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1 **Autodisplay of α -amylase from *Bacillus megaterium* in *E. coli* for the**
2 **bioconversion of starch to hydrogen, ethanol and succinic acid**

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Abstract

24 In this work, the expression of a α -amylase from *Bacillus megaterium* on the cell
25 surface of *E. coli* strains WDHA ($\Delta hycA$, $\Delta ldhA$) and WDHFP ($\Delta hycA$, $\Delta frdD$ and
26 Δpta) by the autodisplay AIDA system was carried out to confer the ability to the *E.*
27 *coli* strains to produce hydrogen, ethanol and succinic acid from starch. For the
28 characterization of the α -amylase, the effect of temperature (30-70°C), pH (3-6)
29 and CaCl₂ concentration (0-25 mM) as well as the thermostability of the enzyme
30 under different temperatures (55-80°C) at several time intervals (0-15 min) were
31 evaluated. The results showed that the α -amylase has a maximum activity at 55°C
32 and pH 4.5. Calcium is required for the activity as well for the thermal stability of
33 the enzyme. The Km and Vmax values calculated were 5.8 mg/mL and 0.0106
34 mg/ml/min respectively. Furthermore, a set of batch fermentations were carried out
35 using 10 g/L plus 1 g/L of glucose as carbon source in 120 mL anaerobically
36 serological bottles using WDHA (succinate producer) or WDHFP (ethanol
37 producer) *E. coli* strains harboring the pAIDA-amyA plasmid. The hydrogen and
38 succinic acid production for WDHA was 1,056.06 mL/L and 6.8 g/L, respectively
39 whereas WDHFP strain produced 1,689.68 mL/L of hydrogen and 2.8 g/L of
40 ethanol. This work represents a promising strategy to improve the exploitation of
41 starchy biomass for the production of valuable compounds without the need of a
42 pre-saccharification process.

43 Keywords: Whole-cell catalysis; α -amylase; starch hydrolysis; biofuels

44 **1. Introduction**

45 The utilization of biomass as feedstock for the production of biofuels and bio-based
46 chemicals has recently become an attractive alternative option. One of the main
47 feedstocks for biofuel and chemicals production is the starch-rich biomass, which is
48 easily depolymerized by amylases to generate high yields of glucose [1]. Starch is
49 contained in many staple foods (e.g. potatoes, wheat, corn, rice, among others),
50 therefore it is abundant and consists of a large number of glucose units conjugated
51 with glycosidic bonds [2]. However, although starchy materials are available in
52 abundance, a previous liquefaction and saccharification process of the biomass is
53 required to hydrolyze polysaccharides into monosaccharides before its use for
54 biofuel or chemical production. Amylases are extracellular enzymes, which
55 hydrolyze starch molecules to give such diverse products as dextrans, and
56 progressively smaller polymers composed of glucose units. In this regard, α -
57 amylases are endoamylases catalyzing the hydrolysis of internal α -1,4-glycosidic
58 linkages in starch in a random manner, preferably in immobilized form [3]. They are
59 usually/mainly produced by bacteria belonging to the genus *Bacillus* such as *B.*
60 *subtilis*, *B. licheniformis*, *B. amyloliquefancies* and *B. stearothermophilus* [4].
61 Several strategies have been adopted for the construction of starch-utilizing
62 system, such as the addition of large amounts of amylases. The use of pure
63 enzymes in biocatalysis has several advantages such as the specificity for selected
64 reactions, simple equipment and procedures [5]. Nevertheless, enzyme production,
65 isolation and purification can be expensive and in addition, the enzymes are often
66 used only once which increases the cost of the process. On the contrary, the use
67 of microorganisms as whole-cell biocatalysts avoids the purification steps and

68 allows the recycling of the enzymes. The advantages of the application of this
69 technology is that the cells themselves provide a natural environment for the
70 enzymes preventing conformational changes in the protein which could result in
71 the loss of the activity [6]. Also, other important advantage is that they can
72 efficiently regenerate the enzymes. Among the different whole-cell systems the
73 AIDA (adhesin involved in diffuse adherence) autodisplay system from *Escherichia*
74 *coli* have favorable features such as modularity and simplicity. This is an efficient
75 surface display system for Gram-negative bacteria and is based on the
76 autotransporter secretion pathway. In general, it consists of a cassette that
77 includes the β barrel of AIDA, the recombinant passenger protein is transported
78 simply introducing its coding sequence in the frame between the signal peptide and
79 the translocator domain [7]. This autodisplay system offers the expression of more
80 than 10^5 recombinant molecules per single cell, permits the multimerization of
81 subunits expressed from monomeric genes at the cell surface and it results in a
82 superior surface exposure of heterologous passenger [8].

83

84 The aim of this study is to carry out the fermentation of starch for the production of
85 hydrogen, succinate or ethanol by two *E. coli* strains with deletions of genes
86 related to carbon metabolism. To make this possible, we used the autodisplay
87 AIDA system to express the α -amylase from *B. megaterium* in the cell surface of *E.*
88 *coli*. The characterization of the enzyme activity, as well as the effectiveness of its
89 ability to hydrolyze starch in batch fermentations was assessed.

90

91 **2. Material and methods**

92 **2.1. Bacterial strain and growth conditions**

93 Strains of *E. coli* WDH were mutated as previously described by Balderas-
94 Hernandez, Maldonado [9]. Briefly, *E. coli* WDH strain [10] ($\Delta hycA$, negative
95 regulator of the formate regulon) was used as parental strain. Gene deletion was
96 carried out by transduction with bacteriophage P1 [11]. Strains from single-gene
97 knockout mutant collection of the nonessential genes of the *E. coli* W3110 were
98 used as donors. Deleted genes were ***ldhA***: D-lactate dehydrogenase; ***frdD***:
99 fumarate reductase; and ***pta***: phosphate acetyltransferase. Gene deletions and
100 resistance loss were confirmed by PCR analysis using standard conditions and the
101 primers described in Table 1.

102

103 *E. coli* Top 10 and pGEM-T easy vector which were used for subcloning of PCR
104 products were obtained from Promega. Cells were routinely grown at 31°C in Luria-
105 Bertani (LB) medium, containing 150 µg/mL of ampicillin. Solid media were
106 prepared by the addition of agar (1.5% w/v).

107

108 **2.2. Construction of artificial AIDA system**

109 The design of the amyA-AIDA fusion gene was carried out to confer the ability of *E.*
110 *coli* to degrade starch. For this, the DNA sequences were assembled with the
111 Snapgene software (GSL Biotech LLC, Version 3.3) and MacVector (MacVector,
112 Inc, Version 10.1). The design of the fusion genes for the “*autodisplay*” of proteins

113 was based on the AIDA sequence reported by Maurer [7]. For the translocation to
114 the internal membrane, the signal peptide of the toxin of the β -subunit of *Vibrio*
115 *cholerae* (CtxB) was selected. The autotransporter gene for AIDA was used, which
116 consists of a peptide and a β -barrel (amino acids from 839 to 1286, GenBank:
117 X65022.1). The nucleotide sequences were optimized for their expression in *E.*
118 *coli* (GenScript, New Jersey, USA). The codon-optimization was performed by
119 evading as many restriction sites as possible; strategic restriction sites were added
120 to be able to exchange protein passenger when required. The gapAP1 promoter of
121 the constitutive *gapA* gene of *E. coli* was selected as the transcriptional regulator of
122 the amyA-AIDA gene, since it works under aerobic and anaerobic conditions. The
123 construction was synthesized by the Biomatik Corp (Delaware, USA). The pUC57
124 plasmid was used as host and the *EcoRV* was used as restriction site as a cloning
125 site. The artificial cassette was called pUC57-AIDA.

126 The *amyA* gene encoding for α -amylase was amplified by polymerase chain
127 reaction of the *B. megaterium* genome (X07261.1). PCR product was inserted into
128 pGEM-T easy vector and then it was digested using the restriction enzymes *AscI*
129 and *XhoI* before ligation into pUC57-AIDA plasmid, which was digested with the
130 same enzymes. This yielded an in-frame fusion protein consisting of the CtxB
131 signal peptide, α -amylase as the passenger, the linker region and the β -barrel
132 autotransporter under the control of the gapAP1 promoter. The plasmid was
133 transformed into *E. coli* WDHA and WDHP by electroporation. The α -amylase
134 gene has a length of 1500 bp. The inserted gene was sequenced before its use in
135 the subsequent experiments.

136 **2.3. α -Amylase activity visualization**

137 The amylase activity was visually detected from the clearing zone around the
138 colonies on starch plates containing 0.3% meat extract, 0.2% soluble starch, 0.5%
139 peptone and 1.5% of agar. The starch agar plate was seeded with individual
140 colonies of *E. coli* WDHA/pAIDA-amyA as well as a negative control *E. coli* WDHA
141 and incubated for 48 h at 37°C. Subsequently, the plate was flooded with iodine
142 reagent (0.01 M I₂-KI solution) and washed with 1 M NaCl.

143 **2.4. Enzymatic reaction**

144 100 mL of LB medium with 200 μ g/mL of ampicillin were inoculated with *E. coli*
145 WDHA or WDHFP. Cells were cultured at 31°C and 180 rpm until an optical
146 density at 600 nm (O.D._{600nm}) of 1 was reached. The cells were centrifuged and
147 washed twice with reaction buffer. The enzymatic reaction was carried out in
148 triplicate, with a biomass O.D._{600nm} of 10 and using 1% soluble starch as substrate.
149 The reactions were stopped by centrifugation at 13,000 rpm for 5 min and the
150 supernatant was used to measure the enzymatic activity.

151 **2.5. Temperature and pH effect on enzyme activity**

152 The effect of temperature on the enzyme activity was determined by incubating the
153 cells in acetate buffer pH 5.5 containing 1% starch and 5 mM CaCl₂ at
154 temperatures ranging from 30 to 70°C for 30 min with vigorous shaking. The
155 enzyme activity was then measured using the 3,5-dinitrosalicylic acid DNS method
156 [12]. Effect of pH on the amylase activity was determined at different pH (3.5-6)
157 using the universal Britton and Robinson's buffer (50 mM phosphoric acid, 50 mM

158 boric acid and 50 mM acetic acid), 1% starch and 5 mM CaCl₂ at 55°C for 30 min.
159 A control without cells was used as a negative control.

160 **2.6. Determination of enzyme thermostability**

161 The thermal stability of the α-amylase activity was determined by measuring the
162 final activities of the enzyme after 15 to 60 min of incubation in acetate buffer pH
163 4.5 and temperature ranging from 55 to 80°C with and without 5 mM CaCl₂.

164 **2.7. Effect of calcium on enzyme activity**

165 To evaluate if the calcium influences the α-amylase activity, cells were incubated in
166 Britton and Robinson's buffer pH 4.5 at 55°C for 30 min with calcium chloride
167 concentration ranging from 0 to 25 mM and vigorous shaking. The negative
168 controls were also evaluated. The activity assayed in the absence of calcium was
169 recorded as 100%.

170 **2.8. Determination of total reducing sugars**

171 The α-amylase activity was determined by measuring the reducing sugars released
172 during starch hydrolysis by DNS method [12]. The reaction mixture contained 50 µl
173 of supernatant from centrifuged samples and 150 µl of DNS reagent, the reaction
174 mixture was boiled for 5 min at 100°C and stopped by cooling to room temperature.
175 The absorbance was measured at 595 nm. Glucose served as the calibration
176 standard for total reducing sugar determination. 1 U was defined as the amount of
177 enzyme that releases 1 µmol of reducing sugars per minute and for the amylase
178 specific activity as one unit of amylase activity per mg of *E. coli* cells.

179 **2.9. Kinetic parameters calculation**

180 The maximum velocity (V_{max}) and the Michaelis constant (K_m) were calculated
181 using the standard activity assay with different substrate concentration (0-3% w/v
182 soluble starch) in Britton and Robinson's buffer (pH 4.5) at 55°C. Kinetic constants
183 (K_m and V_{max}) were calculated by the method of Lineweaver-Burk using standard
184 linear regression techniques.

185 **2.10. Hydrogen, succinate and ethanol production by the *E. coli* using** 186 **soluble starch as substrate.**

187 To conduct the hydrogen production by the *E. coli* WDH strains carrying the
188 pAIDA-amyA plasmid using starch as carbon source, pre-inoculum was grown in
189 LB medium (10 g/L peptone, 5 g/L Yeast extract, 5 g/L sodium chloride)
190 supplemented with 200 µg/mL of ampicillin under aerobic conditions at 31°C for 16
191 h. Cells were harvested, centrifuged at 6000 rpm 10 min, washed and inoculated
192 into 120 mL anaerobic serological bottles containing 110 mL of medium B (12.5
193 mg/L $Na_2MoO_4 \cdot 2H_2O$, 15 mg/L $MnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 8H_2O$ 3 mg/L, 75 mg/L $ZnCl_2$,
194 4500 mg/L $NH_4H_2PO_4$, 11,867 mg/L Na_2HPO_4 , 125 mg/L K_2HPO_4 , 100 mg/L
195 $MgCl_2 \cdot 6H_2O$, 25 mg/L $FeSO_4 \cdot 6H_2O$, 5 mg/L $CuSO_4 \cdot 5H_2O$) [13], 0.5 g/L $CaCl_2$, 1
196 mL/L trace elements solution (ref), 1 g/L yeast extract (Difco), 1 g/L glucose and 10
197 g/L soluble starch. The cultures were started at pH 7.5 and were incubated at 31°C
198 and 180 rpm. The experiments were carried out in triplicate.

199 **2.11. Analytical methods**

200 The amount of hydrogen produced was measured by the acidic water
201 displacement method in an inverted burette connected to serological bottles with

202 rubber and a needle. The hydrogen percentage on the biogas was determined by
203 gas chromatography using a thermal conductivity detector (Agilent Technologies
204 Wilmington, DE, USA) as described elsewhere [10]. Samples of 1 mL were taken
205 at different times during fermentation, and then cells were separated by 5 min
206 centrifugation at 13000 rpm. The supernatants were filtered through a 0.22 μ m
207 membrane (Millipore, Bedford, Massachusetts, USA) [14]. Concentrations of
208 soluble metabolites such as succinic acid, lactic acid, acetic acid, formic acid and
209 ethanol were analyzed by High Performance Liquid Chromatography (HPLC,
210 Infinity LC 1220, Agilent Technologies, Santa Clara CA, USA) using a Refraction
211 Index Detector, a column Phenomenex Rezex ROA (Phenomenex Torrance, CA,
212 USA) at 60°C, and 0.0025 M H₂SO₄ as mobile phase at 0.5 mL/min. An O.D._{600nm}
213 of 1.0 was equivalent to 0.37 g (dry cell weight, DCW) cells/L. Total sugar
214 concentrations were measured using the phenol-sulfuric acid method [15].

215 **3. Results and discussion**

216 **3.1. Design of AIDA system**

217 The fusion gene (Fig. 1) consists of 5' to 3' as follows: *Sma*I restriction site, 5'
218 homologous recombination arm with target to the *frdABCD* gene, *Eco*RI restriction
219 site, promoter of the *gapA* gene of *E. coli*, *Nde*I restriction site, CtxB protein signal
220 peptide, *Asc*I restriction site, passenger gene *amyA* that encodes the α -amylase
221 from *B. megaterium*, *Xho*I restriction site, linker and β -barrel of the AIDA
222 autotransporter of *E. coli*, *Bam*HI restriction site, Rho Independent Terminator and
223 *Sma*I restriction site. The cassette was inserted into the pUC57 plasmid at the

224 *EcoRV* restriction site. The resulting plasmid was called pAIDA-amyA and has a
225 size of 5934 bp.

226 **3.2. Detection of amylolytic activity on plate**

227 An initial plate assay was performed to determine whether the transformants
228 gained amylolytic activity. The amylolytic activity was observed by a halo formation
229 on agar plate. Cells carrying the plasmid pAIDA-amyA or the strain without it as a
230 control were inoculated on a plate of medium containing soluble starch. After
231 incubation for two days at 37°C, the plate was stained with iodine solution. The
232 result shown in figure 2, demonstrated that the cells harboring the plasmid pAIDA-
233 amyA hydrolyzed starch and produced a halo strictly around the colony, while no
234 halo formation was observed around the control cells. This assay indicated that the
235 former cells presented amylolytic activity due to the expression of the α -amylase
236 AIDA system.

237 **3.3. Effect of temperature and pH on enzyme activity**

238 The effect of temperature on the immobilized α -amylase activity was determined by
239 assaying enzyme activity at different temperatures in a range of 30 to 70°C (Fig.
240 3A). The gradual increase in temperature from 30 to 50°C increased the activity of
241 the α -amylase until 55 °C where the maximum activity was achieved. The activity
242 at 50 and 60°C showed over 70% of the maximum activity, however, a further
243 increase in temperature caused a detrimental effect on the activity, in spite of that,
244 the anchored- α -amylase remain active until 70°C. The optimum temperature of
245 amylase in this study was similar to other bacterial α -amylase such as *B.*
246 *stearothermophilus* or *Lactobacillus manihotivorans* LMG 18010 [16]. On the other

247 hand, the influence of pH on the α -amylase activity was evaluated at various pH
248 values in a range of 3 to 6.5. As shown in figure 3B, the enzyme had the maximum
249 activity at 4.5 and α -amylase activity declined rapidly at pH below 5.0 showing only
250 about 30% activity was retained at pH 6. The maximum α -amylase activity at
251 temperature (55°C) and optimum pH (4.5) was 60.17 U with the maximum specific
252 α -amylase activity of 162.63 U/g. The optimum pH of α -amylases produced by
253 several bacterial sources has been reported, including *Bacillus sp*, and they show
254 a variety of pH profiles [17]. Being, the optimal pH of α -amylases in a range from 2
255 to 12 [17]. The maximum activity of most of these α -amylases is in the range of pH
256 6.0-8.0 [18-20] or pH 5.0-7.0 [21], but also has been reported α -amylases with
257 maximum activity at low pH such as those produced by *Bacillus sp.* KR-8104 [22].
258 Acidic, neutral and alkaline α -amylases are suited to different industrial
259 applications. Since optimum activity is obtained at low pH values, this enzyme is
260 highly attractive for industrial process, because several industries applications take
261 place at low pH values. Majority of the α -amylases are unstable at low pH and the
262 liquefaction step in the starch process is currently constrained to operate at pH 5.8-
263 6.2 and natural pH of starch slurry is generally around 4.5 (which is the optimum
264 pH of the enzyme in this study). The extreme conditions required for such
265 pretreatment necessitate the use of an enzyme that is resistant to low pH [23].
266 Since both, the prior and post process steps take place at pH 4.5, therefore, if the
267 α -amylase is stable and active at low pH values one can omit the pH adjustment
268 steps, which is very important in the processing [22].

269 **3.4. Thermostability of the α -amylases**

270 To determine the thermostability of the immobilized enzyme, cells were
271 preincubated at different temperatures (55 to 80°C) with and without 5 mM CaCl₂
272 at different time intervals (15 to 60 min). The reaction mixture without calcium
273 decreased in general around 60% of the maximum relative activity (Fig. 4). When
274 the enzyme was preincubated at 55°C for 60 min of incubation, the activity
275 decreased only 20% in the presence of calcium, interestingly the enzyme without
276 calcium after 60 min of incubation decreased 60% of the maximum activity. At
277 60°C with calcium we observed a decreased of almost 60% after 60 min of
278 incubation. On the other hand, at 60°C without calcium we observed a decreased
279 of almost 60% of the activity after 15 min of incubation. In addition, the α-amylase
280 activity was almost inactivated at 80°C after 60 min of incubation showing a
281 remaining activity of 20%. All these findings indicated that Ca²⁺ ions are important
282 for the folding and stability of the enzyme. It is known that the Ca²⁺ ion strongly
283 influences the α-amylase activity, the positive effect of this metal ion on the
284 thermostability of enzymes has been shown by several authors including on the α-
285 amylases from *B. licheniformis* and *Pyrococcus furiosus* [24, 25]. Most of the α-
286 amylases are reported to have one or two intrinsic Ca²⁺ ions. Secondary calcium
287 binding site have also been reported, which enhanced the thermostability [23]. For
288 instance, Saboury [26], reported the presence of different secondary binding sites
289 for calcium in α-amylase of *B. amyloliquefaciens*, which were responsible for
290 stabilization of the enzyme against thermal denaturation. The stabilizing effect of
291 Ca²⁺ on thermostability can be explained due to the salting out of hydrophobic
292 residues by Ca²⁺ in the protein, thus, causing the adoption of a compact structure
293 [27].

294 **3.5. Effect of calcium on α -amylase activity**

295 The effect of calcium concentration was also evaluated (Fig. 5), the presence of 5
296 mM of CaCl_2 significantly increased the activity almost 50% compared to the
297 control without calcium ($p < 0.05$) and interestingly at 25 mM of CaCl_2 compared to
298 the concentration of 5 mM the relative activity of α -amylase significantly
299 increased 20% ($p < 0.05$). This increase can be explained due to the fact that most
300 of the α -amylases are metal ion-dependent enzymes, such as Ca^{2+} , and it has
301 been reported that the addition of Ca^{2+} ions increases the α -amylase activity, as the
302 alkaliphilic *Bacillus sp.* ANT-6 [23, 24].

303 **3.6. Kinetic parameters of α -amylase**

304 The kinetic parameters (K_m and V_{max}) of α -amylase with soluble starch as
305 substrate were determined by the inverse reciprocal of Lineweaver-Burk. The K_m
306 and V_{max} values were 5.8 mg/mL and 0.0106 mg/mL/min respectively (Fig. 6). So
307 far there are no reports of catalytic constants of α -amylases for whole-cell
308 biocatalysis, but there are reports of α -amylases anchored to nanoparticles or free
309 α -amylases. The K_m value for starch of the α -amylase of this study was within the
310 range of many amylases reported (Table 2) [16].

311 **3.7. Production of hydrogen, succinate and ethanol from soluble starch**

312 To evaluate the effectiveness of the α -amylase-AIDA display system on the
313 production of hydrogen succinate and ethanol by the *E. coli* strain WDHA/pAIDA-
314 amyA, a set of batch cultures was carried out. Cells at an initial biomass
315 concentration of 0.037 g/L were cultivated anaerobically with 10 g/L soluble starch

316 and 1 g/L of glucose as carbon source. The results shown that cells carrying the
317 plasmid pAIDA-amyA, were able to utilize the starch in the medium as carbon
318 source. As it can be seen in figure 7A, *E. coli* WDHA/pAIDA-amyA proliferated to
319 reach a maximum biomass of 0.92 g/L at the 24 h with an adaptation phase of 12
320 h. During this lag phase, it is assumed that the WDHA/pAIDA-amyA strain used the
321 small amount of glucose (1 g/L) available in the medium to support the cellular
322 growth and hence the α -amylase synthesis. After this phase, the cell surface-
323 anchored α -amylases reacted hydrolyzing the starch available releasing the
324 reducing sugars needed for the cellular growth. Also, with the concomitant cellular
325 growth, hydrogen and several soluble metabolites were produced. Figure 7A
326 reveals that hydrogen production began at the 12 h and increased as the total
327 sugar concentration decreased to reach a maximum hydrogen production of
328 1,056.06 mL/L after 82 h of fermentation and a hydrogen production rate of 26.8
329 mL/L/h. *E. coli* WDHA strain has deleted the *hycA* gene which encodes for the
330 negative regulator for the formate regulon [10], as well as the deletion of *ldhA* gene
331 which encodes for a NAD-linked lactate dehydrogenase enzyme responsible for
332 the lactic acid production [28]. In this work, the analysis of the metabolic products
333 formed during the fermentation (Fig. 8) shows that the less abundant product was
334 lactic acid (0.7 g/L) while formic acid was not detected in the medium. On the other
335 hand, succinic acid was the main soluble metabolite (6.8 g/L), followed by the
336 production of acetic acid (1.7 g/L) and ethanol (1.3 g/L). Since, succinic acid
337 production competes with the hydrogen and ethanol production, a WDH *frdD* *pta*
338 mutant was generated and transformed with the pAIDA-amyA plasmid
339 (WDHFP/pAIDA-amyA) to improve the flux of pyruvate to the hydrogen and ethanol

340 production without acetic acid formation. *frdD* gene encodes for the fumarate
341 reductase [29], whereas *pta* gene encodes for the phosphate acetyltransferase
342 which is the first enzyme of the acetate pathway [30]. In figure 7B, it is noted that
343 the hydrogen production and cellular growth by WDHFP/pAIDA-amyA strain
344 presented a longer lag phase compared to the WDHA/pAIDA-amyA strain, this
345 behavior can be attributed to the *pta* gene deletion. Chang et al. [30] reported the
346 same effect in their study, where a *E. coli* JP231 strain which has deleted the *pta*
347 gene showed a slower growth rate on various carbon sources compared to those
348 of the wild type strain, the authors attributed this conduct to the perturbation of the
349 pyruvate and acetyl CoA fluxes in the mutant. It has been previously described
350 that when *pta* is deleted in *E. coli*, pyruvate accumulates in the cell, consequently,
351 the pyruvate accumulation will lower the Phosphoenol pyruvate (PEP)/pyruvate
352 ratio, which results in a lowered substrate consumption of the sugars transported
353 by the phosphotransferase system (PTS) which requires PEP [30-32]. After 35 h,
354 WDHFP/pAIDA-amyA started the hydrogen production and continued until the 116
355 h of fermentation, where the maximum hydrogen production of 1,689.68 mL/L was
356 achieved with a production rate of 33.14 mL/L/h. This hydrogen production is 60%
357 higher than the one attained by WDHA, as well as the production rate. In figure 7B
358 it is observed that the total sugar concentration decreases with the simultaneous
359 hydrogen production increment, hence this data confirms the effectiveness
360 hydrolysis of starch in the medium and its use as carbon source. Regarding to the
361 metabolites produced in the fermentation, in figure 8 it can be observed that the
362 metabolite distribution changed with respect to the profile showed by
363 WDHA/pAIDA-amyA. In this case, the succinic acid reached a concentration of 2.3

364 g/L, whereas acetic acid 2.2 g/L, since *frdD* and *pta* genes were deleted from
365 WDHFP/pAIDA-amyA we expected to observe no succinic and acetic acid
366 production, however, there are alternative routes for the production of these acids
367 in *E. coli*, which involves the glyoxylate shunt where succinic acid is formed from
368 acetyl Co-A by the isocitrate lyase (*aceA*), and acetic acid can be produced directly
369 from pyruvate by the pyruvate oxidase (*poxB*) [33]. Furthermore, the ethanol
370 production reached a concentration of 2.8 g/L which is approximately twice the
371 production achieved by WDHA/pAIDA-amyA. The results showed that the cell-
372 surface α -amylase synthesis by the AIDA system is an effective alternative for the
373 use of a complex polysaccharide such as starch by *E. coli* without the requirement
374 of a saccharification pre-treatment. Moreover, the ability of *E. coli* WDHA and
375 WDHFP carrying the pAIDA-amyA plasmid for the of biofuels production like
376 hydrogen and ethanol, as well as succinic acid which is an important building block
377 in chemical industry shows the potential for these strains to be applied in industrial
378 processes using starch-rich biomass.

379 **4. Conclusions**

380 In this work, we combined a cell surface display system and metabolic engineering
381 for the overproduction of hydrogen, succinate and ethanol using a cheap and
382 abundant polysaccharide such as starch. The expression of α -amylase enzymes
383 on the cell surface of metabolic engineered *E. coli* strains using the AIDA
384 autodisplay system is a novel tool for the hydrolysis of starch and agroindustrial
385 wastes, with this system it is possible to reduce the costs of the production of
386 enzymes and the efficiency of the process.

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392 6. References

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489 **Table 1. *E. coli* strains and primers used in this study**

Strains Name	Genotype description	Reference
WDH	Escherichia coli (lac ⁺ , gal ⁺ , F ⁻ , λ ⁻ , IN (rrnD-rrnE)1, rph-1) Δ <i>hycA</i>	[10]
WDHA	WDH Δ <i>ldhA</i>	This work
WDHFP	WDH Δ <i>frdD</i> Δ <i>pta</i>	This work
Primers Name	Sequence (5' to 3')	Reference
ldhA-FCK	TCGCCATCGGTCTACGGGC	[9]
ldhA-RCK	CATAACACCATTAGCGAAAT	
frdD-FCK	TCTGGTTTCCATACAA	
frdD-RCK	TTAGATTGTAACGACACCAATC	
pta-FCK	CTGCACGTTTCGGCAAATCT	This work
pta-RCK	ATTGCGGACATAGCGCAAAT	This work

490

491 **Table 2. Kinetic parameters of α -amylases by diverse types of**
 492 **immobilizations**

Biocatalysis	Autotransporter	Vmax	Km	Reference
Immobilization	Combi-MOF	6.9881±0.14 μmol/min	0.5889±0.053 μM	[34]
Immobilization	Biopolymer	855 U/mg	15.03 mM	[35]
<i>Bacillus circulans</i> GRS 313	Free enzyme	68.97 U/min	11.66 mg/mL	[36]
Whole cell catalysis	AIDA	0.0106 mg/mL/min	5.8 mg/mL	This work

493

494 **Figure captions**

495 **Figure 1.** Structure of autodisplay AIDA system. It consists of a signal peptide
496 (derived from CtxB), followed by the gene encode for α -amylase. Subsequently,
497 the linker and the β -barrel.

498 **Figure 2.** A starch agar plate showing α -amylase activity by (A) *E. coli*
499 WDHA/pAIDA-amyA strain. (B) Negative control *E. coli* WDHA. The strains were
500 cultured on a starch agar plate for 48 h at 37°C.

501 **Figure 3.** Effect of temperature and pH on α -amylase activity. (A) Temperature
502 dependence of α -amylase. (B) Relative activity profile of α -amylase at different pH
503 conditions. Values are expressed as mean of percentage of relative activity. Bars
504 represent means \pm standard deviations for three replicates.

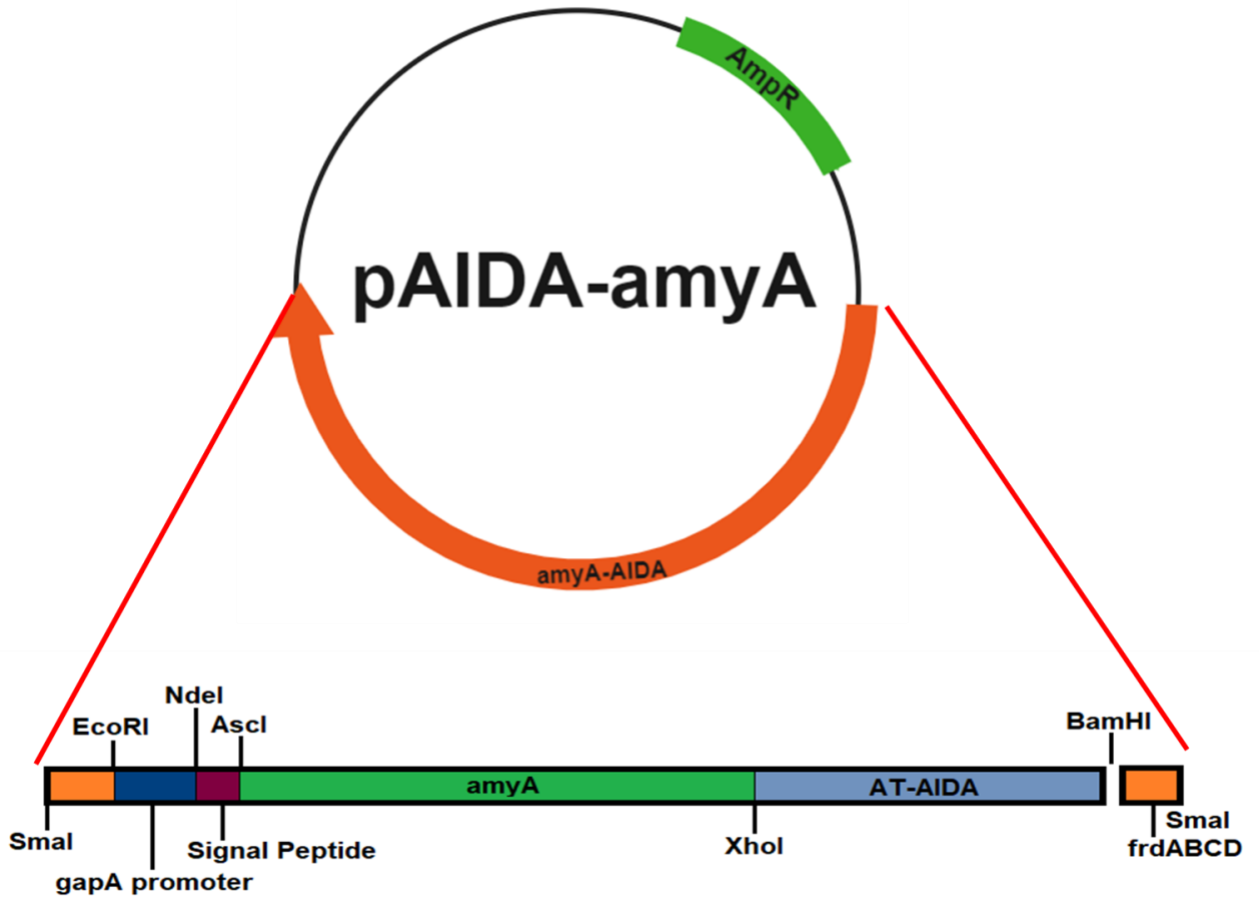
505 **Figure 4.** Thermal stability of α -amylase. For the determination of the
506 thermostability of α -amylase, the enzyme was pre-incubated at different
507 temperatures for 15 to 60 min in the presence or absence of 5 mM CaCl₂ and the
508 remaining activity was determined incubating the enzyme at optimum temperature
509 (55°C for 30 min). Bars represent means \pm standard deviations for three replicates.

510 **Figure 5.** Effect of CaCl₂ concentration on α -amylase activity. Continue line
511 represent reaction mixture with 5 mM CaCl₂; dotted line represents the reaction
512 mixture without CaCl₂ n=3 ($p<0.05$)

513 **Figure 6.** Michaelis-Menten type plot of α -amylase hydrolysis rate at different
514 starch concentration.

515 **Figure 7.** Kinetics of hydrogen production, cell growth and total sugar consumption
516 by (A) WDHA/pAIDA-amyA and (B) WDHFP/pAIDA-amyA using 10 g/L of starch
517 and 1 g/L of glucose incubated at 31°C, initial pH 7.5 and 180 rpm.

518 **Figure 8.** Fermentative metabolites produced at the end of each fermentation.



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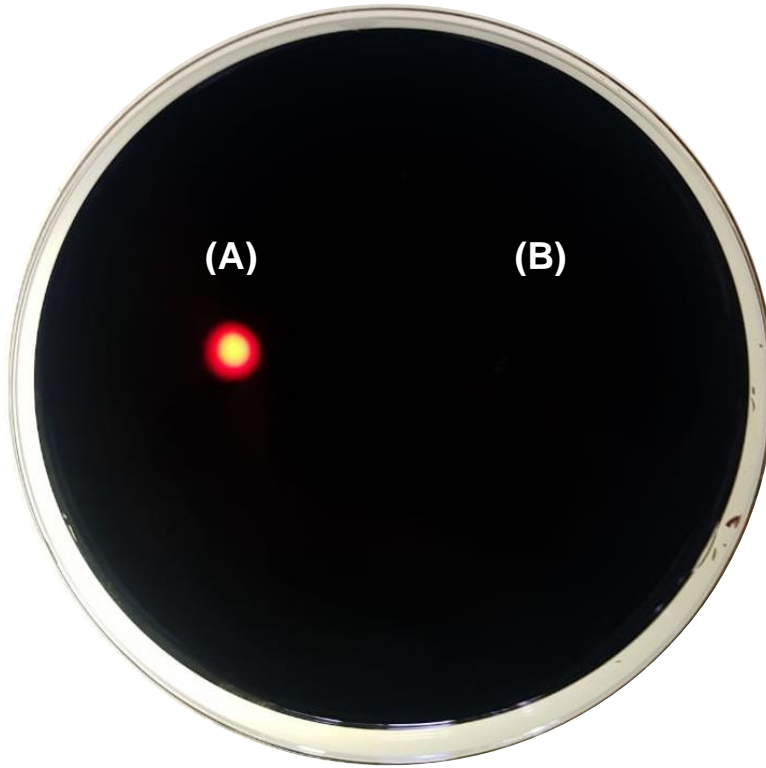
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Fig. 1

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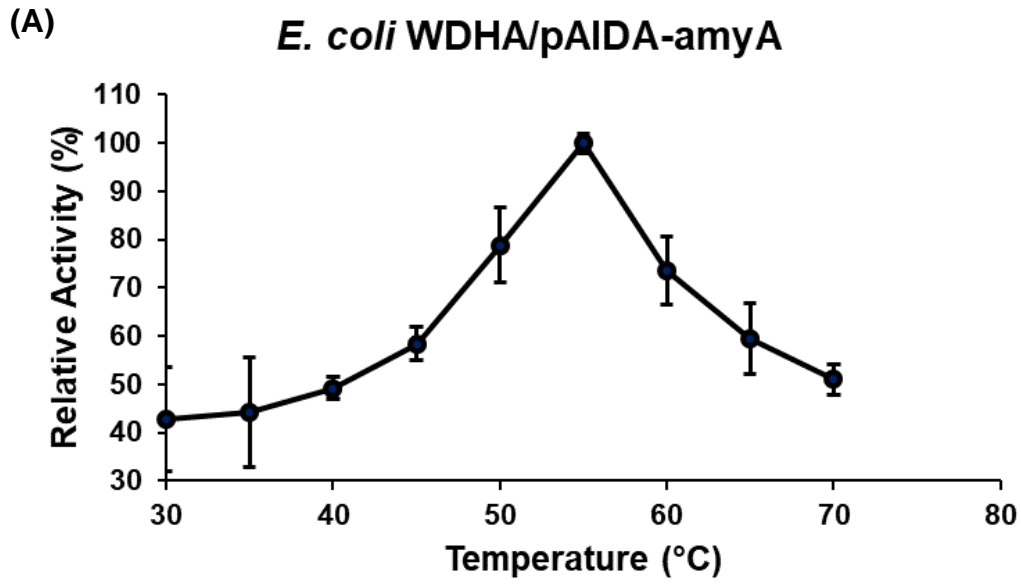


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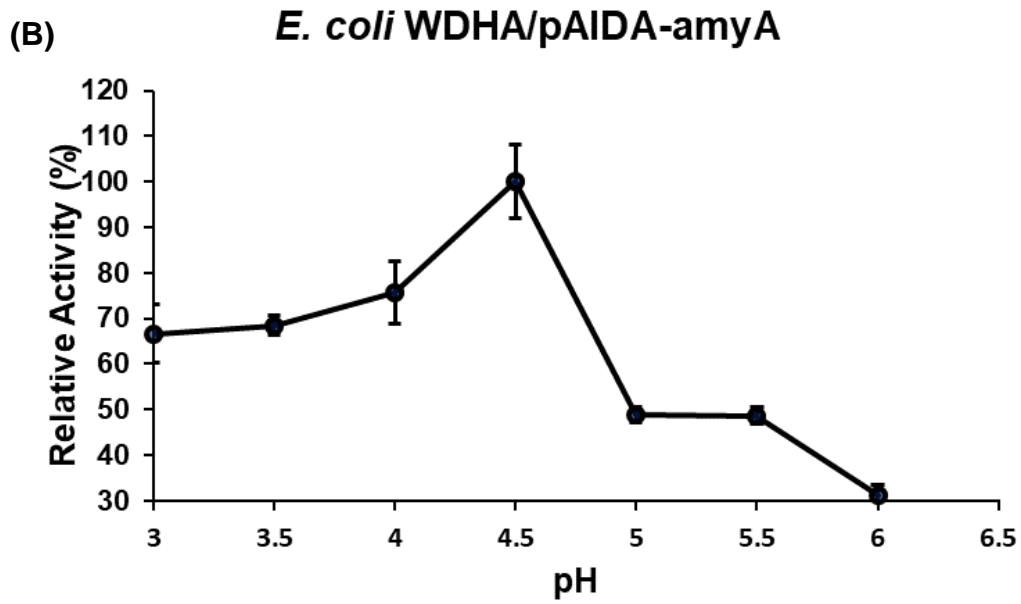
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Fig. 2



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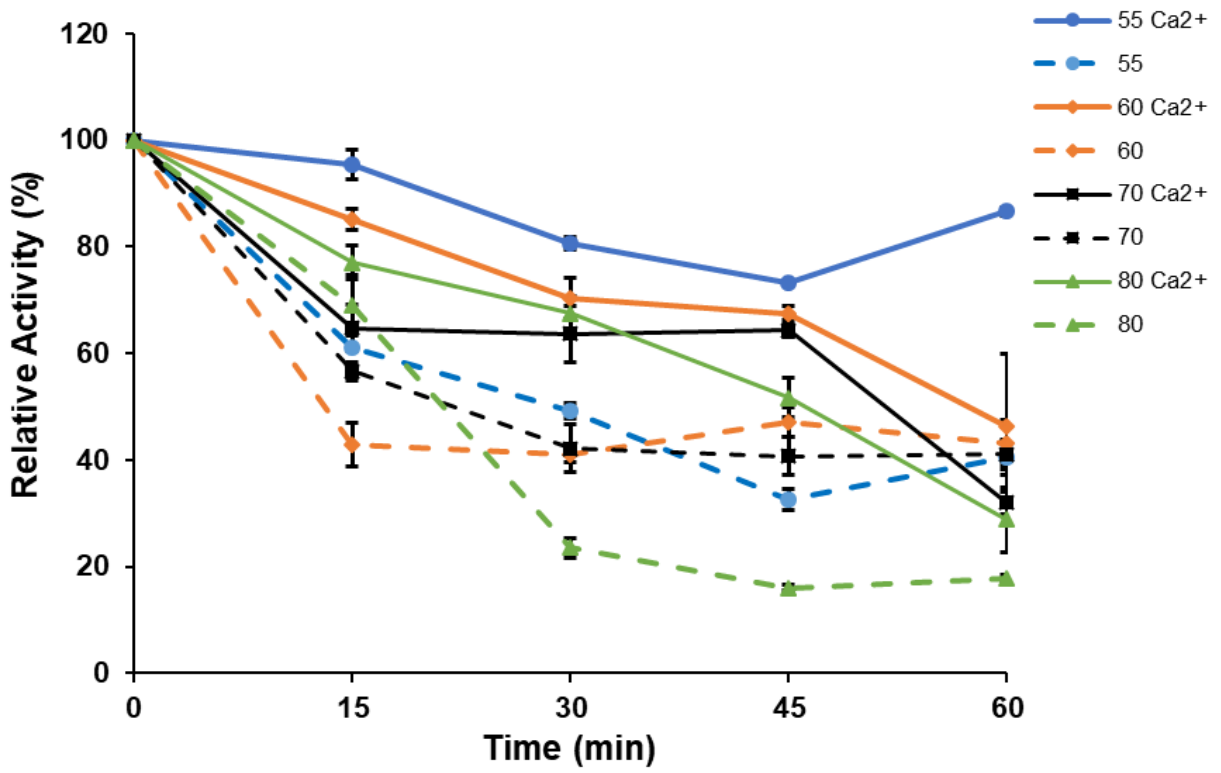
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Fig. 3

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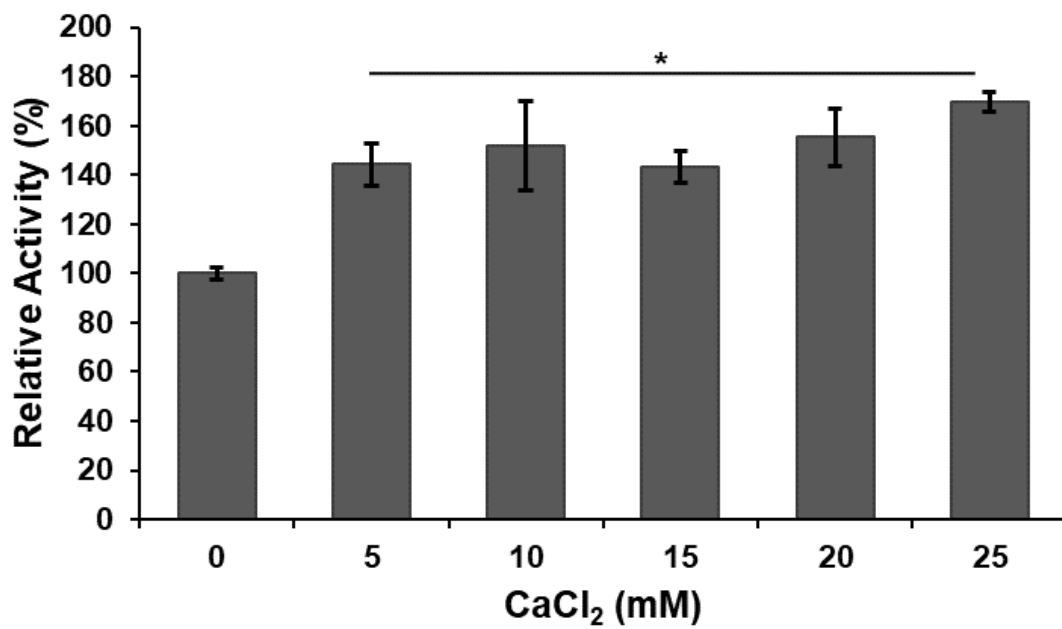
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Fig. 4

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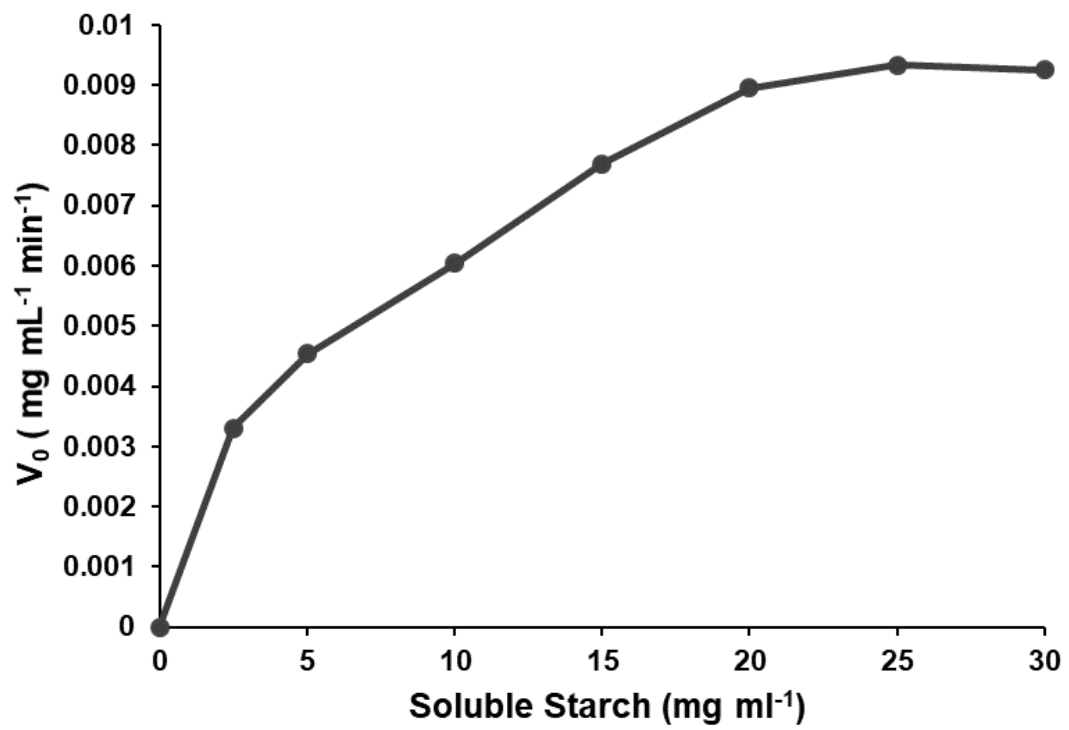


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Fig. 5

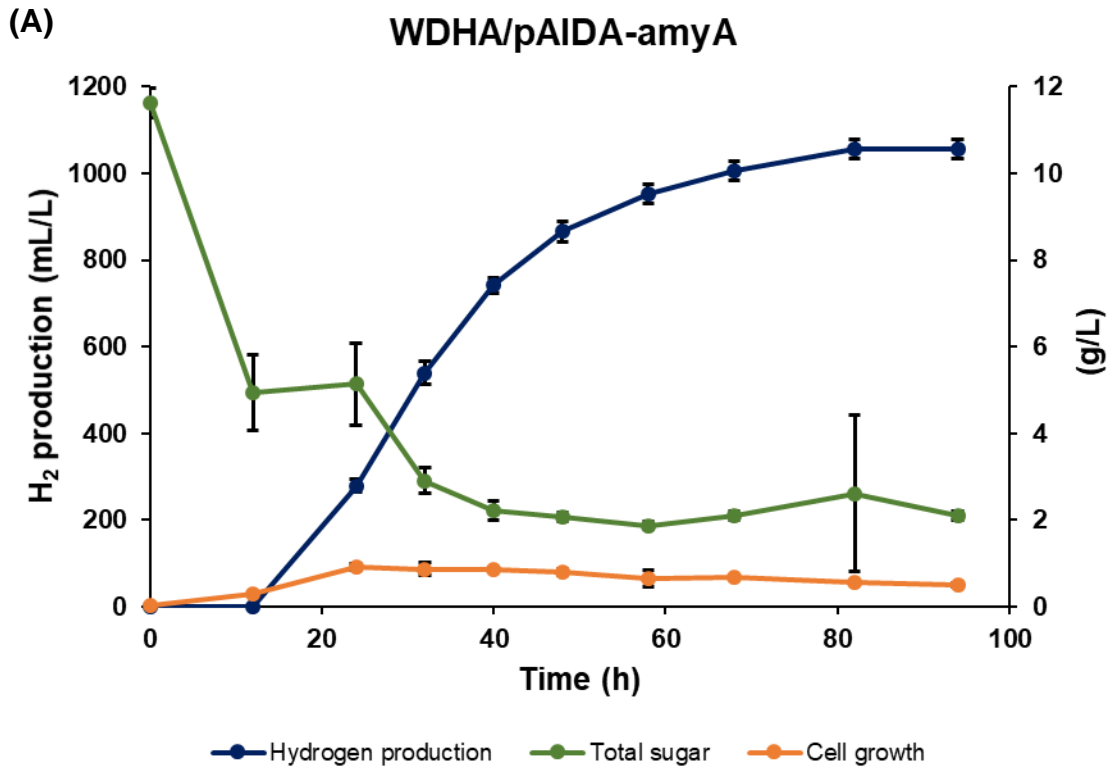
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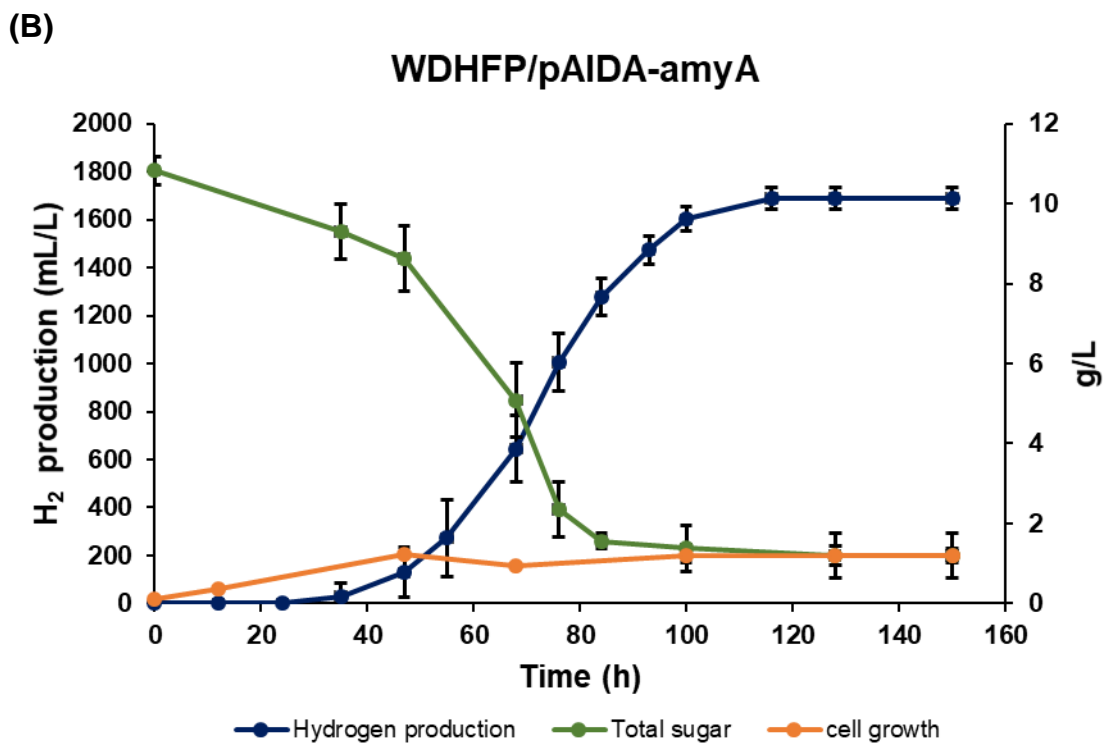
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Fig. 6



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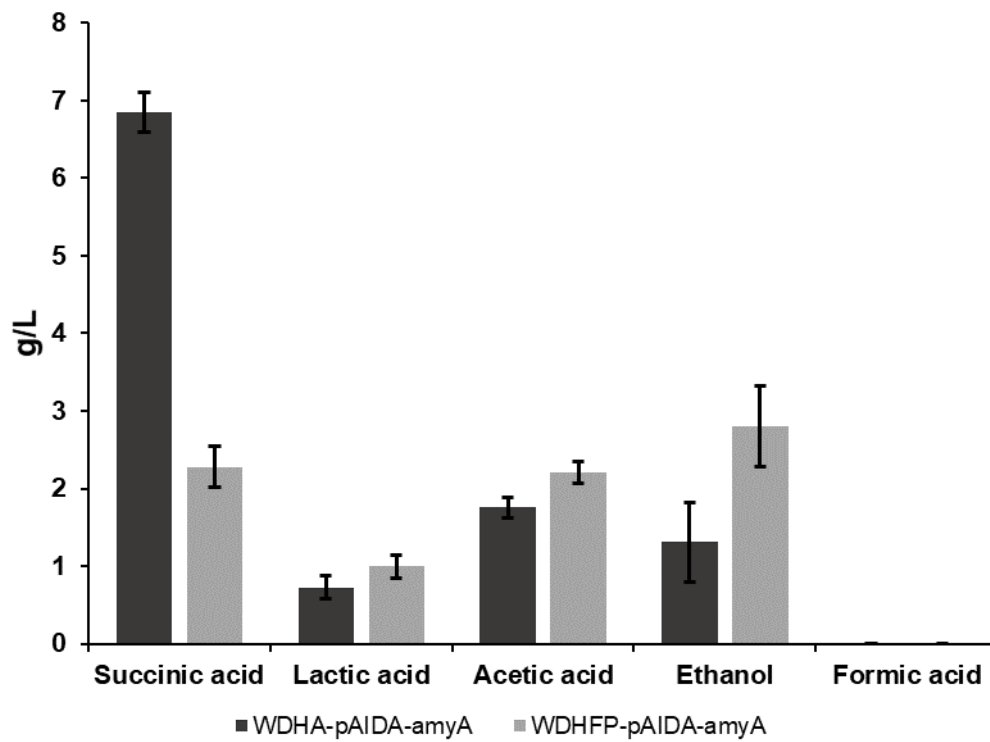


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Fig.7

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Fig.8