Effects of glucose, ethanol and acetic acid on regulation of ADH2 gene from *Lachancea fermentati*

Norhayati Yaacob, Mohd Shukuri Mohamad Ali, Abu Bakar Salleh, Nor Aini Abdul Rahman

Background. Not all yeast alcohol dehydrogenase 2 (ADH2) are repressed by glucose, as reported in *Saccharomyces cerevisiae*. *Pichia stipitis* ADH2 is regulated by oxygen instead of glucose, whereas *Kluyveromyces marxianus* ADH2 is regulated by neither glucose nor ethanol. For this reason, ADH2 regulation of yeasts may be species dependent, leading to a different type of expression and fermentation efficiency. *Lachancea fermentati* is a highly efficient ethanol producer, fast-growing cells and adapted to fermentation-related stresses such as ethanol and organic acid, but the metabolic information regarding the regulation of glucose and ethanol production is still lacking. **Methods.** Our investigation started with the stimulation of ADH2 activity from *S. cerevisiae* and *L. fermentati* by glucose and ethanol induction in a glucose-repressed medium. The study also embarked on the retrospective analysis of ADH2 genomic and protein level through direct sequencing and sites identification. Based on the sequence generated, we demonstrated ADH2 gene expression highlighting the conserved NAD(P)-binding domain in the context of glucose fermentation and ethanol production. **Results.** An increase of ADH2 activity was observed in starved *L. fermentati* (LfeADH2) and *S. cerevisiae* (SceADH2) in response to 2% (w/v) glucose induction. These suggest that in the presence of glucose, ADH2 activity was activated instead of being repressed. An induction of 0.5% (v/v) ethanol also increased LfeADH2 activity, promoting ethanol resistance, whereas accumulating acetic acid at a later stage of fermentation stimulated ADH2 activity and enhanced glucose consumption rates. The lack in upper stream activating sequence (UAS) and TATA elements hindered the possibility of Adr1 binding to LfeADH2. Transcription factors such as SP1 and RAP1 observed in LfeADH2 sequence have been implicated in the regulation of many genes including ADH2. In glucose fermentation, *L. fermentati* exhibited a bell-shaped ADH2 expression, showing the highest expression when glucose was depleted and ethanol-acetic acid was increased. Meanwhile, *S. cerevisiae* showed a constitutive ADH2 expression throughout the fermentation process. **Discussion.** ADH2 expression in *L. fermentati* may be subjected to changes in the presence of non-fermentative carbon source. The nucleotide sequence showed that ADH2 transcription could be influenced by other transcription genes of glycolysis oriented due to the lack of specific activation sites

for Adr1. Our study suggests that if Adr1 is not capable of promoting LfeADH2 activation, the transcription can be controlled by Rap1 and Sp1 due to their inherent roles. Therefore in future, it is interesting to observe ADH2 gene being highly regulated by these potential transcription factors and functioned as a promoter for yeast under high volume of ethanol and organic acids.

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9 **Effects of glucose, ethanol and acetic acid on regulation of ADH2 gene from _Lachancea_**
10 **_fermentati_**

12 Norhayati Yaacob[1,2], Mohd Shukuri Mohamad Ali[1,2] *, Abu Bakar Salleh,[1,2] and Nor 'Aini Abdul
13 Rahman[3]

15 1. *Enzyme and Microbial Technology Research Centre, Universiti Putra Malaysia, 43400 Serdang,*

16 *Malaysia*

17 *2. Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti*

18 *Putra Malaysia, 43400 Serdang, Malaysia*

19 *3. Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences,*

20 *Universiti Putra Malaysia, 43400 Serdang, Malaysia*

22 ***Corresponding author. Tel: +603-89466721**
23 **Fax: +603-89430913**
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25 **Email address: mshukuri@upm.edu.my**
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30   **ABSTRACT**

32   **Background.** Not all yeast alcohol dehydrogenase 2 (ADH2) are repressed by glucose, as reported

33   in *Saccharomyces cerevisiae*. *Pichia stipitis* ADH2 is regulated by oxygen instead of glucose,

34   whereas *Kluyveromyces marxianus* ADH2 is regulated by neither glucose nor ethanol. For this

35   reason, ADH2 regulation of yeasts may be species dependent, leading to a different type of

36   expression and fermentation efficiency. *Lachancea fermentati* is a highly efficient ethanol

37   producer, fast-growing cells and adapted to fermentation-related stresses such as ethanol and

38   organic acid, but the metabolic information regarding the regulation of glucose and ethanol

39   production is still lacking.

41   **Methods.** Our investigation started with the stimulation of ADH2 activity from *S. cerevisiae* and

42   *L. fermentati* by glucose and ethanol induction in a glucose-repressed medium. The study also

43   embarked on the retrospective analysis of ADH2 genomic and protein level through direct

44   sequencing and sites identification. Based on the sequence generated, we demonstrated ADH2

45   gene expression highlighting the conserved NAD(P)-binding domain in the context of glucose

46   fermentation and ethanol production.

48   **Results.** An increase of ADH2 activity was observed in starved *L. fermentati* (LfeADH2) and *S.*

49   *cerevisiae* (SceADH2) in response to 2% (w/v) glucose induction. These suggest that in the

50   presence of glucose, ADH2 activity was activated instead of being repressed. An induction of 0.5%

51   (v/v) ethanol also increased LfeADH2 activity, promoting ethanol resistance, whereas

52   accumulating acetic acid at a later stage of fermentation stimulated ADH2 activity and enhanced

53   glucose consumption rates. The lack in upper stream activating sequence (UAS) and TATA

54   elements hindered the possibility of Adr1 binding to LfeADH2. Transcription factors such as SP1

55   and RAP1 observed in LfeADH2 sequence have been implicated in the regulation of many genes

56   including ADH2. In glucose fermentation, *L. fermentati* exhibited a bell-shaped ADH2 expression,

57   showing the highest expression when glucose was depleted and ethanol-acetic acid was increased.

58   Meanwhile*, S. cerevisiae* showed a constitutive ADH2 expression throughout the fermentation

59   process.

60

61   **Discussion.** ADH2 expression in *L. fermentati* may be subjected to changes in the presence of

62   non-fermentative carbon source. The nucleotide sequence showed that ADH2 transcription could

63   be influenced by other transcription genes of glycolysis oriented due to the lack of specific

64   activation sites for Adr1. Our study suggests that if Adr1 is not capable of promoting LfeADH2

65   activation, the transcription can be controlled by Rap1 and Sp1 due to their inherent roles.

66   Therefore in future, it is interesting to observe ADH2 gene being highly regulated by these

67   potential transcription factors and functioned as a promoter for yeast under high volume of ethanol

68   and organic acids.

69

70   **INTRODUCTION**

71

72   *Lachancea sp*, a type of yeast, is no stranger to the study of alcohol dehydrogenase (ADH) gene

73   isolations and biochemical characterizations. A decade ago, ethanol metabolism in *Lachancea sp*.

74   has been studied by Duarte *et al*. (2004) via enzyme profiling showing the differences in ADH

75   isoenzymes which could be observed across *Lachancea* species. ADH catalyzes the final metabolic

76   step in ethanol fermentation and plays an important role for general metabolic pathways of yeast

77   to yield energy as well as to proliferate under anaerobic conditions (Liang *et al*., 2014).

78

79   *L. fermentati* was a former member of *Kluyveromyces, Saccharomyces* and *Zygosaccharomyces*

80   (Kurtzman. 2003). The identification of *L. fermentati* was based on the evaluation from the

81   perspective of the multigene sequence analysis which resulted in the reassignment of *Lachancea*

82   *sp*. The haploid number of chromosomes in *Lachancea sp.* is reported to be 8, equivalent to half

83   of the haploid chromosome numbers of *Saccharomyces* and *Kluyveromyce*s (Naumova *et al*.,

84   2007).

85

86   To date, *L. fermentati* is known for its efficient role in ethanol fermentation (Natararajan *et al*.,

87   2012). However, there is a lack of information regarding the metabolic and gene regulations that

88   took place during the glucose fermentation of *Lachancea sp*. (Thomson *et al*., 2013). In this study,

89   ethanol, glucose, and acetic acid are investigated for their role in regulating *L. fermentati* ADH2

90   expression. This enzyme would then be compared to the expression of common yeast, *S. cerevisiae*

91   ADH2 in terms of repression and derepression mechanism in relation to carbon sources. Glucose

92   repression and derepression essentially concern genes involved in oxidative metabolism

93   (Weinhandl *et al*., 2014). In fermentation, yeast cells accumulate fermentation products, such as

94   ethanol, from sugars. Then, this was accompanied by an increase in medium acidity, due to the

95   secretion of organic acids at the later stage of fermentation. The pathway associated with stress

96   response in yeast, in line with ethanol and acetic acid production, proved to be deadly, as they

97   demonstrate inhibition of cell growth and represses glucose transport (Burtner et al., 2009).

98   Because ADH2 gene is highly deregulated by glucose, it can control both of the respiration and

99 metabolism function through it's role in aerobic respiration and mitochondrial function. This

100 makes the gene one of the suitable targets for its ethanol and organic acid tolerance. As for this

101 reason, ADH2 can become an important marker in determining the beginning of glucose, ethanol

102 and acetic acid stress activity (Denis et al., 1983; Smidt et al., 2008; Lin et al., 2010).

103

104 The gene can also be linked to the function of hexokinase and other glycolytic genes as it has been

105 demonstrated having a promoter activity based on the glucose stimulation (Lee et al. 2005;

106 Weinhandl et al. 2014). The long-term advantage of utilizing ADH2 gene as a promoter compared

107 to the other common used promoters is that no specific inducer is required. In the case when an

108 increase of biomass concentration was evident, the ADH2 gene has shown a high level of

109 expression which is appropriate in the optimization of bioethanol fermentation (Weinhandl et al.,

110 2014).

111

112   The mechanism of yeast stress tolerance was extensively studied in *S.cerevisiae* as

113 compared to other yeasts. It was reported that *S. cerevisiae* could tolerate various types of stresses

114 in particular of ethanol inhibition and osmotic pressure from acids and sugars from bioethanol

115 production (Zhao and Bai, 2009). The latest development of stress tolerance mechanisms was

116 focused more on the genomics rather than proteomics as it quick to point out the causes of these

117 adverse conditions. However, the mechanism of yeast stress tolerance through correlation of

118 biological response with the flux of gene expression under the presence of stress is equally

119 important as it elaborates the genetic dependence of stress on the external stimuli of fermentation.

120 Hence, by comparing the expression of ADH2 before and after the induction of these stresses, the

121 role of ADH2 can be evaluated. Besides the observation of genes expression to demonstrate

122 environmental liability that potentially leads to the early sign of stress, it is also vital to know the

123 habitual of genes expressed under a favorable condition for example when glucose is in abundance.

124

125   Despite these well–known characteristics, De Smith *et al*., (2012) reported that ADH2 was

126 not fully repressed even in the presence of glucose, as the gene transcription could still be detected

127 in glucose-laden condition. This could be due to the upstream activation sequences (UAS1 and

128 UAS2) located at the C-terminal of ADH2 sequences. Both of them are necessary for the complete

129 derepression of ADH2 which requires activation by trans-acting regulatory element, Adr1p (Smidt

130 *et al*., 2008). For UAS2, the activation (derepression) of ADH2 expression is highly dependent on

131 its orientation and copy numbers. However, when UAS2 is disorientated, a decline of ADH2

132 expression will be observed as it acts synergistically with UAS1 as a binding site for Adr1p to

133 stimulate the expression of ADH2 (Smidt *et al*., 2008; De Smith *et al*., 2012). Thus, the uses of

134 ADH2 as a promoter for bioethanol production could be based on its characteristics in the

135 repression and derepression mechanism (Donoviel *et al*., 1995; Smidt *et al*., 2008). The choice of

136 the right promoter is a crucial point for efficient gene expression, as most regulations take place at

137 the transcriptional level (Weinhandl *et al*., 2014).

138

139   An over-expression of ADH2 is capable of exhibiting a continuous cell growth in ethanol

140 and is important for the control of the glucose uptake. The increase of ADH2 activity also results

141 in the increase of acetic acid production which negatively affects the fermentation, leading to a

142 sluggish or arrested fermentation (Maestre *et al*., 2008). As ADH2 expression increased

143 (derepression), the accumulation of ethanol within the cell is likely to be responsible for the rapid

144 fermentation at 30°C, influencing high cell density and acetic acid production (Nagodawithana *et*

145 *al*., 1976). The transcription of yeast ADH2 in a regulation of the external environment of

146 fermentation is a very interesting subject, as it supports in the description of the mechanism

147 involved in various biotechnological processes (Cho *et al*., 2009; Lin *et al*., 2010). In this study,

148 we analyzed the sequence harboring the ADH2 gene from *L. fermentati*, identify its regulatory

149 genes and transcription binding sites, followed by determination of ADH2 regulation in the

150 presence of glucose, ethanol, and acetic acid.

151

152 **MATERIALS AND METHODS**

153

154 **Yeast strains validation and culture conditions**

155 *S. cerevisiae* obtained from a commercially produced yeast powder was used as a control in this

156 study as it demonstrates ethanol fermentation ability in high glucose content (10%, v/v). This is an

157 important criterion in order to observe the expression or activity of a glucose-regulated ADH2

158 gene. *L. fermentati* was isolated from a fermented nypa sap of *Nypa fruticans* located in Telok

159 Intan, Malaysia (Natarajan *et al*., 2012). The Internal transcribed spacer (ITS) regions (comprising

160 partial sequence 18S rDNA-ITS1-5.8S rDNA-ITS2- partial sequence 28S rDNA) of the selected

161 yeasts were amplified and sequenced for species and strain identification purposes using the

162 methods of Fujita *et al*. (2003). Based on the BLAST analysis, the ITS nucleotide sequences of *S.*

163 *cerevisiae* exhibited 98 to 99% similarity with the *S. cerevisiae* of *Chicha* strain (KC183723.1).

164 As expected, the *L. fermentati* ITS sequence exhibited a 100% similarity with the strain SHM1

165 (accession number JN674449.1). Yeast Peptone Dextrose (YPD) medium (1% yeast extract, 2%

166 peptone, and 2% glucose) was used for the culture maintenance at 30°C.

167

168 **Partial ADH2 amplification and phylogenetic analysis**

169 Both *L. fermentati* and *S. cerevisiae* were subjected to partial ADH2 PCR amplification by using

170 denatured primer (**Table 1**). The ADH2 gene from *L. fermentati* strain SHM1 was designed based

171 on the validated alcohol dehydrogenase sequences of *Saccharomyces* and *Lachancea* origins

172 highlighting the conserved region. The amplification procedure was performed in 20 µl reaction

173 with pre-denaturation of 95°C; 5 min, followed by 30 cycles of denaturation 95°C; 30 sec,

174 annealing 55°C; 30 sec, pre-elongation 72°C; 30 sec, and final elongation 72°C; for 5 min. The

175 912 bp length of ADH2 gene obtained from wild-type *L. fermentati* were excised and purified

176 using QIAquick Gel Extraction Kit (QIAGEN, Germany), before submitted for DNA sequencing

177 (1st Base Sdn Bhd, Malaysia). The deposited nucleotide sequences (accession number: KU203771)

178 were then translated into peptide sequences using a translational tool from ExPASy (SIB,

179 Switzerland) and peptide alignments were performed by using SDSC Biology Workbench 3.2.

180 Phylogenetic analysis based on the model of Pearson and the neighbor-joining analysis was

181 performed with 1000 replicates of bootstrap test by using MEGA 6.06 software.

182

183 **Total RNA extraction and cDNA synthesis**

184 Total RNA was extracted using the Presto™ Mini RNA Yeast Kit (Geneaid, Taiwan) following

185 the protocol provided by the manufacturer. RNA concentration and quality were determined using

186 the A_{260}/A_{280} spectrophotometric absorption ratio (Eppendorf, Germany) and RNA Bioanalyzer

187 2100 Plant Nano chip (Agilent, USA) to determine the RNA integration number (RIN) values. The

188 RNA quality was also tested by electrophoresis on 1% (w/v) agarose gel. cDNA was synthesized

189 from the isolated high-quality RNA by using one-step RT-qPCR iScript™ reverse transcription

190 supermix (BioRad, USA).

191

192 **Real time-PCR primer design**

193 A complete nucleotide sequence of *Saccharomyces cerevisiae* ADH2 was available at the

194 *Saccharomyces cerevisiae* Genome Database (http://www.yeastgenome.org/). The primers for *S.*

195 *cerevisiae* were designed by using the software Primer Express (Applied Biosystems, Foster City,

196 USA), while the primers for *L. fermentati* were designed based on the multi-alignment of ADH2

197 gene sequences from the *Saccharomyces*, *Kluyveromyces,* and *Lachancea* families. Actin1

198 (ACT1) was chosen as the reference gene for normalizations (Ismail *et al.*, 2013). Real-time PCR

199 **Table 1** showed the primer sequences used for gene expression along with their amplicon sizes.

200

201 **Gene expression analysis with real-time PCR**

202 ADH2 gene expression was determined by real-time PCR. An efficiency test was performed using

203 cDNA from *S. cerevisiae* and *L. fermentati* as the template. The PCR efficiency was scaled to 95-

204 105% and above, 0.999 R-squared and a slope of -3.33, as recommended (Bustin *et al.*, 2009).

205 Real-time PCR assays were performed in an iCycler (iQ™5, BioRad, USA), using the standard

206 thermal cycling protocol. The reaction mixture of 20 µl contained 10 µl of iTaq Universal SYBR®

207 Green Supermix (2x) (BioRad, USA), 0.75 nM of forward and reverse primers, 2 µl of 10-fold

208 diluted cDNA, and deionized water to reach the final volume. Real-time PCR experiments were

209 carried out in two replicates, of which each real-time PCR was performed in duplicate on each

210 sample. A negative control without the cDNA template was included. The thermal amplification

211 program used was as follows: 95°C for 30 s; 40 cycles of 95°C for 10 s; and 60°C for 30 s before

212 the melt-curve was collected from 58°C to 90°C. **Figure 1S** displayed melt curve analysis for

213 ADH2 and Act1 genes during the amplification. Gene expression levels were shown as the

214   threshold cycle (C_t) of the studied gene normalized with the C_t of a reference gene, ACT1 (**Table**

215   **1S**).

216

Induction of ADH2

218   In ADH2 protein induction study, 2% (wv^{-1}) glucose (for repression) and 0.5% (vv^{-1}) ethanol (for

219   derepression) were added in 100 ml of YPD medium at 30°C with 200 rpm of agitation after 24 h

220   because there was no glucose remained in the medium. Following the induction, fermentation was

221   extended for one hour before cells were completely harvested and protein was extracted for ADH2

222   activity determination.

223

Intracellular protein extraction

225   The cell was harvested from the culture and washed two times with deionized water. Then, the

226   protein extract was prepared by mixing 20 ml of 50 mM phosphate buffer (pH 7.8). The cell

227   suspension was lysed via the sonication method (Branson, USA) for 2.5 min, with 5 sec of pulses.

228   The supernatant was collected as protein extract after 10 min of centrifugation at 8000 X g with a

229   temperature of 4°C. Total protein concentration was determined by the method of Bradford (Quick

230   Start™ Bradford, BioRad), as specified by the manufacturer.

231

ADH2 screening assay

233   The unpurified soluble protein extracted from *S. cerevisiae* and *L. fermentati* cells were used in

234   the measurement of ADH2 activity. The reaction for ADH2 assay was performed in 0.3 ml of total

235   volume containing 20 mM of sodium pyrophosphate buffer (pH 7.8), 3.3% (v/v) of ethanol, 7.5

236   mM of NAD$^+$ and <10 µg of ADH (protein), with 7 to 8 min of reaction time. ADH2 activity was

237   measured by increasing 340 nm absorbance in response to NADH or acetaldehyde production. The

238   units/ml of ADH2 activity was calculated by using 6.22 of the millimolar extinction coefficient of

239   β-NADH at 340 nm. One unit of NAD^+ converts 1.0 μmole of ethanol to acetaldehyde per minute

240   at pH 7.8 and 25°C (Kägi *et al*., 1960). ADH activity was also qualitatively measured via native

241   PAGE by adding ADH activity buffer containing 4.0 mg of phenazine methyl sulfate (Sigma-

242   Aldrich, Germany), 10 mg of nitroblue tetrazolium (Sigma-Aldrich, Germany), 50 mg of NAD^+

243   (Sigma, Germany) and 0.05 ml of absolute ethanol dissolved in 50 ml of 0.1 M Tris-HCl buffer

244   (pH 8.5) until the desired purple staining was formed (Young *et al.,* 2000).

245

246   **Shake flask fermentation**

247   Seed cultures of *S. cerevisiae* and *L. fermentati* were grown at 30°C, 200 rpm agitation speed for

248   24 h in a universal tube containing 10 ml of the YPD medium and then inoculated into the

249   fermentation medium at a level of 1% (v v^{-1}). The fermentation medium of 2% YPD (1% yeast

250   extract, 2% peptone, and 2% glucose) was prepared at 100 ml in a 500 ml Erlenmeyer flask, where

251   the fermentations were all carried out in similar condition. Before the fermentation, the starting

252   density (OD_{600}) of all cultures was 0.5. Over the course of the 30 h fermentation, samples were

253   collected every 6 h to monitor the growth, ethanol, organic acid and residual glucose

254   concentrations. Repeats were performed in triplicate. The fermentation products were centrifuged

255   at 8000 X g for 10 min to separate the cells from the analytes. The supernatants were stored at -

256   20ºC until used for the ethanol and glucose assays while the precipitants were used in the RNA

257   extraction and ADH assays.

258

259   **Determination of ethanol and glucose concentration**

260   The content of the ethanol in the fermentation samples was determined based on the 96-microwell

261   plate system using the ethanol extraction method (Seo *et al*. 2009). The sugar concentration was

262   determined using the 3, 5-dinitrosalicylic acid (DNS) method (Bernfeld., 1955).

263

264   **Acetic acid production determination**

265   Analyzes of organic acids, such as ethanoic acid (acetic acid) were performed by High-

266   Performance Liquid Chromatography, using an Agilent Poroshell 120 EC-C18 column. The

267   column and mobile phase line of the instrument were thoroughly washed with 50%

268   Acetonitrile/50% water of mobile solution. The running buffer or eluent that was used to detect

269   weak acids contained 1 mmol/l of sulfuric acid and 8 mmol/l of Na_2SO_4, with pH 2.8. The flow

270   rate of the mobile phase was adjusted to 0.9 ml/min and the temperature was fixed at 25º C.

271

272   **RESULTS AND DISCUSSION**

273

274   **Derepression of ADH2 by glucose induction**

275   While the era of yeast synthetic biology began in the well-characterized model organism *S.*

276   *cerevisiae*, other non-conventional yeast production systems such as *Hansenula polymorpha*,

277   *Kluyveromyces lactis*, *Pichia pastoris*, and *Yarrowia lipolytica* have been regarded as a eukaryote

278   model with growing importance (Wagner and Alper. 2015). These yeasts including *L. fermentati*

279   have roles in the manufacture of vaccines, therapeutic proteins, food additives, and renewable

280   chemicals, but recent synthetic biology advances have the potential to greatly expand and diversify

281   their impact on biotechnology, which is how the potential of ADH2 gene from *L. fermentati*

282   (LfeADH2) can provide an alternative biomanufacturing platforms primarily as a promoter. To

283   date, *L. fermentati* has not yet been widely considered for industrial usages.

284

285   During induction at the glucose-limiting period, ADH2 from *L. fermentati* exhibited higher affinity

286   for glucose and ethanol, as shown by a 2.5-fold increase in 0.5% (vv^{-1}) ethanol and 0.5-fold

287   increase in 2% (wv^{-1}) glucose (**Figure 1**). However, *S. cerevisiae* ADH2 activity was increased by

288   1.3-fold following the addition of 2% glucose, and was repressed by 0.5-fold with the addition of

289   0.5% ethanol. Based on the results obtained, ADH2 protein from *Saccharomyces cerevisiae* did

290   not exhibit a strong glucose-repressible activity.

291

292   ADH2 activity staining using the ADH2 protein confirmed the outcomes of ADH2 activity after

293   the sugar and non-sugar-based induction. The degradation of ethanol as a substrate by this protein-

294   entrapped in native polyacrylamide gel can be measured by its intensity after staining. Based on

295   these results, it can be implied that both glucose and ethanol shared significant roles in the

296   derepression or activation mechanism of *L. fermentati* ADH2 promoter.

297

298   This experiment demonstrated that both ADH2 enzyme activity and protein expression shifted

299   with the addition of ethanol or glucose into the medium. In previous work by Irani *et al*. (1987),

300   ADH2 from *S. cerevisiae* was undetectable in the presence of a fermentable carbon source such as

301   glucose, but when glucose was exhausted from the medium or when the cells are grown on a non-

302   fermentable carbon source such as ethanol, ADH2 enzyme activity, and mRNA are present in large

303   amounts. The results were connected to Denis *et al*. (1983), which described contradicting changes

304   in *S. cerevisiae* ADH1 expression resulting an increase of mRNA levels when ethanol grown-cells

305 were transferred into medium containing glucose (Tornow and Santangelo., 1990). Therefore, it is

306 not surprising that both ADH1 and ADH2 have been widely accepted as a promoter in yeast for

307 their regulation according to a specific carbon source. In this study, ADH2 gene from *L. fermentati*

308 strain SHM1 has been found to be regulated by both glucose and ethanol, an interesting concept

309 of a two-in-one promoter system.

310

311 Previously, *S. cerevisiae* ADH2 promoter is not the only alcohol dehydrogenase promoter

312 that has been used in the study of glucose-related expression. Fission yeast *S. pombe* ADH2

313 promoter which showed high homology at the protein level to *S. cerevisiae* ADH2 also was

314 frequently utilized as a promoter. Unlike *S. cerevisiae*, *S. pombe* ADH2 was constitutively

315 expressed that resulted in the up-regulation of ADH2 transcription in the presence of glucose

316 (Russel and Hall., 1983). Contradict to that, *K. marxianus* strain IFO 1802 ADH2 (KmADH2)

317 exhibited a low expression level in high glucose and ethanol throughout the 72 h of 150 g/L glucose

318 fermentation (Liang *et al.*, 2014). Overall, the stringency of yeast ADH2 activation and expression

319 towards the glucose availability was rather obscure. There are mutants that allowed ADH2

320 expression to escape glucose-repression in which, a rare-semidominant Adr1 allele (constitutive)

321 is involved. The constitutive Adr1 showed mutations between amino acids 227 and 239 was said

322 to be caused by a post-translational inhibition (Ratnakumar *et al.*, 2009). Inclusive to our findings,

323 the glucose induction reflects the possibility of ADH2 to be express in the presence of glucose. It

324 means that glucose and ethanol would have the ability to promote ADH2 expression in *L.*

325 *fermentati*. This result was contradicted to *K. marxianus* ADH2 where the protein was unable to

326 be expressed in the presence of either of this compound.

327

328 Based on these findings, we performed some retrospective analysis on sequences bearing *L.*

329 *fermentati* ADH2-like sequences highlighting on the presence of transcription binding sites which

330 probably involved in the activation of ADH2 transcription.

331

332 **Multiple alignments of partial ADH peptide sequences**

333 The partial ADH gene amplifications were initiated by using a pair of denatured primer (**Table 1**).

334 The primer sequences were designed based on the multi-alignment of verified ADH2 nucleotide

335 sequences from *Saccharomyces* and *Lachancea* species. To date, there are no alcohol

336 dehydrogenase sequences available in NCBI database for *L. fermentati*. Thus, we amplified and

337 sequenced ADH2 gene from *L. fermentati* (previously known as *Zygosaccharomyces fermentati*)

338 for the first time. The 912 bp-length ADH2 nucleotide sequences amplified from *L. fermentati* was

339 very different from the well-known yeast ADH2 sequences. The highest similarity score of ADH2

340 sequences amplified from *L. fermentati* strain SHM1 (KU203771) was 88.6% with *L. kluyveri*

341 ADH2 (AAP51047.1). The nucleotide sequences and motifs observed in ADH2 gene in *L.*

342 *fermentati* were highly conserved. But the analysis showed that there were a few variations found

343 to the other existing ADH2 sequences from different species. **Figure 2** showed the multiple

344 alignments of various ADH2 protein sequences together with ADH2 gene from *L. fermentati*. The

345 analysis of ADH2 sequences from *L. fermentati* successfully identified the highly preserved amino

346 acids and the zinc-binding motifs. Based on the protein alignments, several residues were believed

347 to have a species-related function as observed in Iso-37, Glu-117, Glu-175, Cys-183, Thr-228,

348 Val-231 and Gly-276. It is, therefore, important to study on the role of these residues toward the

349 expression of this gene as they could provide be a useful indication on the nature of regulation of

350 this protein. In this assignment, Thr-228 and Val-231 were the residues included in sequences for

351   gene expression throughout the yeast fermentation study. Typically the HXXXH motifs containing

352   imidazole ring has been well described in yeast as the site where metal ion exchange happens in

353   zinc-containing enzymes. Currently, both *S. cerevisiae* and *L. fermentati* exhibited conservation

354   of this zinc binding sites at residues 48-55 based on the HXXXH motif and residues 64-74

355   according to the Interpro analysis (Obradors *et al.*, 1998).

356

357   The neighbor-joining phylogenetic analysis of ADH2 nucleotide sequences from *L. fermentati*

358   strain SHM1 exhibited highest similarity to ADH2 gene belonged to *L. kluyveri* followed by the

359   *Saccharomyces* family. A slight distance from *L. fermentati* observed in the phylogenetic tree

360   indicated the distribution of *Kluyveromyces* and another different *Lachancea* cluster family, where

361   this isolated cluster of *Lachancea* group contained the uncharacterized ADH2 sequence. Hence,

362   *L. fermentati* ADH2 was presumed to inherit considerable genomic characteristics from the

363   *Saccharomyces* and *Kluyveromyces* origins **(Figure 3)**.

364

365   **The transcription binding site in partial ADH2 sequences of *L. fermentati***

366   Yeast ADH2 was known to repress growth on fermentative carbon sources and have a lower

367   affinity for glucose (Kachevrovsky *et al.*, 2008). The results of glucose-inducible ADH2

368   contradicted the normal regulation of glucose-repressible ADH2, as observed in real-time PCR,

369   indicating that ADH2 transcription was not glucose-dependent. To date, it is known that the

370   glucose-regulated ADH2 gene was activated by several regulating genes. Among these genes is

371   Adr1, which binds to the ADH2 promoter site at upper-activating sequence (UAS). When glucose

372   was added, the established Adr1-UAS1 binding weakened, as Adr1 was inhibited by glucose,

373   which, in turn, repressed ADH2, causing minimal expression or activity (Irani *et al.*, 1987; Young

374   *et al.*, 2000). However, Young *et al.*, (2008) reported that Adr1 competently binds to the specific

375   UAS sequence even when glucose levels are still high, which may be a possible reason for the

376   high ADH2 activity observed after glucose was added. Alternatively, the increase in Adr1

377   synthesis could also cause ADH2 to express constitutively during growth on glucose. This

378   condition allows ADH2 expression to escape glucose repression (Irani *et al.*, 1987). However so,

379   this type of regulation had only been studied in *S. cerevisiae* and has not been evaluated for other

380   kinds of yeast species. **Figure 4** demonstrates that the conservation of Adr1-binding site was not

381   always significant for glucose-regulated genes as most of the ADH2 sequences from yeast which

382   belonged to non-*Saccharomyces* group possessed no inherent Adr1-binding sites. A multiple

383   sequence alignment of ADH2 proteins from *P. stipitis* (Y13397.1), *S. cerevisiae* S288c

384   (NM_001182812.1), *K. marxianus* (KF678866.1) and *L. fermentati* (KU203771) showed some

385   irregularities in the conservation sites of UAS and TATA sequences (**Figure 4**). Nucleotide

386   sequence analysis showed that *P. stipitis* and *S. cerevisiae* possessed UAS and TATA repeats while

387   *K. marxianus* and *L. fermentati* did not.

388

389   In *S. cerevisiae*, the presence of large excess of competing promoter elements such as UAS

390   and TATA regions were important to ensure the competency of ADH2 promoter (Irani *et al.*,

391   1987). The involvement of Adr1 transcription factor to promote ADH2 expression was primarily

392   based on the conservation of UAS1 and UAS2 regions. The ADH2 promoter which lacked the

393   upstream regulatory region has been reported to cause in inactivation, while the missing of TATA

394   box in the sequence was reported to contribute to an intermediate level of binding competition

395   (Irani *et al.*, 1987). It is worth to note that the control of Adr1 binding does not only occur at the

396   gene transcriptional level but also during the post-translational modification of the protein as well

397 (Irani *et al.*, 1987; Kachevrovsky *et al.*, 2008). Following the preliminary genomic studies, ADH2

398 gene transcription in *L. fermentati* might not work according to its' approach (glucose-repressible)

399 in the absence of UAS and TATA.

400

401   The presence of Adr1-binding sequence at nucleotides 99-108, 228-237, 828-839 showed

402 that ADH2 gene from *L. fermentati* would be readily transcribed. The lack in adjacent upper

403 stream-activating sequences, however, left the sequence incomplete for Adr1 binding.

404 Nonetheless, within the sequence of ADH2, there are other potential regulatory genes which made

405 up the complex transcriptional network in yeast. These genes which exist in abundance can

406 promote ADH2 regulation inadvertently providing suitable condition which stimulates them. In *L.*

407 *fermentati*, regulatory genes or activator-binding protein such as the zinc finger transcription factor

408 (Sp1) and repressor/activator site binding protein (Rap1) are believed to equally participate in

409 ADH2 gene regulation. Sp1 contains a highly conserved DNA-binding domain composed of three

410 zinc fingers close to the C-terminus with serine/threonine- and glutamine-rich domains in their N-

411 terminal regions. This protein is an extremely versatile protein involved in the expression of many

412 different genes which differs between the cell types during development. Given that there are at

413 least 11 sites for the binding of Sp1 in *L. fermentati* ADH2 gene and its importance in many genes

414 activation, it is predicted that the cells would not survived without their presence. So far, little is

415 known how Sp1 act on natural promoters in combination with other transcription factors *in vivo*

416 (Suske., 1999; Bouwman and Philipsen., 2002).

417

418   The presence of RAP1 transcriptor factor which was known to activate most ribosomal

419 protein and glycolysis enzymes can function as a strong key promoter to ADH2 gene in the

420   presence of glucose. The Rap1 site appears to mediate the response of these genes to the

421   appearance or disappearance of glucose; for example, their expression is coordinately down-

422   regulated upon depletion of glucose at the diauxic shift (Santangelo., 2006). The presence of

423   regulatory genes shown in **Figure 5** was predicted via *in-silico* –based software (TRANSFAC

424   Database, Biobase, GmbH) and was found to be conserved in the nucleotide sequence of ADH2

425   from *L. fermentati*. Therefore, ADH2 gene could harbor its function through various promoters

426   and activators without having the UAS and TATA regions to activate it through Adr1.

427

428   In general, ADH2 has a broader role in the yeast cell physiology and growth than

429   previously reported, as this gene can either be involved in the utilization of non-fermentable carbon

430   sources, carbohydrate and nitrogen metabolism or protein synthesis depends on its type of species

431   (Cheng *et al*., 1994). In the quest to determine which carbon elements contributes to ADH2

432   activation in the presence of glucose, ethanol, and by-product (acid), a relative gene expression of

433   ADH2 from *L. fermentati* and *S. cerevisiae* were performed.

434

435   **ADH2 gene regulation**

436   In analyzing ADH2 expression, cells from *S. cerevisiae* and *L. fermentati* were harvested at

437   different growth stages. The cell harvest obtained from the basal (zero h), exponential (6th, 12th h),

438   mid-exponential (18th h) and stationary (30th h) phases, along the 30 hr length of 2% (w/v) glucose

439   fermentation. The regulations of ADH2 in both yeasts were influenced by cell growth, ethanol

440   production as well as glucose consumption as shown in **Figure 6.**

441

442   It is true that when glucose gets depleted and ethanol started to build up, the expression of

443   *S. cerevisiae* ADH2 improved showing its activation during exponential growth phase. The trend

444   of ADH2 expression follows the order of consumption and ended with ethanol as well as by-

445   products production. The availability of glucose can influence the repression of ADH2 in the

446   waking phase of fermentation. But once the glucose had been completely used up, ethanol or even

447   acetate would take up the role of ADH2 derepression. It has been reported that at the transcriptional

448   level, the up-regulation of glucose-repressible ADH2 is a result of an increase in ethanol or acetate

449   production. (Ida *et al*., 2012; Weinhandl *et al*., 2014).

450

451   In LfeADH2, the sluggishness expression in conjunction with the glucose consumption

452   was expected. When ethanol was high, LfeADH2 expression was derepressed. The derepression

453   state of LfeADH2, however, did not last to the end of fermentation, as ADH2 was immediately

454   repressed through the oxidation of ethanol due to its decline or with the presence of other common

455   fermentation by-products such as acetic acid. In this study, *L. fermentati* ADH2 derepression by

456   ethanol was pursued with the increase of ADH2 activity. However, the reason of ADH2

457   derepression in glucose remained unclear, as glucose was expected to inhibit the transcription of

458   ADH2. One of the reasons would be because of its high rate in glucose consumption that would

459   contribute to the major decline in ADH2 expression rather than the concentration itself. In returns,

460   the expression or activity of ADH2 are more likely to increase when the glucose consumption rate

461   declined and not due to the limitation of glucose in the medium (**Table 1S**).

462

463   The outcomes of ADH2 gene expression from *L. fermentati* ADH2 showed that the gene

464   has an indispensable function for being tolerate to a higher concentration of ethanol and to maintain

465   cell growth in ethanol. Apparently, the production of ethanol has not improved following the up-

466   regulations of LfeADH2 expression. The regulation of ADH2 could be influenced by other co-

467   expressed genes, such as pyruvate decarboxylase (PDC). PDC plays a role in acetaldehyde

468   formation during the conversion of pyruvate to acetaldehyde, where it may have led to the

469   reduction of ethanol concentration (Ida *et al.*, 2012; Milanovic *et al.*, 2012). This would further

470   promote the down-regulation of ADH2 expression. Together, the involvement of PDC and ADH2

471   were reported to synergistically promote growth in yeasts by glucose metabolism, as these

472   enzymes were capable of efficiently converting intracellular pyruvate and NADH into ethanol.

473   This event typically occurred in a homoethanol fermentation pathway, also known as the Entner-

474   Doudoroff (ED) pathway (Piriya *et al.*, 2012). The main difference in the gene expression of ADH2

475   derived from *S. cerevisiae* and *L. fermentati* has been the result of ethanol. This could be explained

476   by *L. fermentati* ADH2 affinity for ethanol which exhibited lower K_m compared to *S. cerevisiae*

477   ADH2 (**Table 2**).

478

479   In this observation, the activity of ADH2 was identical to the transcriptional profiles of

480   ADH2 exhibited over the fermentation course. The bell-shaped curve of ADH2 activity in *L.*

481   *fermentati* again indicated that ADH2 transcription was not compelled to the regulation of glucose-

482   ethanol alone. The increased level of acetic acid might also involve in the regulatory mechanism

483   of ADH2 gene expression, in which the function of this weak acid was known to retain the redox

484   potential under fermentative condition.

485

486   **Influence of acetic acid production on ADH2 activity**

487   Acetic acid co-production can influence yeast ADH2 activity during cell growth via the metabolic

488   oxidation of carbon sources that generates large amounts of organic acids. To manage the cytosolic

489   pH homeostasis, the production of acids must be kept in equilibrium with their utilizations

490   (Zdraljevic *et al.*, 2013). As ADH2 involved in the oxidation of ethanol and acetic acid production,

491   it was relevant to determine the correlation between the production of organic acid, particularly of

492   acetic acid, and the changes of ADH2 activity. The presence of this acid can contribute to the rate-

493   limiting steps in ethanol fermentation although they are produced to retain intracellular pH in

494   response to glucose availability and to prevent direct inhibition of ethanol via their buffering

495   capacity (Thomas *et al.* 2002; Ullah *et al.* 2012).

496

497   Subsequent experiments performed in glucose fermentation also showed that ADH2

498   activity was very much regulated by acetic acid produced by *L. fermentati* (**Figure 7A**). However,

499   *S. cerevisiae* ADH2 activity appeared not to be regulated by acetic acid (**Figure 7B**).

500

501   According to Maestre *et al.*, (2008), the overexpression of the ADH2 gene resulting in its

502   high activity during alcohol fermentation would affect the glucose uptakes, cell growth and

503   increased production of acetic acid. It was also reported that this condition was associated to

504   detoxification by removal of acetaldehyde (oxidized product) to restore the intracellular redox

505   potential. Judging by the importance of ADH2 expression in *L. fermentati* to prevent cell-induced

506   apoptosis, the role of ADH2 could not be ruled out. Acetic acid can easily penetrate the cell wall

507   and induced cell death and one way to decelerate this process is through promoting ADH2

508   expression or activity (Sousa *et al.*, 2012). Hence, we proposed that acetic acid could be the non-

509   fermentative carbon source, capable of triggering the regulation of LfeADH2 in glucose

510   fermentation as well as to promote cell growth.

511

512   ADH2 activity in yeasts come in handy as it has the advantage to promote organic acid and

513   ethanol tolerance and to maintain the relative production of ethanol. In **Table 1S**, ADH2 activity

514   in *L.fermentati* was found high in the presence of acetic acid and ethanol. These concentrations

515   were equivalent to 0.835 mmol/L of acetic acid and 10.62 g/L of ethanol when *L. fermentati* ADH2

516   activity reached its highest level. Subsequently, ADH2 activities subsided when acetic acid was

517   suppressed. Based on these results, we described that the approach of LfeADH2 in gene

518   transcription was not strictly glucose-regulated, as specifically observed in previous experiments

519   performed in most of the wild-type *S. cerevisiae* (Vallari *et al.*, 1992).

520

521   **CONCLUSION**

522   Many of the cellular and metabolic features of *L. fermentati* ADH2 showed protective roles toward

523   intracellular processes during ethanol fermentation. The up-regulation of *L. fermentati* ADH2 in

524   the presence of glucose and ethanol showed that ADH2 enabled both glucose utilization and

525   ethanol production to occur simultaneously. Acetic acid also is a precursor to ADH2 activity, and

526   *L. fermentati* has showed to demonstrate a higher tolerance to acetic acid prior to the increase in

527   ADH2 activity. Figure 8 showed a schematic diagram of ADH2 sequence from *L. fermentati* which

528   plays important role to the cause of gene transcription influencing protein expression during

529   glucose fermentation. Generally, ADH2 has become of practical importance for bioethanol

530   production from *L. fermentati*, as this gene was highly regulated at the highest level of ethanol

531   fermentation. The mechanism of Adr1−ADH2 binding in *L. fermentati* can be unique but the

532   absence of UAS and TATA elements would mean that the gene was unable to be transcribed based

533   on the glucose-repressible condition. Alternatively, the presence of other well-described regulating

534   genes such as Rap1 and Sp1 could be of importance to the activation of *L. fermentati* ADH2. Based

535   on this preliminary study of ADH2 from *L. fermentati*, it is of great interest to describe the

536   mechanism of ADH2 activation and which type of gene regulations that mostly affect the

537   transcription process. Through this knowledge, the probable use of the ADH2 gene in *L. fermentati*

538   as a promoter in a facilitating ethanol synthesis from highly rich acetic acid medium of

539   lignocellulosic hydrolysates could be of worth significance.

540

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661

662 **Figure legend**

663

664 **Figure 1.Glucose and ethanol induction on ADH2 from *S. cerevisiae* and *L. fermentati* .** ADH2

665 activity in *S. cerevisiae* (Sce) and *L. fermentati* (Lfe) after induction with 2% (wv⁻¹) glucose (A)

666   and 0.5% (vv⁻¹) ethanol (B). Qualitatively, the ADH2 activity staining was performed on the non-

667   denaturing polyacrylamide gels with respective lanes indicated as 1 (before induction) and 2 (after

668   induction). All experiments have been repeated twice for validation.

669

670   **Figure 2 Multiple ClustalW alignment of ADH2 peptide sequences of *Lachancea fermentati***

671   **SHM1 (KU203771), Kluyveromyces marxianus (BAP71734.1), Kluyveromyces lactis**

672   **(CAA45739.1), *Lachancea kluyveri* (AAP51047.1), *Lachancea lanzarotensis* (CEP60577.1),**

673   ***Lachancea thermotolerans* CBS 6340 (XP_002555715.1), *Saccharomyces bayanus***

674   **(AAP51043.1), *Saccharomyces cerevisiae* YJM451 (AJS66970.1), *Saccharomyces kudriavzevii***

675   **(EJT43588.1) and *Saccharomyces pastorianus* (AAP51051.1).** The red box showed amino acid

676   alignment of zinc-binding site (HXXXH) present in the medium-chain NAD (P)-dependent

677   dehydrogenase. While the blue-box contained the sequences of NAD (P)-binding domain having

678   the motifs of SLEGGEV and KATNGG used for gene expression. The N-terminal of ADH2 gene

679   from *Lachancea fermentati* strain SHM1 starts at residue 29-137, while the C-terminal starts at

680   residue 180-304 based on Interpro (EMBL-EBI) analysis.

681

682   **Figure 3 Neighbor-joining phylogenetic analysis separating the sequences of ADH2 from *L.***

683   ***fermentati* in *Lachancea* ADH2 family and *Saccharomyces/Kluyveromyces* ADH2 family**

684   **groups.** Clustering was performed via the neighbour-joining method using Pearson model with

685   the software package, MEGA (Molecular Evolutionary Genetics Analysis) version 6.06. The

686   percentages at the nodes indicate the levels of bootstrap support for the branch point based on 1000

687   bootstrap replicates.

688

689   **Figure 4 Multiple nucleotide sequence alignment of *Pichia stipitis* (Y13397.1), *Saccharomyces***

690   ***cerevisiae* S288c (NM_001182812.1), *Kluyveromyces marxianus* (KF678866.1) and *Lachancea***

691   ***fermentati* SHM1 (KU203771).** The presence of UAS sequences (5'-GGAGA-3') was identified

692   in a purple box and TATA elements were captured in blue box. Three transcription genes were

693   identified as Sp1 (11) in orange-colored box, Rap1 (3) in red-box and Adr1 (3) represented in

694   green-colored box.

695

696   **Figure 5. The fractionation of regulatory transcription genes present in ADH2 gene from**

697   ***Lachancea fermentati* strain SHM1 (in percentage).**

698

699   **Figure 6. The profiles of ADH2 gene expression measured by real time PCR and ADH2**

700   **activity determination based on protein reaction with ethanol on both *S. cerevisiae* and *L.***

701   ***fermentati***. Fig 6A and 6B exhibited the relative ADH2 gene expression, whereas Fig 6C and 6D

702   exhibited the ADH2 activity of each protein extracted from *S. cerevisiae* and *L. fermentati*

703   respectively, under similar fermentation state.

704

705   **Figure 7. Effect of acetic acid production on the ADH2 activity from *L. fermentati* (A) and**

706   ***S. cerevisiae* (B) glucose fermentation**

707

708   **Figure 8.Schematic diagram describing the role of ADH2 gene from *Lachancea***

709   ***fermentati* strain SHM1 in the regulation of glucose, ethanol and organic acid in bioethanol**

710   **fermentation**

711

712 **Tables**

713

714 **Table 1 List of the primers for ADH2 gene amplifications and one reference gene for the**

715 **normalization of the gene expression studies.** ACT1* was selected as the reference gene for

716 normalization.

717

718 **Table 2 Enzyme kinetics of crude alcohol dehydrogenase (ADH) from *L. fermentati* and *S.***

719 *cerevisiae*. Assays were carried out at 25°C and the absorbance at 340 nm was measured. Assays

720 were performed in triplicates per single run. $V_{Max \ and}$ K_M were calculated based on the half-

721 reciprocal, Hanes-woolf equation (Ritchie and Prvan, 1996).

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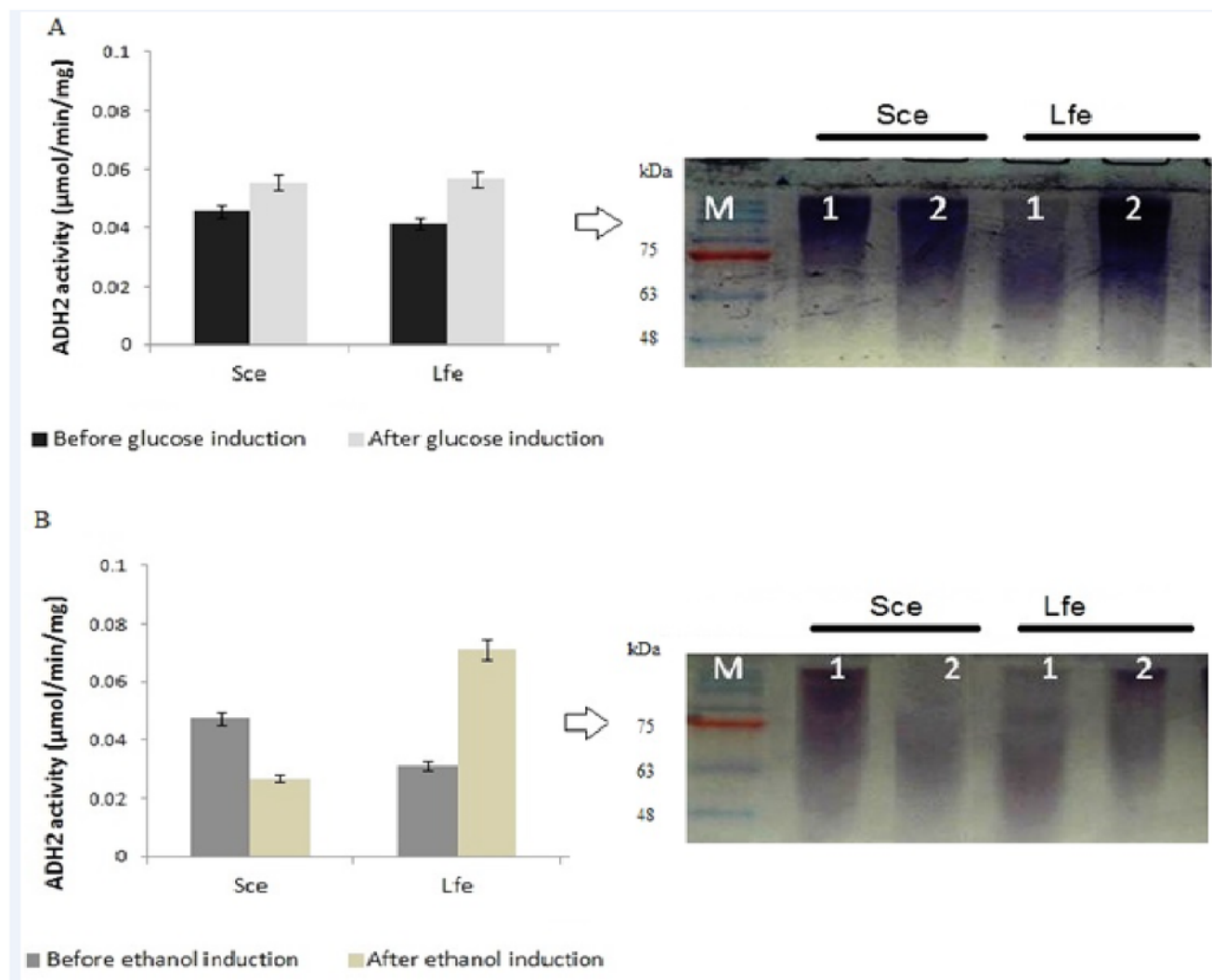
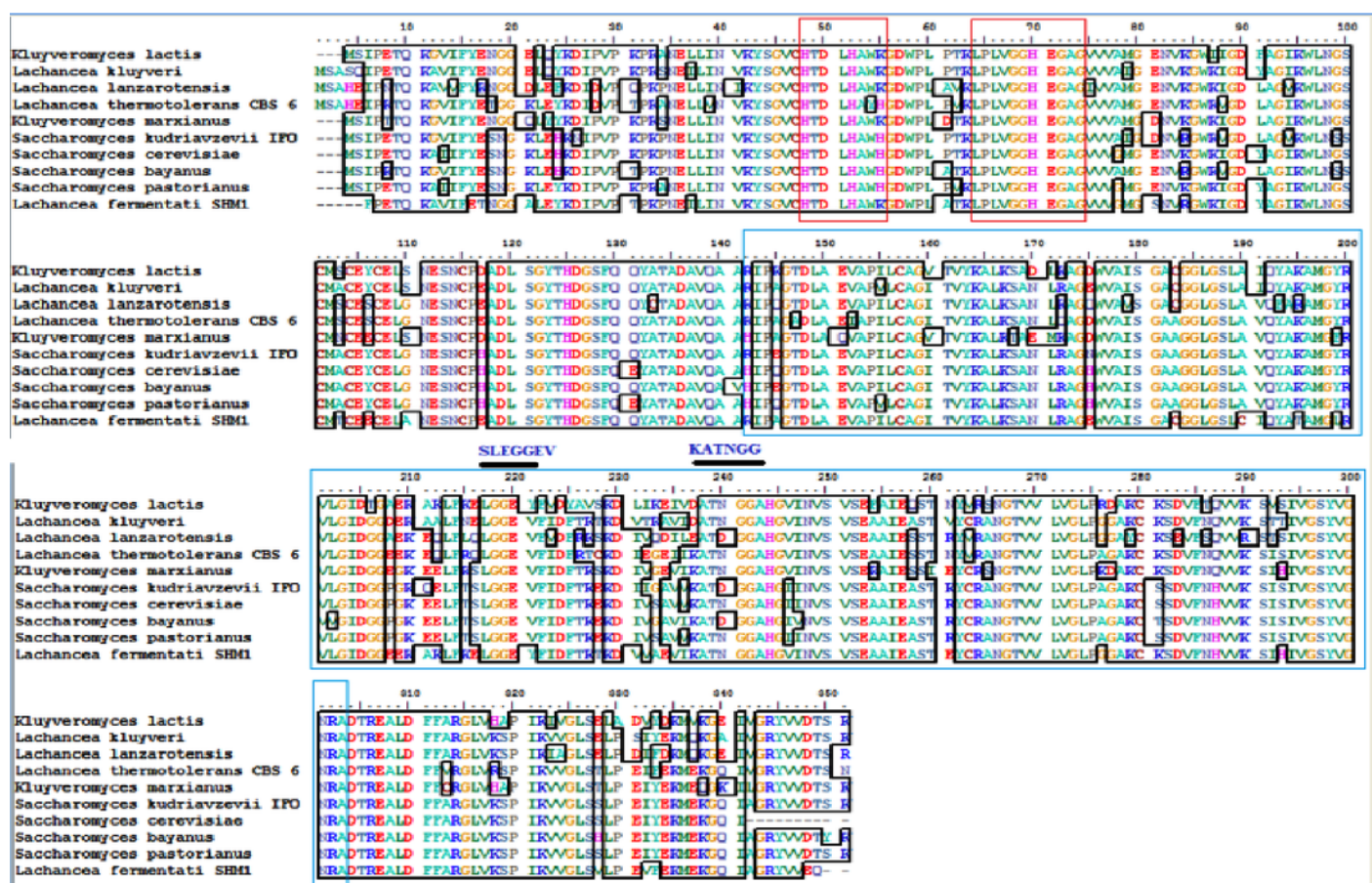
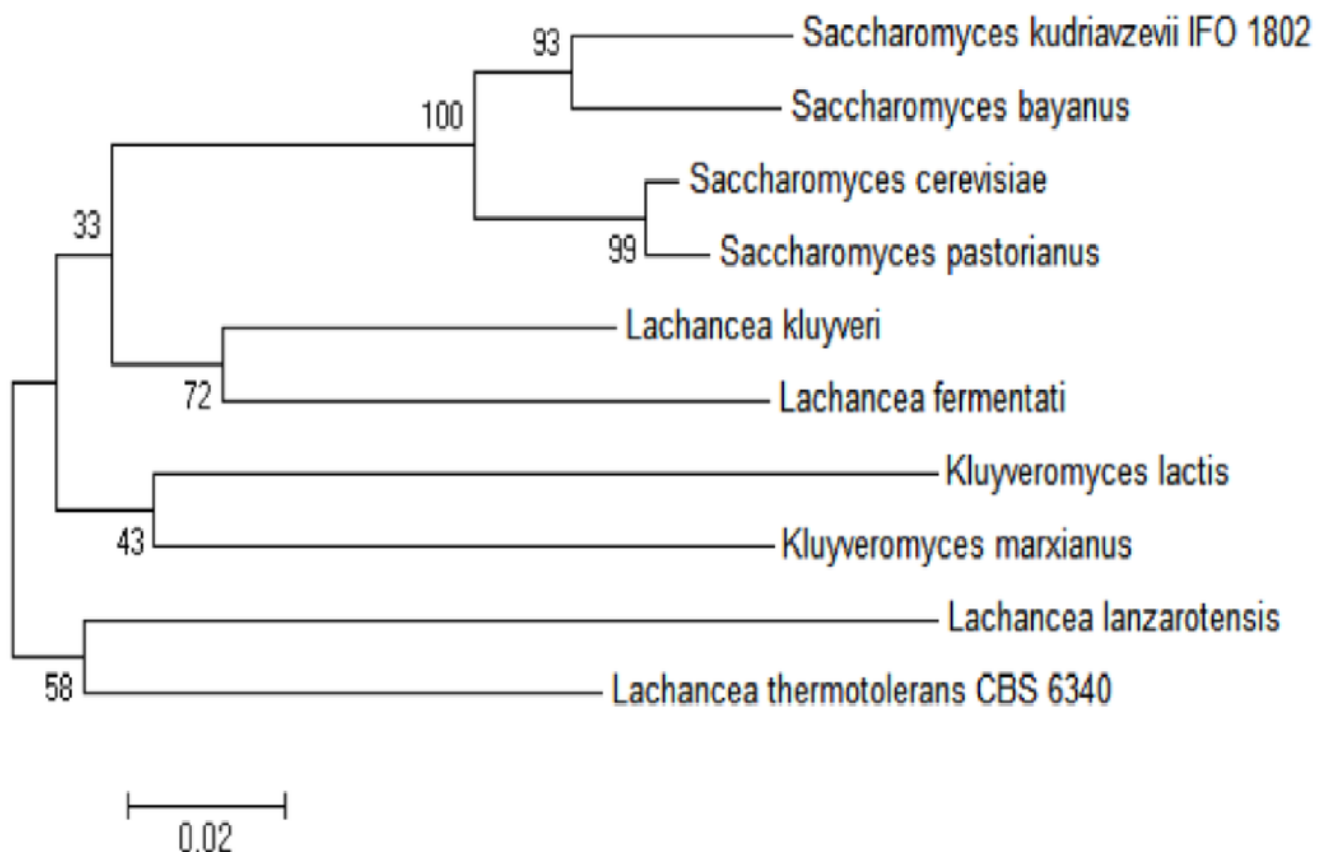
Glucose and ethanol induction on ADH2 from *S. cerevisiae* and *L. fermentati*.

ADH2 activity in *S. cerevisiae* (Sce) and *L. fermentati* (Lfe) after induction with 2% (wv^{-1}) glucose (A) and 0.5% (vv^{-1}) ethanol (B). Qualitatively, the ADH2 activity staining was performed on the non-denaturing polyacrylamide gels with respective lanes indicated as 1 (before induction) and 2 (after induction). All experiments have been repeated twice for validation.

Multiple ClustalW alignment of ADH2 peptide sequences

The red box showed amino acid alignment of zinc-binding site (HXXXH) present in the medium-chain NAD (P)-dependent dehydrogenase. While the blue-box contained the sequences of NAD (P)-binding domain having the motifs of SLEGGEV and KATNGG used for gene expression. The N-terminal of ADH2 gene from *Lachancea fermentati* strain SHM1 starts at residue 29-137, while the C-terminal starts at residue 180-304 based on Interpro (EMBL-EBI) analysis.

3

Neighbor-joining phylogenetic analysis separating the sequences of ADH2 from *L. fermentati* in *Lachancea* ADH2 family and *Saccharomyces/Kluyveromyces* ADH2 family groups.

4

Multiple sequence alignment of *Pichia stipitis* (Y13397.1), *Saccharomyces cerevisiae* (NM_001182812.1), *Kluyveromyces marxianus* (KF678866.1) and *Lachancea fermentati* (KU203771).

The presence of UAS sequences (5'-GGAGA-3') was identified in a purple box and TATA elements were captured in blue box. Three transcription genes were identified as Sp1 (11) in orange-colored box, Rap1 (3) in red-box and Adr1 (3) represented in green-colored box.

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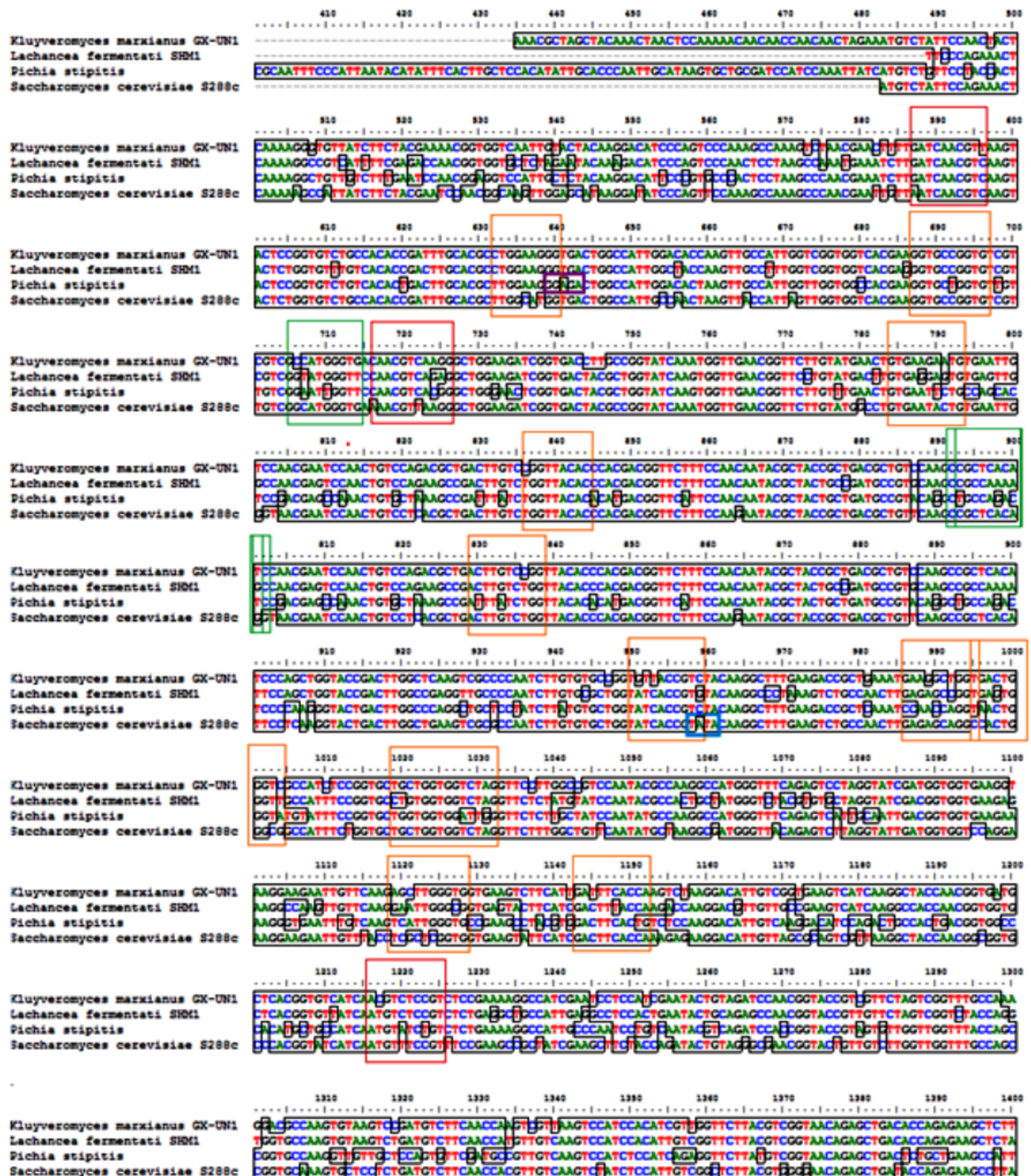
The fractionation of regulatory transcription genes present in ADH2 gene from
Lachancea fermentati strain SHM1 (in percentage)

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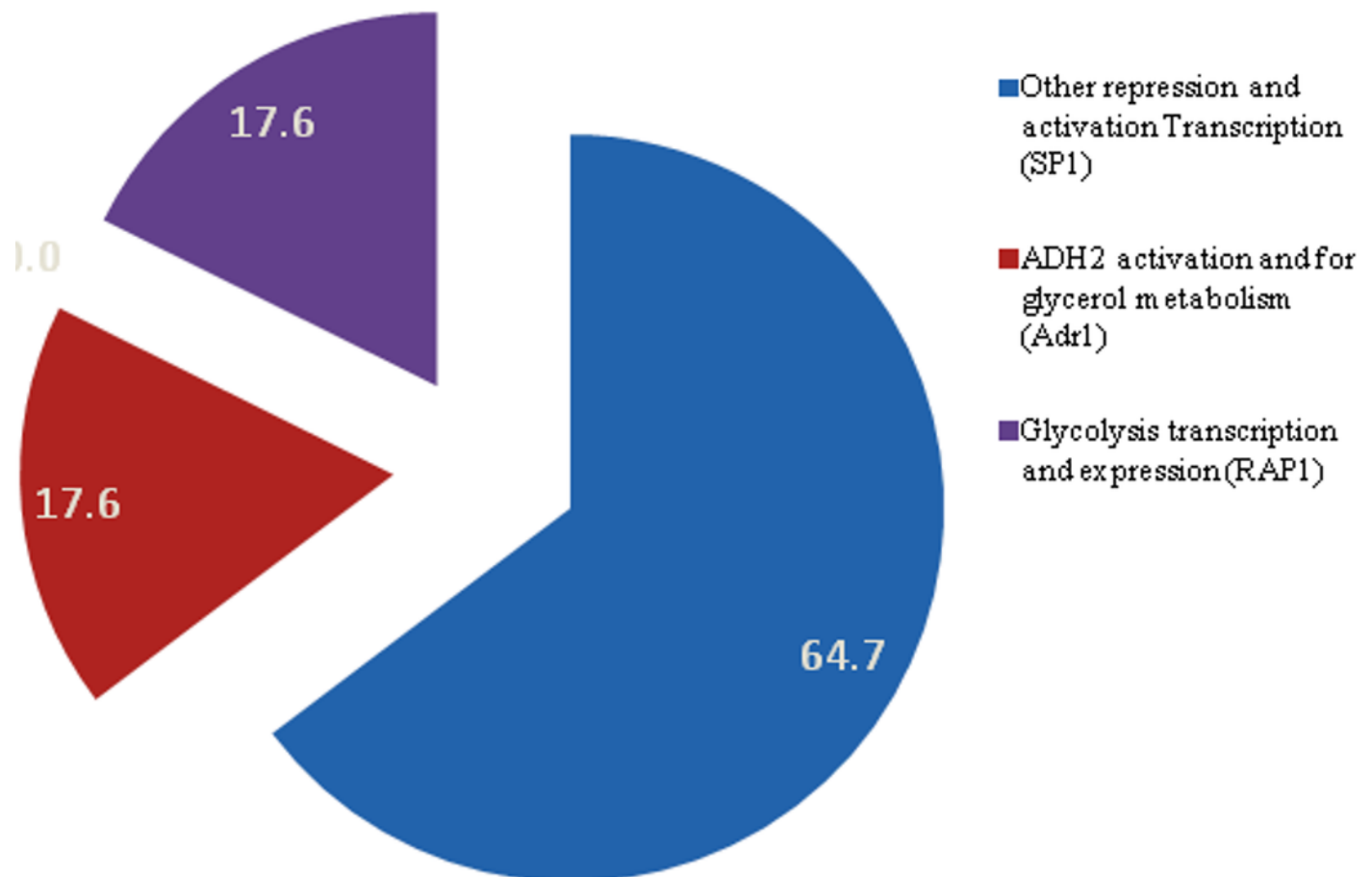
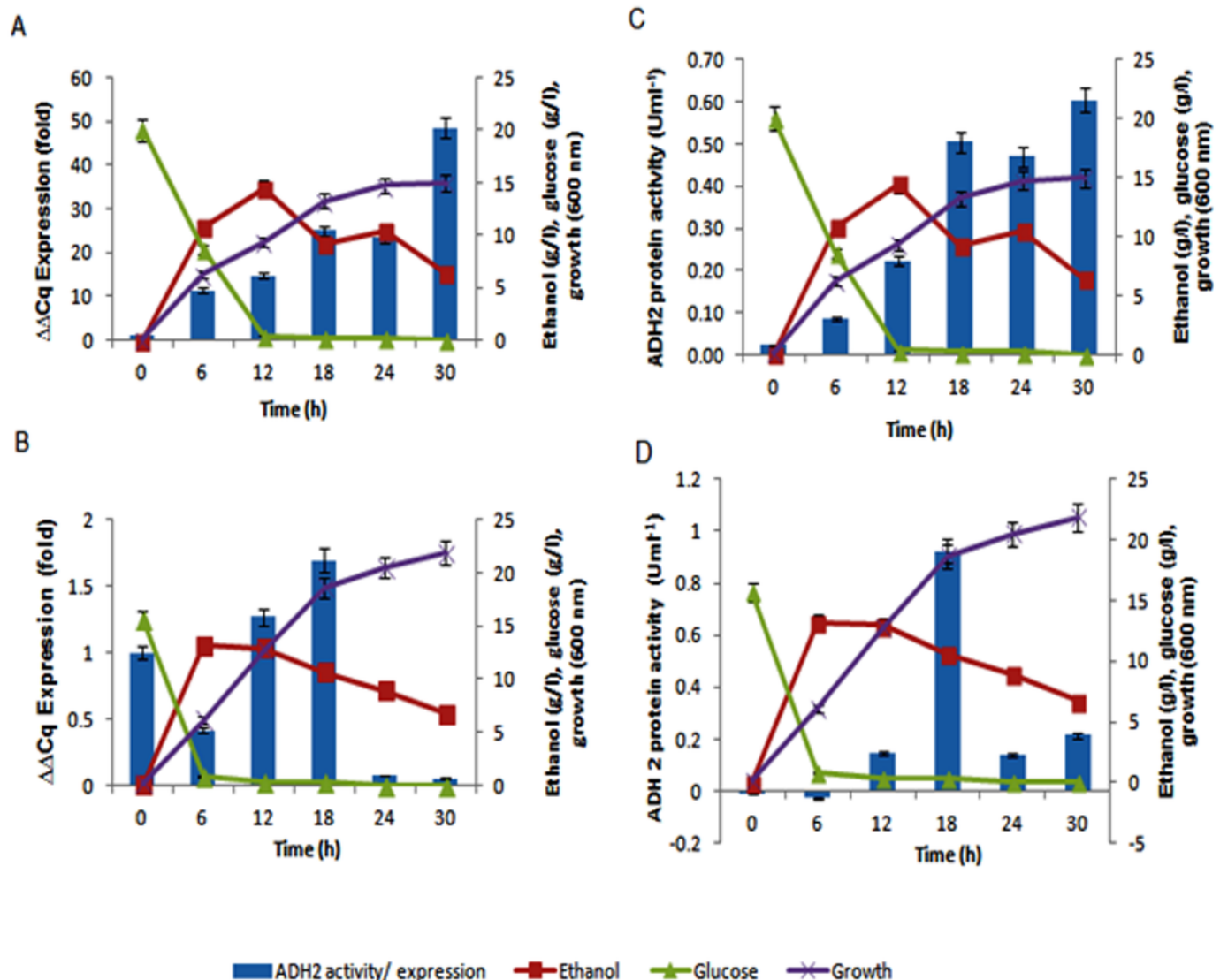
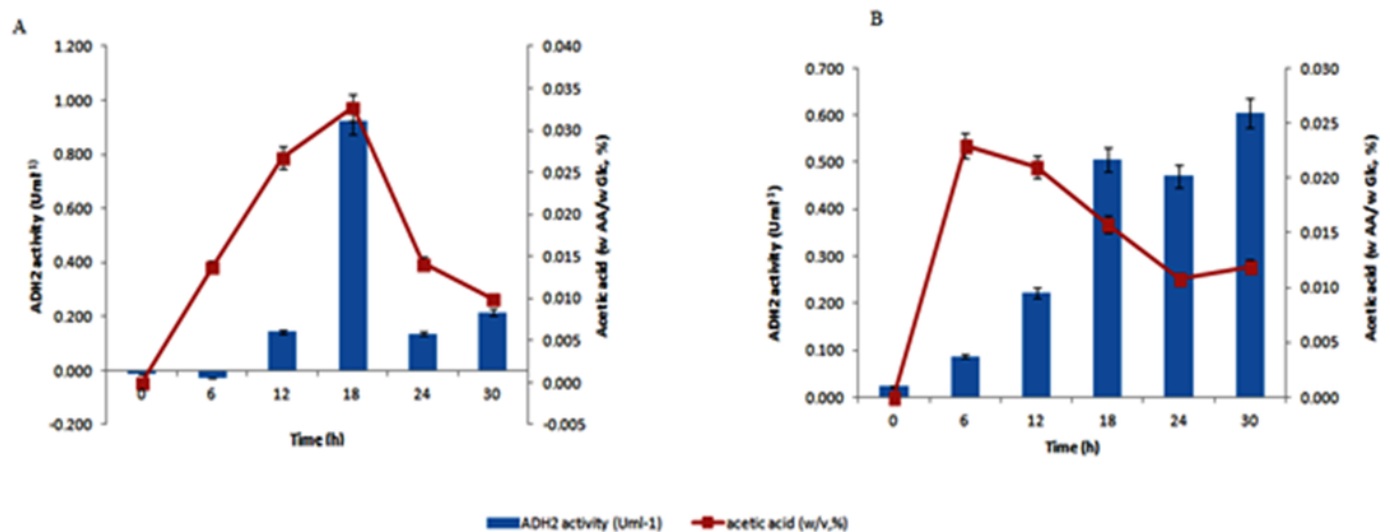
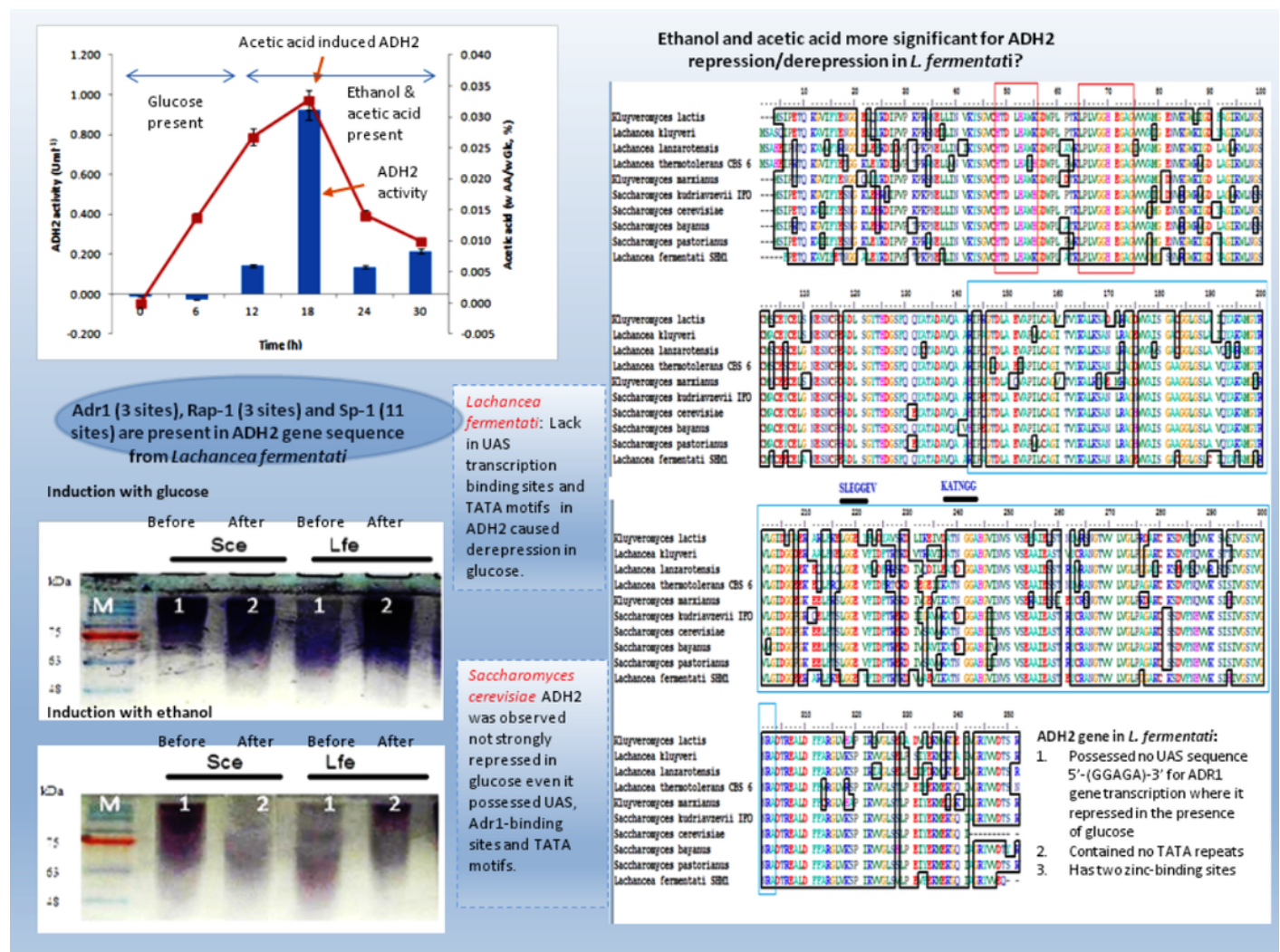

The profiles of ADH2 gene expression measured by real time PCR and ADH2 activity determination based on protein reaction with ethanol on both *S. cerevisiae* and *L. fermentati*.

Fig 6A and 6B exhibited the relative ADH2 gene expression, whereas Fig 6C and 6D exhibited the ADH2 activity of each protein extracted from *S. cerevisiae* and *L. fermentati* respectively, under similar fermentation state.

Effect of acetic acid production on the ADH2 activity from *L. fermentati* (A) and *S. cerevisiae* (B) glucose fermentation.

Schematic diagram describing the role of ADH2 gene from *Lachancea fermentati* strain SHM1 in the regulation of glucose, ethanol and organic acid in bioethanol fermentation

List of the primers for ADH2 gene amplifications and one reference gene for the normalization of the gene expression studies.

ACT1* was selected as the reference gene for normalization.

1   Table 1

No.	Primer	Sequence (5'-3')	Start (bp)	Stop (bp)	Tm (°C)	Length (bp)
1.	Degenerate primer amplification based on *Lachancea kluyveri* ADH2 (accession no. AAP51047.1) gene sequence for unknown ADH2 gene isolation from *L. fermentati* strain SHM1 (to provide sequence template for selected gene targets)					
	ForADH2	ATYCCAGAAACTCAAAARGCCRTTA	538	565	52.5	912
	RevADH2	TGTCRACAACGTATCTACCRRCAAT	1547	1572	59.3	
2.	Real time RT-PCR primer sequences (derived from sequences from partial amplified ADH of respective strain)					
i	SceADH2_F	CGCAGTCGTTAAGGCTACCAA	690	710	59	70
	SceADH2_R	CGATAGCGGCTTCGGAAAC	760	742	59	
ii	LfeADH2_F	GACTTTACCAAGACCAAGG	655	673	62	61
	LfeADH2_R	ACCTTGAGCACCACCGTTGGTG	720	699	66.3	
iii	ACT1_F *	TGGATTCCGGTGATGGTGTT			56	72
	ACT1_R*	TCAAAATGGCGTGAGGTAGAGA			56	

Enzyme kinetics of crude alcohol dehydrogenase (ADH) from *L. fermentati* and *S. cerevisiae*.

All assays were carried out at 25°C and the absorbance at 340 nm was measured. Assays were performed in triplicates per single run. $V_{Max\ and}$ K_M were calculated based on the half-reciprocal, Hanes-woolf equation (Ritchie and Prvan, 1996).

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3   **Table 2**

	Substrate	K_M (mM)	Vmax (umol mg^{-1} sec^{-1})	K_{cat} (s^{-1})	K_{cat}/K_M (M^{-1} s^{-1})
SceADH2	ethanol	0.28×10^2	0.8×10^{-2}	1.18×10^{-2}	42.54×10^{-2}
LfeADH2	ethanol	5.08×10^2	1.3×10^{-2}	3.86×10^{-2}	7.60×10^{-2}