct were grown in YPD medium, a decrease in Yx/s yields was observed. The lowest values were observed in the A5 strain in YPD and the A1 strain in Agave medium (0.027 and 0.026 respectively) whereas the highest yields were quantified in the wild type AR5 strain (0.033 and 0.031).

## Transcription level

The degree of *ADH1* and *HXT1* overexpression varied between the different recombinant strains. Transcript levels were increased when *ADH1* and *HXT1* genes were overexpressed individually, in the transformant strains A1 and A3 respectively. On the contrary, overexpression levels decreased when both genes (particularly *ADH1* gene) were expressed simultaneously in the A5 strain (Fig. 2).

## Alcohol dehydrogenase activity

While A1 and A5 strains containing the *ADH1* construct grown in YPD medium showed the highest enzyme activities ($3.04 \pm 0.09$ and $2.29 \pm 0.02$ U per mg of protein), the A3 strain containing only the *HXT1* construct showed enzymatic activity similar to the AR5 strain (Table 2). Similar behavior was

**Table 2** Ethanol, glycerol and biomass yields as well as Adh activity of recombinant strains and wild type *S. cerevisiae* growth on YPD and Agave medium

| Strain | Cell mass formation g $l^{-1}$ | Ethanol production g $l^{-1}$ | Glycerol production g $l^{-1}$ | Y p/x g ethanol g biomass$^{-1}$ | Y p/s g ethanol g sugar consumed$^{-1}$ | Y x/s g biomass g sugar consumed$^{-1}$ | Y gly/s g glycerol g sugar consumed$^{-1}$ | Efficiency (%) | Act Adh1p U mg prot$^{-1}$ |
|---|---|---|---|---|---|---|---|---|---|
| AR5-Y | 3.76 ± 0.17$^{a}$ | 46.16 ± 2.97$^{a}$ | 3.01 ± 0.14$^{c}$ | 12.27 ± 0.16$^{d}$ | 0.411 ± 0.005$^{c}$ | 0.033 ± 0.000$^{a}$ | 0.027 ± 0.000$^{c}$ | 80.33 ± 0.22$^{d}$ | 1.84 ± 0.00$^{a}$ |
| A1-Y | 3.50 ± 0.14$^{bc}$ | 45.92 ± 2.19$^{a}$ | 2.93 ± 0.16$^{c}$ | 13.22 ± 0.11$^{c}$ | 0.420 ± 0.006$^{bc}$ | 0.032 ± 0.000$^{b}$ | 0.027 ± 0.001$^{c}$ | 82.25 ± 0.31$^{c}$ | 3.04 ± 0.09$^{c}$ |
| A3-Y | 3.24 ± 0.15$^{c}$ | 44.56 ± 3.18$^{a}$ | 3.40 ± 0.13$^{b}$ | 13.99 ± 0.25$^{b}$ | 0.425 ± 0.002$^{ab}$ | 0.030 ± 0.000$^{b}$ | 0.032 ± 0.001$^{b}$ | 83.27 ± 0.25$^{b}$ | 1.86 ± 0.00$^{a}$ |
| A5-Y | 2.89 ± 0.17$^{d}$ | 45.37 ± 3.03$^{a}$ | 3.75 ± 0.11$^{a}$ | 15.72 ± 0.54$^{a}$ | 0.432 ± 0.006$^{a}$ | 0.027 ± 0.001$^{c}$ | 0.036 ± 0.002$^{a}$ | 84.57 ± 0.35$^{a}$ | 2.29 ± 0.02$^{b}$ |
| AR5-A | 2.95 ± 0.15$^{a}$ | 43.65 ± 2.05$^{a}$ | 3.86 ± 0.12$^{b}$ | 14.78 ± 0.40$^{b}$ | 0.456 ± 0.006$^{b}$ | 0.031 ± 0.001$^{a}$ | 0.040 ± 0.002$^{b}$ | 89.40 ± 0.33$^{c}$ | 0.408 ± 0.00$^{a}$ |
| A1-A | 2.40 ± 0.10$^{b}$ | 35.85 ± 2.45$^{b}$ | 3.79 ± 0.11$^{b}$ | 14.87 ± 0.31$^{b}$ | 0.393 ± 0.005$^{c}$ | 0.026 ± 0.000$^{b}$ | 0.041 ± 0.001$^{b}$ | 76.88 ± 0.99$^{d}$ | 0.524 ± 0.00$^{d}$ |
| A3-A | 2.50 ± 0.13$^{b}$ | 43.03 ± 1.98$^{a}$ | 4.12 ± 0.12$^{a}$ | 17.19 ± 0.46$^{a}$ | 0.473 ± 0.003$^{a}$ | 0.028 ± 0.002$^{b}$ | 0.045 ± 0.001$^{a}$ | 92.61 ± 0.14$^{b}$ | 0.467 ± 0.00$^{b}$ |
| A5-A | 2.45 ± 0.12$^{b}$ | 41.77 ± 2.01$^{a}$ | 4.19 ± 0.09$^{a}$ | 17.06 ± 0.71$^{a}$ | 0.482 ± 0.007$^{a}$ | 0.028 ± 0.000$^{b}$ | 0.048 ± 0.003$^{a}$ | 94.29 ± 0.35a | 0.506 ± 0.00$^{c}$ |

Data are expressed as the mean of two independent experiments run in duplicate ± standard deviation. Values in the same column with the same superscripts are not statistically different ($P > 0.05$)

observed when recombinant strains were cultivated in Agave medium. Strains A1 and A5 showed an increase in enzymatic activity from 65% and 24% respectively in comparison to the AR5 strain; on the contrary, Adh activity in the A3 strain was similar to that of wild type AR5. When enzymatic activities in both media were compared, an increase up to threefold was observed in YPD medium with respect to Agave medium (Table 2). It was found in both culture media that Adh activity was higher in the A1 strain (transformed only with *ADH1* gene) when compared to the A5 strain (transformed with both genes).

## Ethanol production

No significant difference in ethanol production was observed between the AR5 strain and the recombinant strains cultivated on YPD medium, on the contrary ethanol production was significantly lower in the A1 strain on Agave medium (Fig. 1). Ethanol yields expressed per gram of biomass produced and substrate consumed showed significant differences. The Yp/x was increased in recombinant strains A3 and A5 cultivated in YPD medium (14.02 and 28.11% respectively) as well as in the Agave medium (16.31 and 15.43% respectively); contrariwise, the A1 strain showed a lower yield than *S. cerevisiae* AR5 in Agave medium (Table 2). Likewise the Yp/s was slightly higher in the three recombinant strains than in the AR5 strain when cultivated in YPD medium and Agave medium except for the A1 strain, which showed a lower yield than the AR5 strain in Agave medium. The highest fermentative efficiency was detected in A5 strain grown in YPD and Agave medium (84.57 and 94.29% respectively).

## Glycerol production

Glycerol production increased when *HXT1* gene was overexpressed in the transformant strains A3 and A5 cultivated on YPD and Agave medium (Fig. 1). On the contrary, no significant difference in glycerol production was observed between the AR5 strain and the recombinant strain A1 cultivated on YPD and Agave medium (Table 2). Glycerol yields expressed per gram of substrate consumed showed significant

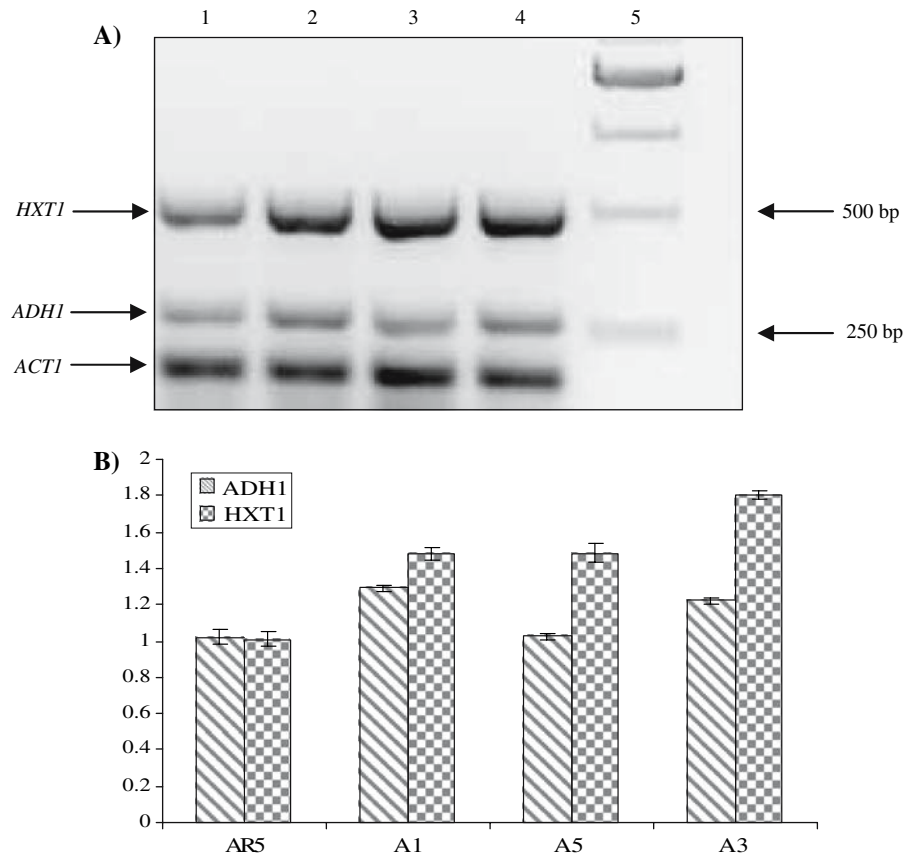


**Fig. 2** Level expression of *ADH1* and *HXT1* genes. (**a**) RT-PCR analysis the alcohol dehydrogenase I and hexose transporter I transcripts accumulation in *S. cerevisiae* cell grow in YPD: lane 1, strain AR5; lane 2, transformant strain A1; lane 3, transformant strain A5; lane 4, transformant strain A3; lane 5, molecular weight marker. *HXT1* (497 bp), *ADH1* (265 bp) and *ACT1* (175 bp). (**b**) *ADH1* and *HXT1* mRNA level. Images of agarose gel obtained in (**a**) were digitalized and intensity of each band quantified by densitometry with the Quantity one program (BioRad). The experiment was repeated on at least two independent occasions; figure shows result from a representative experiment

differences. The Ygly/s was increased at the recombinant strains A3 and A5 cultivated in YPD medium (18.52 and 33.33% respectively) as well as in the Agave medium (12.5 and 20% respectively); in contrast, the A1 strain showed a similar yield than *S. cerevisiae* AR5 in Agave medium (Table 2).

## Discussion

This study was conducted to investigate whether overexpression of *ADH1* and *HXT1* genes could lead to improved fermentative efficiency of *S. cerevisiae* AR5.

Diverse strategies have been used for the purpose of increasing the conversion rate of sugar to ethanol (Smits et al. 2000; Qing-Xue et al. 2007). By placing their genes on multicopy vectors, individual or simultaneous overproduction of some glycolytic enzymes in yeast increases their specific activities but has no effect on the rate of ethanol production (Schaaff et al. 1989; van de Aar et al. 1990; Hauf et al. 2000). In this work *ADH1* and *HXT1* genes were overexpressed individually and simultaneously in one *S. cerevisiae* strain isolated from tequila factory by cloning into a yeast centromere plasmid. The Hxt1p transporter was selected on the hypothesis that the increase of transporters during fermentation, especially at the beginning, may result in high fermentative efficiency. Additionally *ADH1* overexpression could increase the glycolytic flux and therefore the ethanol synthesis. In order to increase the *HXT1* expression, this gene was fused to the truncated *HXT7* promoter, which is constitutively active in ethanol as well as in glucose medium (Hauf et al. 2000). The best yields and fermentative efficiency were observed in the A3 and A5 strains with both recombinants harboring the *HXT1* gene, suggesting a major role of Hxt1p transporter than the alcohol dehydrogenase I. Glucose transport has been suggested to be one of the most important control points of glycolytic flux (Ye et al. 1999; Reijenga et al. 2001; Otterstedt et al. 2004). However, *ADH1* overexpression could contribute to increase the

fermentative efficiency when *ADH1* was expressed individual (A1) and simultaneously (A5) in the transformant strains (Table 2). Unfortunately, we had problems during quantitation of transcript levels of *ADH1* of the strains grown on Agave medium due to diverse pigments and other substances that difficulted the obtention of a clean cellular pellet.

Nevertheless, when Adh1p activity was assessed, an increment of the enzymatic activity in A1 and A5 strains cultivated on YPD and Agave media was observed. This increment was associated with an increment in the yields and fermentative efficiency in comparison with AR5 strain (Table 2). This suggests that the *ADH1* overexpression improves the fermentative efficiency in the transformant strains. Nevertheless, further increments of the *ADH1* overexpression could take place fusing this gene to a strong promoter instead of using the cognate sequence.

The recombinant strains obtained had reduced growth when compared to their host strain, which is indicative of a protein burden effect. This effect was more marked in the A5 strain harboring *ADH1* and *HXT1*. Protein burden may be ascribed to the dilution of essential enzymes necessary for protein production and to an increase in energy demand for the additional production of protein (Snoep et al. 1995). Additionally, the effect burden on enzymatic activity in the strains harboring multiple overexpressed constructs has been reported (Hauf et al. 2000). Effect burden was not observed in the recombinant strain A1; this strain showed a higher level of transcript and enzymatic activity. On the contrary, the A5 strain harboring *ADH1* and *HXT1* genes showed a slight decrease in Adh activity, probably caused by the simultaneous overproduction of both proteins. Most laboratory *S. cerevisiae* strains are either haploid or diploid. However, industrial yeast strains are predominantly diploid or aneuploid and occasionally polyploidy (Pretorius 2000). Effect of aneuploidy or polyploidy could enhance dosage of important genes improving fermentation such as *ADH1* (Dilorio et al. 1987). We ignore the ploidy of the AR5 strain and, therefore, effect of the number of copies of *ADH1* and *HXT1* genes present in the cell on the fermentative efficiency.

Nevertheless that the copy number of the centromere plasmid is one per cell (Cottarel et al. 1989; Sikorski and Hieter 1989), simultaneous overexpression of *ADH1* and *HXT1* genes improved the fermentative efficiency. The higher fermentative efficiency observed in the Agave medium in comparison with YPD medium, could be the result of the presence in the juice of nonreducing sugars (di and trisaccharides) as result of an incomplete hydrolysis of insulin. This partial hydrolysis increased the available sugar, which could be utilized by the yeast. There are no genetically engineered yeasts are currently used in the commercial production of tequila. The successful commercialization of transgenic yeasts for the tequila industry will depend on a multitude of technical, economic, marketing, safety, regulatory, and ethical issues. Self-cloning yeast will be the most likely candidate for the first commercial applications of yeasts genetically modified in food and beverage industries (Akada 2002).

## Conclusions

The simultaneous overexpression of Adh1p and Hxt1p proteins resulted in increased ethanol yields and fermentative efficiency, when the recombinant strains were cultivated in YPD and Agave media. This is the first work reporting an increase of fermentative efficiency caused by simultaneous overexpression of *ADH1* and *HXT1* genes in one strain isolated from tequila must. However, further studies will have to be carried out previous to the introduction of these strains to the tequila elaboration process.

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