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1 **Section: Biofuels and Environmental Biotechnology**

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3 **Identification of reference genes suitable for normalization of RT-qPCR expression data in**
4 ***Saccharomyces cerevisiae* during alcoholic fermentation.**

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22 **Abstract**

23 Expression data from RT-qPCR (reverse transcription quantitative PCR) needs to be normalized to
24 account for experimental variability among samples caused by differential yields of the transcripts in
25 RNA extraction or in the reverse transcription. The most common method is to normalize against one
26 or more reference genes (RG). We have selected RGs suitable for normalization of RT-qPCR raw data
27 in *Saccharomyces cerevisiae* during alcoholic fermentation. The RGs were evaluated by three different
28 statistical methods. The suitability of the selected RG sets was compared with *ACT1*, a commonly used
29 non-validated single RG, by normalizing the expression of two target genes. Expression profiles of the
30 target genes revealed the risk of misleading interpretation of expression data due to an unreliable RG.

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47 **Introduction**

48 RT-qPCR (reverse transcription quantitative PCR) has become a reference technique in quantitative
49 mRNA analysis. It offers the possibility to quantify gene expression by measuring transcripts over a
50 wide range of concentrations, combining robustness and simplicity with a greater sensitivity (Bustin et
51 al. 2005) than the classical mRNA analysis tools such as Northern blot analysis (Dean et al. 2002).

52 As in other mRNA analysis methodologies, results must be normalized to account for the experimental
53 variability among samples caused by differential yields in transcripts during RNA extraction or in the
54 delicate step of the reverse transcription to produce cDNA. Several methods have been proposed for
55 normalization of the RT-qPCR results including the addition of exogenous RNA, normalization with
56 respect to total RNA or cell number (Hugget et al. 2004)

57 In the most common normalization strategy, the RT-qPCR output of target gene (TG) expression is
58 compared with that of one or more endogenous genes, called reference genes (RG), by using
59 calculation approach such as $\Delta\Delta C_t$ corrected or not with the amplification efficiency. This approach
60 assumes that RG expression is stable throughout the experiment. Several studies have reported
61 expression data normalized with RGs, such as those encoding actin, glyceraldehyde-3-phosphate
62 dehydrogenase and other genes, assumed their stability without confirming their transcriptional
63 behavior during the experiment. However, an increasing number of studies demonstrate that there is no
64 universal, stable RG and even the commonly used RGs are subjected to regulation under certain
65 physiological conditions (Schmittgen and Zakrajsek 2000). Therefore, the candidate RGs must be
66 validated, confirming the stability of their expression during the experiments, prior to their use in
67 normalization of expression data for a TG.

68 Establishing a reliable RG is difficult because the RG is also subjected to the same experimental errors
69 as the TG. To resolve this circular problem several methods have been proposed (Vandesompele et al.
70 2002; Pfaffl et al. 2004; De Kok et al. 2005; Andersen et al. 2004).

71 In *Saccharomyces cerevisiae*, studies have focused on validation of RGs under a particular
72 physiological condition, such as glucose stimulation or dehydration (Ståhlberg et al. 2008; Vaudano et
73 al. 2009). Teste et al. (2009) validated a set of RGs suitable for *S. cerevisiae* growing in a synthetic
74 minimal medium with 2% (w/v) glucose or galactose and pH 5.0. However, there is no established set
75 of RGs suitable for normalizing expression data of *S. cerevisiae* during alcoholic fermentation, such as
76 production of beer and wine, i.e. fermentation of grape must or malt wort, under conditions
77 characterized by low pH, high sugar concentration (120-250 g/l) and steadily increasing ethanol
78 concentration.

79 In this study we report the identification and validation of a set of RGs suitable for normalization of
80 raw data of RT-qPCR in *S. cerevisiae* during alcoholic fermentation. Using three statistical methods,
81 we evaluated the stability of ten candidate RGs in four *S. cerevisiae* strains during fermentation of a
82 synthetic medium simulating grape must, until complete sugar consumption. The RG sets were
83 experimentally validated by analysis of expression data of two TGs, *ADH1* and *GPD2*, encoding
84 enzymes involved in alcoholic fermentation and in its regulation.

85 **Materials and methods**

86 *Yeast strains and media*

87 *Saccharomyces cerevisiae* strains, ISE128, ISE77, ISE24, ISE117 from the collection of the CRA-
88 Centro di Ricerca per l'Enologia (Asti, Italy) were grown at 20°C under anaerobic conditions in 2 l
89 flasks, using 1.4 l synthetic must, pH 3.2, of the following composition (per liter): 90 g glucose; 90 g
90 fructose; 3 g yeast extract; 3 g malt extract; 1 g ammonium sulphate; 1 g ammonium phosphate; 3.5 g
91 tartaric acid; and 1.7 g yeast nitrogen base.

92 The yeasts were first propagated in YPD medium for 48 h and then inoculated at 10^6 cells /ml in the
93 MNS2 synthetic must. Cell for RNA extraction were harvested by centrifugation of a pre-chilled
94 aliquot of must at 5000 x g for 10 min. pellet was washed with cold water and stored at -80°C until
95 further use.

96 The cell growth was followed at 600 nm and the progress of fermentation was monitored by HPLC
97 analysis of medium aliquots using a refractometric detector and a Rezex RCM-monosaccharide
98 column (Phenomenex).

99 *RNA extraction and cDNA synthesis*

100 RNA extraction was from 2×10^7 cells using a commercial kit (Omega Biotek). DNase treatment was
101 carried out and RNA quality and concentration were assessed spectrophotometrically and on a
102 Experion microcapillary electrophoresis system (Biorad). cDNA was synthesized from 0.3 μ g total
103 RNA using the two step iScript Select cDNA Synthesis kit (Biorad) as the RT enzyme, with RNase
104 treatment. The cDNA was conserved at -80°C .

105 *Primers and real-time PCR*

106 Primers were designed using Primer 3 software with the exception of the *18S* primers that were
107 designed by Martinez et al. (2004), and *TAF10*, *TFCl*, *UBC6* designed by Teste et al. (2009). Primer
108 specificity was tested *in silico* by BLAST analysis and by agarose gel electrophoresis. Amplification
109 efficiency was calculated by the dilution method (Rasmussen et al. 2001). Primer characteristics are
110 given in the Online resource 1.

111 Real-time PCR was performed in 96-well plates on a Biorad ICycler instrument (Biorad) using SYBR
112 Green as fluorophore. Reactions were carried out in 20 μ l that contained 2.5 μ l cDNA, 0.5 μ M forward
113 and reverse primers and 10 μ l 2x EVA Green master mix (Biorad). Each sample was analyzed twice
114 and a no-template control for each primer was included in all real-time plates. Amplifications were
115 performed under the following conditions: 95°C for 3 min, 40 cycles of 95°C for 10 s, 63°C for 30 s,
116 72°C for 30 s and a final extension at 72°C for 5 min. At the end of the amplification cycle, a melting
117 analysis was carried out to verify the absence of non-specific amplification.

118 The expression level of a given gene was reported as quantification cycle (Cq), corresponding to the
119 number of cycles required to reach a predetermined threshold fluorescence. The threshold values were

120 obtained by using the automated setting of the instrument software (base line subtracted curve fit data).
121 The data expressed as Cq were imported into a Microsoft Excel data sheet for subsequent analysis.

122 *Analysis of reference genes*

123 We tested 10 genes previously reported in literature as potential RGs, including those that are widely
124 used in normalization studies on *S. cerevisiae* such as genes encoding 18S rRNA, actin (*ACT1*) and
125 glyceraldehyde-3-phosphate dehydrogenase, isoenzyme 2 (*TDH2*). Expression variability of the
126 candidate RG was measured in all samples taken during fermentation with the four strains treated as
127 the homogeneous group. Three statistical parameters were applied to evaluate variability: (i) standard
128 deviation and coefficient of variation of Cq calculated with Bestkeeper software (Pfaffl et al. 2004);
129 (ii) M value after stepwise exclusion performed with geNorm software version 3.3 for Microsoft Excel
130 (Vandesompele et al. 2002); and (iii) two times the standard deviation of the variation from the global
131 mean as described by De Kok et al. (2005).

132 *Analysis of target genes*

133 The expression analysis of target genes *GPD2* and *ADH1* was performed by normalizing the
134 expression value transformed into copy numbers, to the geometric mean of the RGs, using GeneEx
135 Software (MultiD Analyses AB). In the calculation of copy number from raw Cq, amplification
136 efficiencies were considered.

137 **Results**

138 *Choice of reference genes*

139 We used three statistical approaches to analyze the expression values and select the best RGs. The first
140 one was based on the coefficient of variance (CV) and standard deviation (SD) of Cq. Table 1 shows
141 the Cq values determined for each gene that was analyzed using the Bestkeeper software. The most
142 abundantly transcribed gene was 18S rRNA with mean Cq values of 9.62 followed by *TDH2* and
143 *PGK1* with a mean Cq of 18.85 and 19.55, respectively. The least abundant transcripts were of *TFC1*,
144 with a mean Cq value of approximately 32.00. Only 18S, *QCR9*, *TDH2* and *TFC1* showed a SD value

145 lower than 1, which is the upper limit above which a gene must be considered unfit as a RG according
146 to Pfaffl et al. (2004). Considering the CV values, the most stable gene was *TFC1* with a value of 2.87
147 followed by *QCR9* and *18S* with values of 3.70 and 3.88, respectively.

148 In the second statistical method employed here, the GeNorm algorithm (Vandesompele et al. 2002),
149 genes were classified according to the M value, which represents the average of pairwise variation, V,
150 intended as the standard deviation of the ratio between the expression of a particular gene and all other
151 candidate genes. Next, the software excludes the least stable gene (with the higher M value) and
152 recalculates the M value, identifying, at the end of this stepwise exclusion, the two steadily expressed
153 ones.

154 After stepwise exclusion using GeNorm, the best reference genes were found to be *TDH2* and *PGK1*
155 followed by *QCR9* on the basis of the M values (Fig. 1). Based on these results *QCR9* was used as the
156 third reference gene for normalization.

157 The third method (De Kok et al. 2005) is based on the assumption that the mean expression of all
158 candidate RGs (global mean) corresponds to the best normalization, provided that the genes have
159 independent functions. The stability of the RGs was evaluated by calculating two times the standard
160 deviation (2SD) of the differences in Cq of a gene with respect to the global mean expression. Based
161 on this parameter, the best ranked genes were *18S*, *QCR9* followed by *TDH2* while *LSC2* was
162 classified in the last position (Table 2).

163 *Validation of reference gene sets*

164 With the aim to validate the sets of RGs, we normalized the expression of two TGs during
165 fermentation by RT-qPCR. The performance of the normalization to the RG sets identified with the
166 above three statistical methods was compared with that of *ACT1*, a commonly used single RG. For
167 this, expression data of two yeast strains, ISE117 and ISE24, were used as example. Production of
168 ethanol and glycerol were monitored by HPLC analysis of medium aliquots (Fig. 2).

169 The first gene studied was *GPD2*, homolog of *GPD1*, encoding glycerol-3-phosphate dehydrogenase
170 isoenzyme involved in the first step of glycerol biosynthesis. In both the strains, normalization with the
171 validated sets of RGs produced similar results (Fig. 3). *GPD2* expression decreased steadily during the
172 initial 24 h of fermentation to 30 to 50% of the value at time 0. After 24 h no significant variations
173 were observed. However, when normalization was performed with *ACT1*, the expression profile
174 obtained was entirely different in both strains (Fig. 3). In ISE117, *GPD2* was transcribed at a steady
175 rate up to 72 h and then upregulated between 72 and 120 h to reach 2.5-fold induction levels; whereas
176 in ISE24 the trend was similar with 4-fold induction levels reached by 216 h.

177 The second gene studied was *ADHI*, encoding the major alcohol dehydrogenase, a key enzyme
178 responsible for the reduction of acetaldehyde to ethanol, the final step in the alcoholic fermentation..
179 The expression profile of this gene in ISE24 and ISE117 strains obtained after normalizing with the
180 different RG sets showed steadily decreasing levels of expression during the initial 48 to 72 h of
181 fermentation (Fig. 4). There was good agreement among the three sets of RGs used for normalization.
182 At the end of fermentation the gene was down-regulated 5-fold in ISE117 strain and about 10-fold in
183 ISE24 strain, compared with time 0. Again, in contrast to these results obtained with the RG sets, the
184 expression profile of *ADHI* obtained after normalization with *ACT1* indicated wide fluctuations in the
185 levels of transcription during the course of fermentation, and with larger variability and dispersion.

186 **Discussion**

187 During the course of alcoholic fermentation of media with a high sugar content such as during wine
188 and beer fermentations, the yeast undergoes metabolic adjustments in response to a continuously
189 changing environment. Thus, for example during fermentation by *S. cerevisiae* selected strains, cells
190 that are produced aerobically in media of low sugar content, are inoculated in grape must or malt wort
191 that have a high sugar content (100-250 g/l) and low O₂. These fermentation conditions demand a
192 rapid switch from aerobic to anaerobic metabolism. Throughout the fermentation, the growing yeast
193 cells transform sugars into ethanol until stressful conditions are created due to accumulating ethanol

194 that in certain productions (for example “Passito” in Italy or “Eiswein” in Germany, wines made from
195 dried grapes) can reach 15-17% (v/v). The yeast cells adapt to these stressful variations of osmotic
196 pressure and nutrient availability by modulating gene expression that results in the appropriate cellular
197 responses. In this conditions, the monitoring of transcription using RT-qPCR requires a stable set of
198 RGs to enable normalization of the raw data. Validation of a reliable set of RG is thus fundamentally
199 important in such studies.

200 Here, we studied the transcriptional variability of 10 candidate RGs during fermentation with four *S.*
201 *cerevisiae* strains. Depending on the method used the genes *TDH2*, *PGK1*, *QCR9*, *TFC1* and *18S*
202 ranked in the first three positions while the *LSC2* expression was always the least stable, and hence
203 unequivocally unsuitable as a RG. *TDH2* and *18S* are among the genes widely used as RGs in RT-
204 qPCR literature and the results presented here justify their use. Nevertheless, the use of *18S* as a RG
205 should be carefully evaluated because of its peculiar characteristics of being a final product and not a
206 translatable mRNA, and requiring the addition of a random primer in the retro transcription step.
207 Moreover due to its high abundance, the *18S* transcript may not be suitable for the normalization of a
208 low abundant transcript. However, in our study, the *18S* was seen to be highly stable and, when used as
209 a RG, yielded expression profiles similar to those obtained with other RGs. Only *QCR9* was selected
210 by all the three calculation approaches used here, confirming its validity as a RG for *S. cerevisiae*
211 during growth in different metabolic conditions (Vaudano et al. 2009). The *ACT1*, frequently used as
212 RG in RT-qPCR experiments, appeared to be unfit for normalizing the expression data during
213 fermentations, ranking only in the fifth position with the geNorm method and in the seventh position
214 with the other two statistical methods.

215 The normalization of two target genes *GPD2* and *ADH1* with a single *ACT1* as a RG in comparison
216 with the normalization with the geometric average of statistically identified RGs, validates the findings
217 discussed above. The results showed the reliability of the three different sets of RGs that exhibit
218 minimal variation among them. On the contrary, the profile generated with *ACT1* revealed a risk of

219 misinterpretation of expression data as it indicated an induction of transcription throughout the
220 fermentation instead of a repression observed with the normalization based on the RGs identified by
221 statistic approaches. This apparent overestimation of *GPD2* and *ADHI* expression is due to a
222 repression of the *ACT1* itself during the fermentation when cells reach the stationary phase and the
223 growth rate decreases (Monje-Casas et al. 2004; Teste et al. 2009), which was also observed in our
224 experiments (data not shown). The interpretation of induction of a TG may thus be due to repression of
225 the RG. The statistical analysis and experimental data presented here confirm that the use of a non-
226 validated RG is unacceptable and that a prior evaluation of RG is an essential step in RT qPCR, as
227 recently proposed in the guidelines, MIQE (*Minimum Information for Publication of Quantitative RT-*
228 *PCR Experiments*) (Bustin et al. 2009).

229 Regarding the two target genes studied, *ADHI* and *GPD2*, we observed a weak albeit significant
230 repression during the fermentation with respect to the level of expression at time 0. It is interesting to
231 note that, observing raw mRNA data, *ADHI* was expressed at a level that is two orders of magnitude
232 higher than that of *GPD2* (data not shown). With respect to time 0, the *ADHI* expression showed a
233 tendency to decrease but remained elevated reflecting its importance in glycolysis during the last step
234 of reduction of acetaldehyde in ethanol. Also *GPD2* exhibited a weak repression throughout the
235 fermentation confirming the gene to be less regulated in comparison with the homologous *GPD1*, as
236 reported by Rossignol et al. (2003) using microarray, and by Pigeau and Inglis (2007) with Northern
237 blot analysis. It probably plays a minor role in glycerol formation during fermentation.

238 **In conclusion**, the present work describes the selection of the most suitable RGs specific for
239 normalization of RT-qPCR expression data in *S. cerevisiae* during alcoholic fermentation. These
240 findings are important in expression studies in order to understand critical events during industrial
241 fermentation processes, such as sluggish or stuck fermentations. This work also highlights the risk of
242 misleading interpretations based on normalization of the data with a non-validated RG. Therefore,

243 evaluation of stability of expression of RGs under given experimental conditions is a crucial
244 prerequisite to obtain reliable information about gene expression.

245 **Acknowledgements**

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248

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Tables

Table 1 Statistic distribution of 10 candidate reference genes based on Cq (cycle of quantification) values, using BestKeeper software version 1.0 (Pfaffl et al., 2004).

	<i>18S</i>	<i>ACT1</i>	<i>TUB2</i>	<i>QCR9</i>	<i>TDH2</i>	<i>PGK1</i>	<i>LSC2</i>	<i>UBC6</i>	<i>TAF10</i>	<i>TFC1</i>
GM [Cq]	9,61	23,00	26,42	21,96	18,82	19,50	28,67	28,28	29,19	32,02
AM [Cq]	9,62	23,05	26,49	21,99	18,85	19,55	28,83	28,32	29,24	32,04
Min [Cq]	8,40	20,20	22,80	20,20	16,30	16,00	23,20	25,70	26,40	30,20
Max [Cq]	11,10	26,40	31,50	24,90	21,00	22,60	38,30	32,10	32,90	35,30
SD [\pm Cq]	0,37	1,20	1,54	0,81	0,91	1,16	2,50	1,28	1,40	0,92
CV [% Cq]	3,88	5,23	5,82	3,70	4,83	5,93	8,66	4,50	4,80	2,87
Min [x-fold]	-2,31	-4,35	-8,76	-2,47	-4,64	-7,52	-21,97	-5,99	-6,90	-3,53
Max [x-fold]	2,82	5,95	20,90	4,51	3,79	5,98	232,00	14,10	13,12	9,70
SD [\pm x-fold]	1,30	2,31	2,91	1,76	1,88	2,23	5,65	2,42	2,65	1,89

GM [Cq]: geometric mean of Cq; AM [Cq]: arithmetic mean of Cq; Min [Cq]: minimum value of Cq; Max [Cq]: maximum value of Cq; SD [\pm Cq]: standard deviation of Cq; CV [% Cq]: coefficient of variation of Cq expressed as percentage; Min [x-fold]: minimum value expressed as folds of expression; Max [x-fold]: maximum value expressed as folds of expression SD [\pm x-fold]: standard deviation in folds of expression

Table 2 Evaluation of the candidate reference genes, using the statistical approach proposed by de Kok et al. (2005).

genes	2xSD ^a
<i>18S</i>	1,0
<i>QCR9</i>	2,1
<i>TDH2</i>	2,2
<i>TFC1</i>	2,4
<i>PGK1</i>	2,9
<i>UBC6</i>	3,1
<i>ACT C</i>	3,1
<i>TAF10</i>	3,4
<i>TUB2</i>	3,8
<i>LSC2</i>	6,4

^a Two time the standard deviation of the variation from the global mean expressed in Cq.

Figures

Figure 1 Average expression stability values (M) of the remaining control genes during stepwise exclusion of the least stable gene, performed by GeNorm software on the candidate reference genes in all fermentations.

Figure 2 Ethanol (a) and glycerol (b) production during fermentation of synthetic must performed with ISE117 and ISE24 strains. Error bars represent standard deviation (n = 2)

Figure 3 *GPD2* gene expression during fermentation with ISE117 (a) and ISE24 (b) strains. mRNA are normalized with geometric mean of the reference genes expression values transformed in copy numbers. Normalization was performed with *ACT1* alone and with different gene sets obtained from statistic calculation: *18S*, *TDH2* and *QCR9* (SET A: method proposed by De Kok et al. (2005); *18S*, *TFC1* and *QCR9* (SET b: coefficient of variation (Pfaffl et al. 2004); *TDH2*, *PGK1* and *QCR9* (SET C: GeNorm calculation (Vandesompele et al. 2002); Expression values of time 0 was consider as calibrator (fold expression =1) Error bars represent standard deviation (n = 4)

Figure 4 *ADH1* gene expression during fermentation with ISE117 (a) and ISE24 (b) strains. mRNA are normalized with geometric mean of the reference genes expression values transformed in copy numbers. Normalization was performed as described in Fig 3. Expression values of time 0 was consider as calibrator (fold expression =1) Error bars represent standard deviation (n = 4)

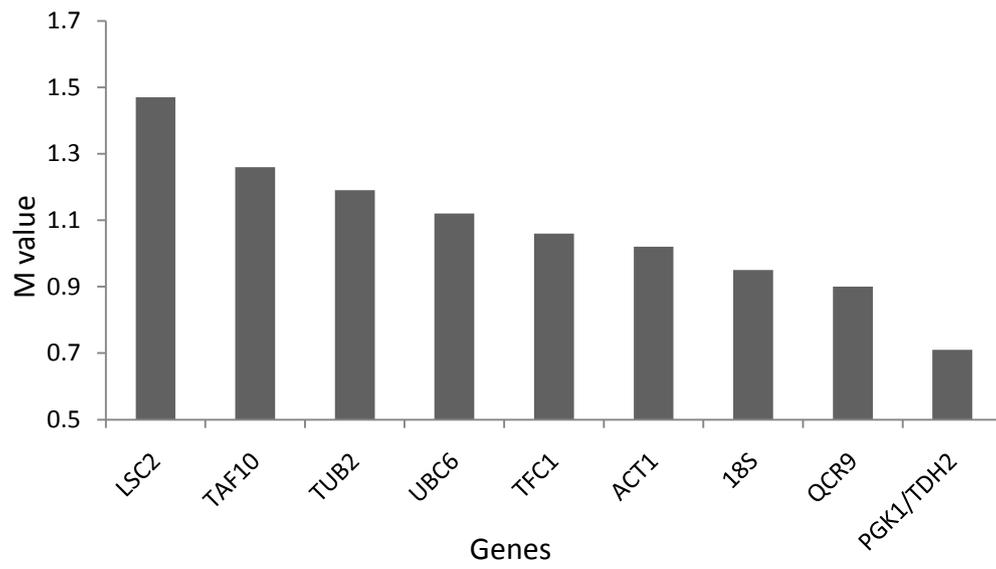


Fig. 1

Vaudano E., Noti O., Costantini A., Garcia-Moruno E.

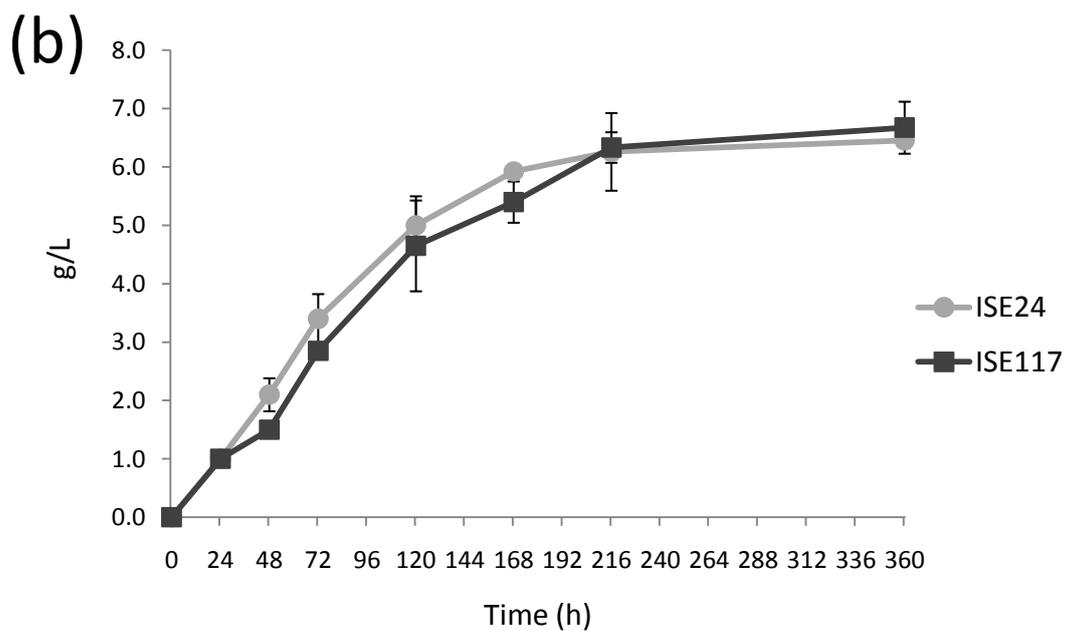
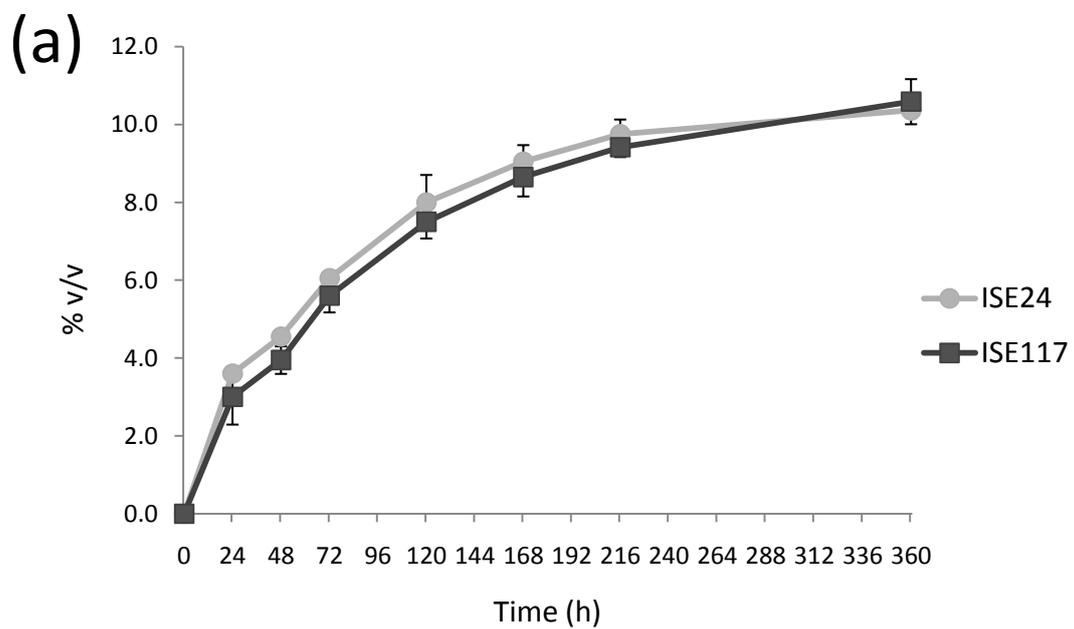


Fig.2

Vaudano E., Noti O., Costantini A., Garcia-Moruno E.

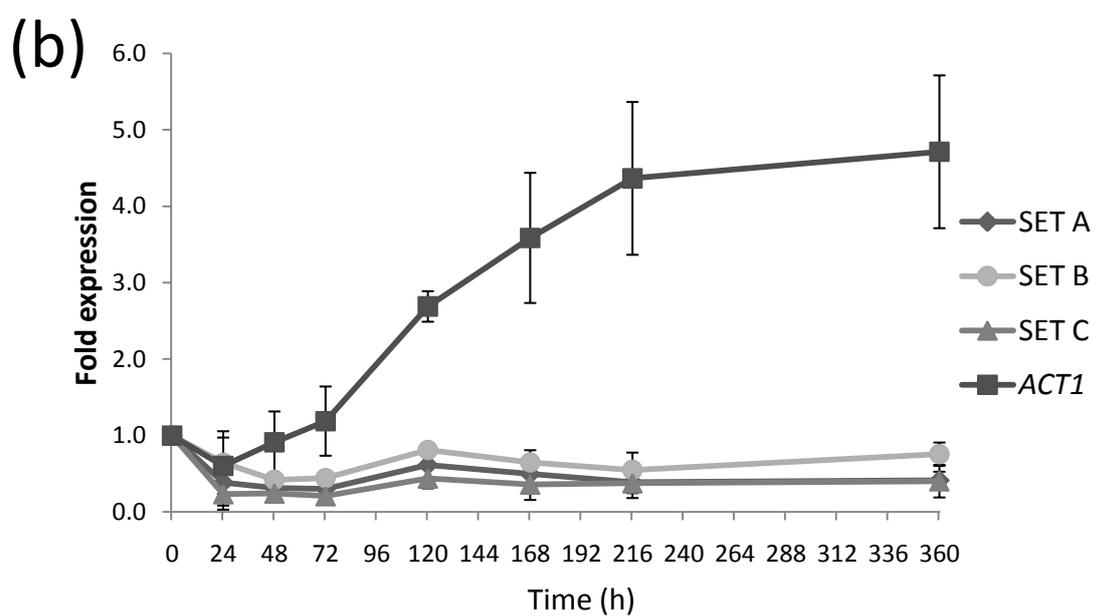
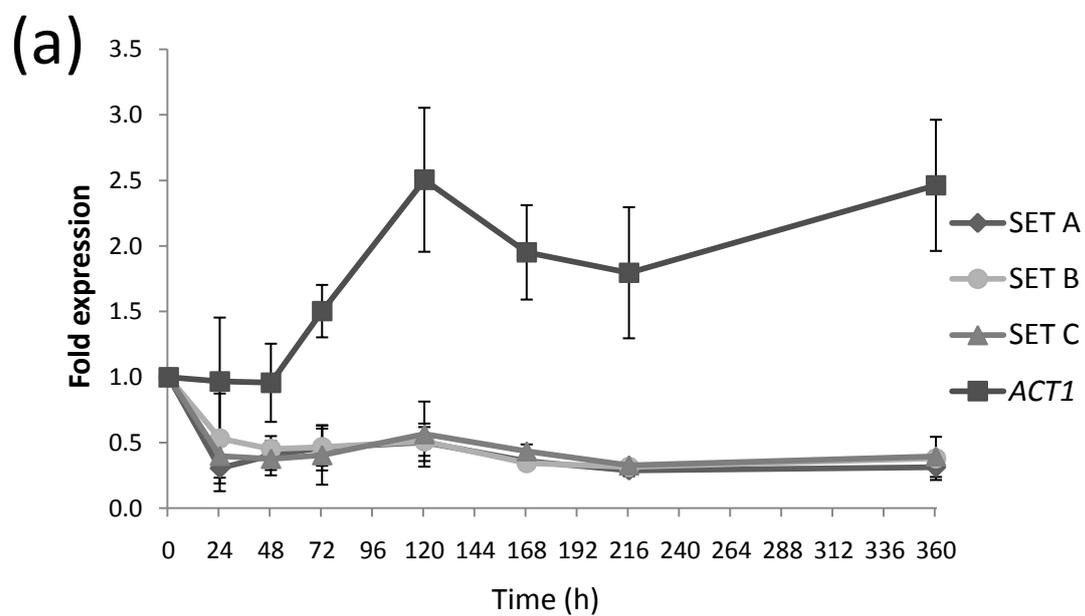


Fig. 3

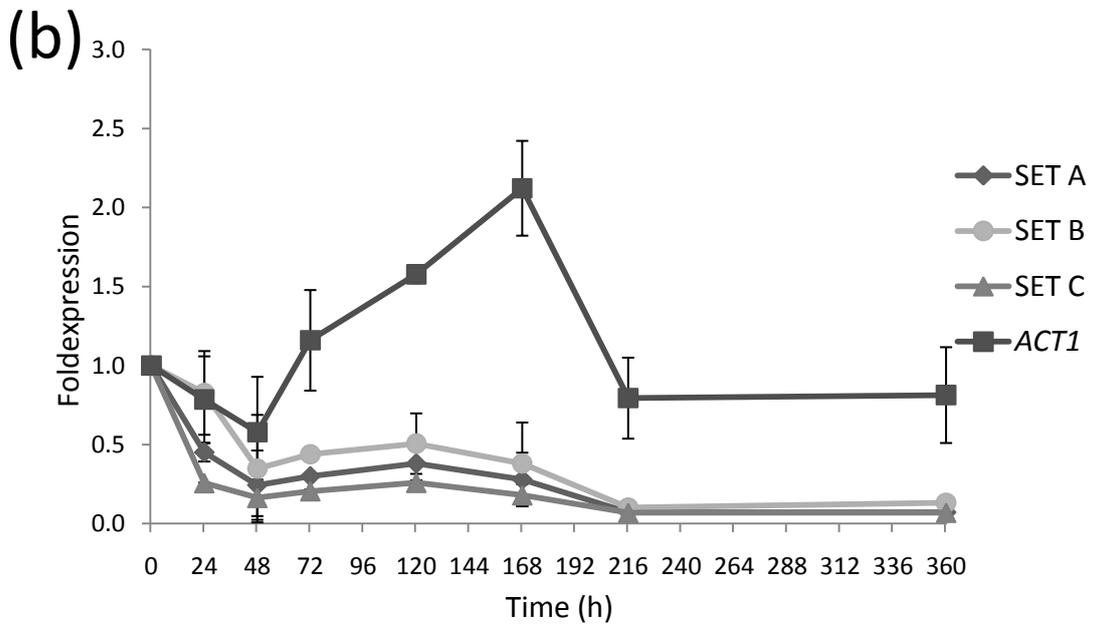
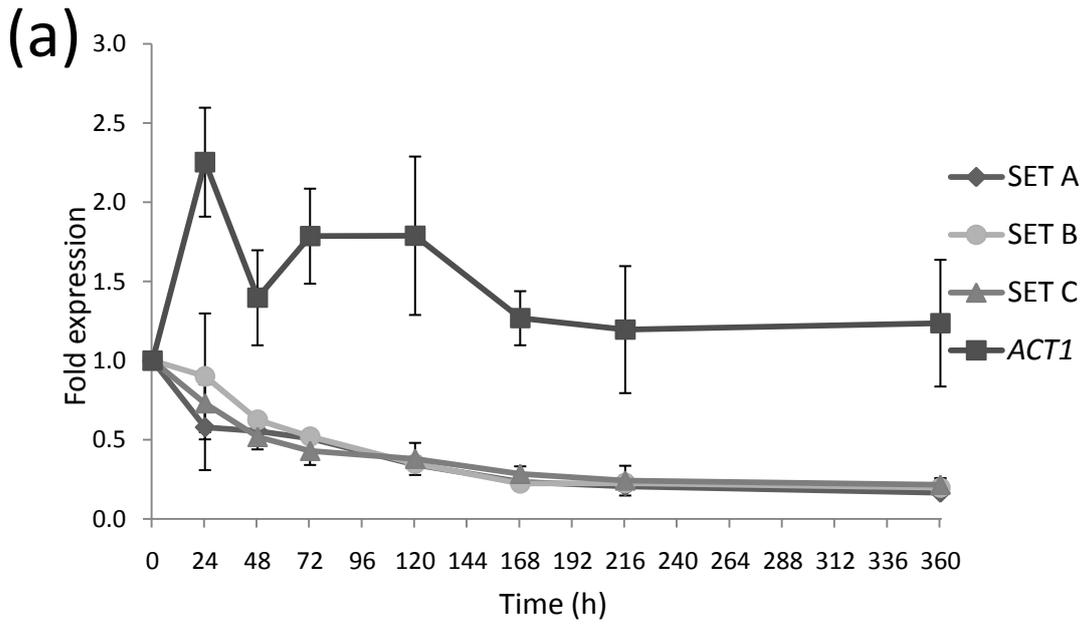


Fig. 4

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