

# *Microbial production of D-lactic acid from dried distillers grains with solubles*

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1 Research Article

2 **Microbial production of D-lactic acid from Dried Distillers Grains with Solubles (DDGS)**

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16

17 **Keywords:** D-Lactic acid, DDGS, Separate hydrolysis and fermentation (SHF), Simultaneous  
18 saccharification and fermentation (SSF)

19

20 **Abbreviations:** DDGS, Dried Distillers Grains with Solubles; SHF, Separate hydrolysis and  
21 fermentation; SFF, Simultaneous saccharification and fermentation

## 22 **Practical application**

23 D-lactic acid is an important monomer for the synthesis of biodegradable plastics, where  
24 mixtures of poly-D-lactic acid (PDLA) and poly-L-lactic acid (PLLA) generate heat stable  
25 polylactic acid (PLA) suitable for high temperature processing applications. However, research  
26 on D-lactic acid is limited whereas the optical purity of lactic acid is one of the crucial factors  
27 towards the production of highly crystalline PLA. This study demonstrated the potential of  
28 producing D-lactic acid with high optical purity from alkaline pretreated DDGS, a cheap and  
29 renewable resource produced in large amounts by the bioethanol industry. The SSF approach  
30 resulted in faster and higher production of D-lactic acid, with a higher conversion yield of  
31 glucose to lactic acid (84.5%) compared to conventional SHF (72.9%). The SSF process  
32 demonstrated good scalability as similar fermentation characteristics were obtained between the  
33 small (100 ml) and larger scale (2-L) fermentation vessels.

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44 **Abstract**

45

46 D-Lactic acid production is gaining increasing attention due to the thermostable  
47 properties of its polymer, poly-D-lactic acid (PDLA). In this study, *Lactobacillus coryniformis*  
48 subsp. *torquens*, was evaluated for its ability to produce D-lactic acid using Dried Distillers  
49 Grains with Solubles (DDGS) hydrolysate as the substrate. DDGS was first subjected to alkaline  
50 pretreatment with sodium hydroxide to remove the hemicellulose component and the generated  
51 carbohydrate-rich solids were then subjected to enzymatic hydrolysis using cellulase mixture  
52 Accellerase® 1500. When comparing separate hydrolysis and fermentation (SHF) and  
53 simultaneous saccharification and fermentation (SSF) of *L. coryniformis* on DDGS hydrolysate,  
54 the later method demonstrated higher D-lactic acid production (27.9 g/l, 99.9% optical purity of  
55 D-lactic acid), with a higher glucose to D-lactic acid conversion yield (84.5%) compared to the  
56 former one (24.1 g/l, 99.9% optical purity of D-lactic acid). In addition, the effect of increasing  
57 the DDGS concentration in the fermentation system was investigated via a fed-batch SSF  
58 approach, where it was shown that the D-lactic acid production increased to 38.1 g/l and the  
59 conversion yield decreased to 70%. In conclusion, the SSF approach proved to be an efficient  
60 strategy for the production of D-lactic acid from DDGS as it reduced the overall processing time  
61 and yielded high D-lactic acid concentrations.

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## 66 **1 Introduction**

67 Lactic acid ( $C_3H_6O_3$ ) is considered one of the most useful chemical products and has  
68 attracted a great attention worldwide due to its widespread applications in food, chemical,  
69 cosmetic, textile and pharmaceutical sectors. It has also emerged into the bioplastics industry,  
70 where lactic acid serves as the building block for polylactic acid (PLA) synthesis. PLA is a  
71 biodegradable polymer that holds great potential in replacing petroleum-based polymers.  
72 Because of its degradability and biocompatibility, PLA is extensively used in the biomedical  
73 field as a surgical suture, drug-delivery material and bone fixation material [1, 2]. In addition,  
74 PLA received a Generally Recognized as Safe (GRAS) status from the US Food and Drug  
75 Administration (FDA) in 2002, which allowed the expansion of its applications within the food  
76 industry. PLA can be utilised as a food contact material, e.g. for the production of cutlery, cups,  
77 plates and containers, or as food packaging material [2, 3]. At the moment, the PLA market  
78 demand accounts for 11.4% of the total bioplastic production worldwide, and is equal to  
79 approximately 180,000 metric tonnes per year. In addition, the PLA demand is estimated to grow  
80 significantly, by 28% per year until 2025, as a result of the expansion of the bioplastics market  
81 [4, 5].

82 PLA can be manufactured by utilising either the D- or L- forms of lactic acid, or its  
83 racemic mixture [6, 7]. Poly-L-lactic acid (PLLA) polymer has a low melting point (180 °C) and  
84 low crystallisation ability [8]. On the other hand, polymer blends of purified PLLA and purified  
85 poly-D-lactic acid (PDLA) produce racemic crystals called stereo-complexes which have higher  
86 melting point (230 °C) and distortion temperatures, and as such, offer significant advantages for  
87 a number of applications such as high heat packaging materials [9-12]. The optical purity of

88 lactic acid is one of the crucial factors towards the production of highly crystalline PLA. To this  
89 end, the microbial lactic acid production route offers advantages compared to the chemical  
90 production route, as specific isomers of D- or L- lactic acid can be produced depending on the  
91 selected bacterial strain.

92 Over 90% of the commercially produced lactic acid is derived from microbial  
93 fermentation utilising glucose, sucrose or corn starch as carbon sources [13]. However, the  
94 relatively high cost of pure sugars has driven research on industrial fermentation towards the use  
95 of alternative resources, which can be obtained through the valorisation of cheap, renewable  
96 agricultural biomass [13-17]. Apart from no interference with food industry, the other advantages  
97 of utilising renewable sources is the possibility of producing cheaper fermentation medium at  
98 higher nutrient content to support bacterial growth. Specifically, agricultural residues such as  
99 corn stover [18, 19], rice bran [20], peanut meal [21], broken rice [22] and unpolished rice [23]  
100 have been studied as potential carbon sources for lactic acid production. However, the hydrolysis  
101 of biomass materials that are rich in cellulose/hemicellulose produces a mixture of sugar  
102 monomers such as glucose, xylose, mannose, arabinose and galactose. Most of the  
103 homofermentative D-lactic acid producers (i.e. *Lactobacillus* sp. and *Sporolactobacillus* sp.) are  
104 unable to ferment sugars other than glucose, thus, leave the non-utilised sugar to accumulate in  
105 the fermentation broth at the end of fermentation process [18, 19]. Besides, fermentation broths  
106 derived from renewable sources also contain a mixture of compounds, including a variety of  
107 sugars and proteins, degraded compound from pretreatment, polyphenols and organic acids, and  
108 thus require an effective downstream processing for the recovery of the lactic acid [24, 25].  
109 Several methods such as precipitation, extraction, crystallization, ion exchange, adsorption,

110 membrane filtration, distillation and nanofiltration can be used to recover lactic acid from  
111 fermentation broth [17, 25].

112 Agricultural biomass needs to be treated either chemically (with acid or alkali) or be  
113 enzymatically hydrolysed in order to be converted into fermentable sugars. The enzymatic  
114 approach is preferred to chemical hydrolysis, as the reactions are more specific and less  
115 hazardous [25, 26]. The overall production process can consist either of two steps operated  
116 sequentially, i.e. Separate Hydrolysis and Fermentation (SHF) or concurrently, i.e. Simultaneous  
117 Saccharification and Fermentation (SSF) [25]. In SHF, enzymatic hydrolysis and fermentation  
118 take place separately, and each process is conducted at its optimal conditions. The major  
119 disadvantage of SHF is that the accumulation of sugars after hydrolysis can reduce the activity of  
120 enzymes, particularly cellulase and  $\beta$ -glucosidase, by 60 – 75% [26-28]. In contrast, in SSF,  
121 enzymatic hydrolysis and fermentation process are carried out simultaneously, allowing for the  
122 direct assimilation of monomeric sugars by the microbial cells, thus reducing the risk of sugar  
123 accumulation in the medium. Additional advantages of SSF include shorter production times for  
124 the targeted product, higher production yields (% g product / g of substrate) and lower  
125 production costs due to the lower amount of energy and labor required [26, 29, 30].

126 Dried Distillers Grains with Solubles (DDGS) is a by-product of bioethanol production  
127 from wheat or corn, as well as of the distillery industry, and is currently used as animal feed due  
128 to its high protein (29 - 38%) and fibre content (40 - 46%) [31-33]. However, DDGS has to  
129 compete with other protein sources such as soybean meal and rapeseed meal within the animal  
130 feed market which are considered of a better quality [31]. Moreover, the possible high levels of  
131 mycotoxins (3-fold compared to the original sources, i.e. wheat or corn grains) in DDGS have  
132 become a concern for the farming industry [34]. In terms of fibre composition, DDGS is



133 primarily composed of cellulose and hemicellulose, which mainly consist of the  
134 monosaccharides glucose, xylose and arabinose as the main sugars. As DDGS is also  
135 characterised by a high fibre content, it could potentially be used as an alternative carbon source  
136 for lactic acid fermentation [35]. In commercial lactic acid production, glucose and corn starches  
137 have been widely used as substrates for fermentation. However, this is economically  
138 unfavourable as pure sugars have a higher economic value than the lactic acid produced [13, 36].

139 The main objective of this study was to develop a fermentation process for the production  
140 of optically pure D-lactic acid from wheat DDGS hydrolysates using *Lactobacillus coryniformis*  
141 subsp. *torquens*. Two fermentation approaches, SHF and SSF, were evaluated in terms of lactic  
142 acid yield, productivity and purity.

143

## 144 2 Materials and Methods

145

### 146 2.1 Microorganisms and culture conditions

147 *Lactobacillus coryniformis* subsp. *torquens* (DSM 20004) was obtained from the German  
148 Collection of Microorganisms and Cell Cultures (DSMZ). The stock of the bacterial culture was  
149 kept in mixtures of commercial MRS broth and glycerol and stored at -80 °C. Bacterial strains  
150 were cultivated in 250 ml Erlenmeyer flasks containing 50 ml MRS broth at 37 °C and 150 rpm  
151 agitation speed for 18 h, and were subsequently used as inoculum. The cell growth was  
152 monitored by optical density (OD) using a Biomate 3 UV/VIS Spectrophotometer (Thermo  
153 Spectronic, Rochester, NY) at 600 nm wavelength.

154

155 2.2 Raw material and enzyme

156 Dried Distillers Grains with Solubles (DDGS) was supplied from a bioethanol plant  
157 (Vivergo, Yorkshire, UK). It was ground using a coffee grinder (DeLonghi, Australia) into a fine  
158 powder (particle smaller than 0.85 mm) and stored at room temperature (25 °C) prior to alkaline  
159 pretreatment. The commercial cellulase mixture Accellerase® 1500 was kindly provided by  
160 Danisco US Inc. (Genencor, Leiden, Netherlands); it consisted of multiple enzyme activities  
161 including endoglucanase (2200 – 2800 CMC U/g),  $\beta$ - glucosidase (450 - 775 pNPG U/g),  
162 exoglucanase and hemicellulase. The enzyme was kept at 4 °C before use.

163

164 2.3 Growth of *L. coryniformis* in semi-defined MRS-based media

165 The microbial growth of *L. coryniformis* was initially studied in a 100 ml fermentation  
166 vessel containing 50 ml of deMan Rogosa Sharpe (MRS) as the basal medium [glucose, 16 g/l  
167 (commercially present in MRS media); casein peptone, 10.0 g/l; meat extract, 10.0 g/l; yeast  
168 extract, 5.0 g/l; tween 80, 1.0 g/l; potassium phosphate dibasic ( $K_2HPO_4$ ), 2.0 g/l; sodium acetate  
169 ( $CH_3COONa$ ) 5.0 g/l; di-ammonium hydrogen citrate ( $C_6H_6O_7$ ), 2.0 g/l; magnesium sulphate  
170 heptahydrate ( $MgSO_4 \times 7H_2O$ ), 0.2 g/l; and manganese (II) sulphate monohydrate ( $MnSO_4 \times$   
171  $H_2O$ ), 0.05 g/l]. The fermentation system consisted of a 100 ml glass vessel connected to a  
172 temperature controlled water bath (GD 120, Grant, Cambridge) set at 37 °C, a FerMac 260 pH  
173 controller (Electrolab, Hertfordshire) and a Stuart stirrer; no nitrogen or air addition was included  
174 in the system. The strain was inoculated at a similar starting OD (OD ~ 0.05) for all  
175 fermentations. Three fermentation runs were conducted: (i) with the pH controlled at pH 5, (ii)  
176 with the pH controlled at pH 6, and (iii) with uncontrolled pH. The maximum specific growth

177 rate ( $\mu_{\max}$ ) was calculated from the slope of the plot depicting the natural logarithm (ln) of the  
178 OD against time. Using the best pH for *L. coryniformis* growth, the strains were then cultivated  
179 at 37 °C in modified MRS basal medium (outsources carbon) supplemented with a single carbon  
180 source (20 g/l of glucose, xylose or arabinose) as well as mixed sugars (10 g/l glucose and 10 g/l  
181 xylose). For all fermentations, samples were taken at regular time intervals and analysed for cell  
182 growth by OD measurement, lactic acid and acetic acid concentration, residual sugar and optical  
183 purity of D-lactic acid (%), as described in Section 2.7.

184

#### 185 2.4 Alkaline pretreatment of DDGS

186 DDGS was pretreated with 5% (w/v) NaOH at 121 °C (~16 psi) for 15 minutes at 10%  
187 (w/v) DDGS loading. After pretreatment, the mixture was cooled down to room temperature and  
188 centrifuged at 17,105  $\times g$  (Heraeus Multifuge X3R, Thermo Fisher, USA) for 20 minutes at 4 °C.  
189 The obtained solids were extensively washed with distilled water until the pH reached around 8,  
190 and the pH neutralised with HCl (6 M) to a final pH between 5 - 5.5. The solids were frozen (-20  
191 °C), freeze-dried (VisTis Sentry 2.0, Warminster, PA) and stored in a closed container at room  
192 temperature (25 °C) until further use.

193

#### 194 2.5 Separate hydrolysis and fermentation (SHF) of DDGS hydrolysate

195 Alkaline pretreated DDGS solids (3.3 g) were hydrolysed with Accellerase® 1500 (5 ml)  
196 at a ratio of 1 ml enzyme : 0.33 g cellulose; the cellulose content of DDGS pretreated solids was  
197 approximately 50% w/w. The enzymatic hydrolysis was conducted at 50 °C for 24 h with 300  
198 rpm speed in a shaking incubator (SciQuip, Shropshire, UK), followed by heat inactivation at 95

199 °C for 10 minutes. The mixture was centrifuged at 17,105  $\times g$  for 20 minutes (4 °C) and the  
200 supernatant was collected and filter sterilised using 0.22  $\mu\text{m}$  sterile vacuum filter (EMD Milipore  
201 Stericup<sup>TM</sup>). 5 ml of sterile concentrated yeast extract (200 g/l) were then added aseptically into  
202 the 100 ml fermentation vessel (final concentration 20 g/l). *L. coryniformis* was inoculated into  
203 50 ml of DDGS hydrolysate at a similar starting OD of ~0.05. Lactic acid fermentation was  
204 carried out at 37 °C for 54 h; no nitrogen or air was passed through the fermentation medium.  
205 The pH of the cultures was maintained at 6 through the addition of NaOH (2 M). Samples were  
206 taken at regular time intervals and kept at -20 °C until further analysis.

207

## 208 2.6 Simultaneous saccharification and fermentation (SSF) of DDGS hydrolysate

209 The SSF experiments were conducted in a 100 ml fermentation vessel using the  
210 fermentation system described in Section 2.3; the pH was controlled at pH 5 at 37 °C. 3.3 g of  
211 alkaline pretreated DDGS were steam-sterilised inside the fermentation vessel by autoclaving the  
212 vessel at 121 °C for 15 minutes. After cooling, sterile distilled water and yeast extract (20 g/l)  
213 were added into the fermentation vessel. The SSF process was initiated by the addition of  
214 Accellerase® 1500 into the DDGS hydrolysate at a loading rate of 1 : 0.33 (ml enzyme : g  
215 cellulose), followed by inoculation with *L. coryniformis* at a starting OD of approximately 0.05.  
216 In certain runs, 1.1 g of pretreated DDGS (resulting in 11 g/l and 22 g/l of glucose, respectively)  
217 were aseptically added, with the aid of a portable Bunsen burner, when the glucose in the  
218 fermentation medium was depleted (fed-batch SSF); this was approximately after 24 h of  
219 fermentation. No nitrogen or air was passed through the fermentation medium. For all SSF  
220 experiments, samples were taken at regular time intervals and kept at -20 °C for further analysis.

221 The SSF process was also studied in a 2L stirred tank bioreactor (Biostat B, Sartorius,  
222 Germany) with 1.5 L working volume. 99 g of alkaline pretreated DDGS solids were added into  
223 the bioreactor and steam-sterilised by autoclaving at 121 °C for 30 minutes. After cooling the  
224 bioreactor, 1500 ml of sterile distilled water were added plus yeast extract (final concentration 20  
225 g/l). Accellerase® 1500 was added at a loading rate of 1:0.33 (ml enzyme : g cellulose), followed  
226 by the addition of *L. coryniformis* inoculum at a starting OD of ~0.05. The fermentation was  
227 carried out at 37 °C with an initial agitation speed of 250 rpm. The minimum dissolved oxygen  
228 (DO) level was kept at 20% by controlling automatically the stirrer speed and the pH was  
229 maintained at 5 with 5M NaOH and HCl. Antifoam 204 (10%, v/v, Sigma) was added to prevent  
230 foaming during fermentation.

231

## 232 2.7 Analytical methods

233 Sugars (xylotriose, xylobiose, glucose, xylose and arabinose) and organic acids (lactic  
234 acid and acetic acid) were analysed by high performance liquid chromatography (HPLC) in an  
235 Agilent Infinity 1260 system (Agilent Technologies, USA) equipped with an Aminex HPX-87H  
236 column (Bio-rad, Hercules, CA) at 0.6 ml/min flow rate, with 5 mM H<sub>2</sub>SO<sub>4</sub> as mobile phase. The  
237 temperature of the column was set at 65 °C and the sugars and organic acids were detected using  
238 a refractive index detector. The presence of D- and L-lactic acid in the fermentation broth was  
239 determined by using the D- and L-lactate dehydrogenase enzyme kit (K-DLATE 07/14,  
240 Megazyme, Ireland). The optical purity (%) of D-lactic acid was calculated as follows [21, 37]:

$$241 \quad \text{Optical purity (\%)} = \frac{D - \text{lactic acid} \left( \frac{g}{l} \right)}{D - \text{lactic acid} \left( \frac{g}{l} \right) + L - \text{lactic acid} \left( \frac{g}{l} \right)} \times 100 \%$$

242

243 The nitrogen consumption during fermentation was determined by the Free Amino Nitrogen  
244 (FAN) method as described by Lie [38] with some modifications. 0.5 ml of diluted sample was  
245 mixed with 0.25 ml of colour reagent (49.71 g of  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 5 g of ninhydrin, 3 g of  
246 fructose and ~ 40 g of  $\text{KH}_2\text{PO}_4$  dissolved in 1 l of distilled water; pH 6.6 – 6.8) in a 2 ml  
247 Eppendorf tube. The mixture was heated at 100 °C in a thermal block (Grant, Cambridge) for 16  
248 minutes and immediately cooled in an ice bath. 1.5 ml of dilution reagent (2 g potassium iodate,  
249  $\text{KIO}_3$ , in 616 ml distilled water and 384 ml 96% ethanol) was added and the free amino nitrogen  
250 content was measured at 570 nm. A calibration curve was constructed using glycine as standard  
251 at various concentrations (0.25 – 2 mg/l).

252

### 253 3 Results and Discussion

254

#### 255 3.1 Effect of growth conditions on *L. coryniformis* fermentation in MRS-based media

256 The results from the growth experiments of *L. coryniformis* in the MRS-based medium  
257 with glucose as the carbon source under pH controlled (pH 5 and 6) and non pH controlled  
258 conditions are shown in Table 1. The optimum pH value for the cell growth of *Lactobacillus* sp.,  
259 including the species used in this work is normally between 5.0 and 7.0 [39, 40]. The results are  
260 in line with the above as good cell growth was obtained in the pH values tested. In the  
261 uncontrolled pH culture, the pH dropped to ~4.1 after 24 h of growth, and the maximum specific  
262 growth rate ( $\mu_{\text{max}}$ ) for *L. coryniformis* was 0.30  $\text{h}^{-1}$ . Approximately 3 g/l glucose was left  
263 unutilised in the media after fermentation. When the pH of the medium was controlled, the  $\mu_{\text{max}}$

264 increased to  $0.36 \text{ h}^{-1}$  at both pH 5 and 6, indicating improved microbial growth with all glucose  
265 being utilized during fermentation. This indicated that pH is one of the controlling factors that  
266 promote high lactic acid production [36].

267 Another factor that plays an important role for lactic acid fermentation is the type of  
268 carbon source used. Glucose, xylose and arabinose (at 20 g/l) were tested as the carbon source in  
269 a MRS based medium (Table 1), as these sugars are most likely present in the DDGS  
270 hydrolysates. Among the sugars, *L. coryniformis* only consumed glucose, with 98 - 99.9% of  
271 glucose being converted to lactic acid and did not consume xylose and arabinose. This finding  
272 shows that *L. coryniformis* is a homolactic acid producer which utilizes the Embden-Meyerhof-  
273 Parnas pathway (EMP) to convert one molecule of glucose into two molecules of lactic acid [39,  
274 41]. *Lactobacillus* species can produce L-or D- lactic acid, depending on the type of lactate  
275 dehydrogenase (nLDH) present in their cluster (*ldhL* or *ldhD*, respectively) [42-44]. In the case  
276 where both D- and L- lactic acid are produced from a single strain, this is likely due to the  
277 presence of both *ldhD* and *ldhL* [45]. Moreover, in all the growth experiments *L. coryniformis*  
278 produced exclusively D-lactic acid (99.9% optical purity) which is in line with previous studies  
279 with this particular microorganism [9].

280

### 281 3.2 Separate hydrolysis and fermentation (SHF) of *L. coryniformis* on DDGS hydrolysate

282 Alkaline pretreated DDGS consisted of 52.6 g glucose, 25.0 g xylose, 10.3 g arabinose  
283 and 0.04 g protein per 100 g of dried material. In the first part of the work, alkaline treated  
284 DDGS solids were hydrolysed to simple sugars using the Accellerase® 1500 enzyme at 50 °C  
285 for 24 h. The sugar composition in the DDGS hydrolysate was: glucose ~27.0 g/l, xylose ~6.1  
286 g/l, xylobiose ~5.9 g/l, and arabinose ~0.8 g/l. 86.5% cellulose was successfully hydrolysed to

287 glucose during hydrolysis. The hydrolysate was then used as fermentation medium for lactic acid  
288 production by *L. coryniformis*. The fermentation pH for *L. coryniformis* was set to 6, based on  
289 the results in Table 1.

290 Figure 1 shows the fermentation characteristics (cell growth, nitrogen and sugar  
291 consumption, lactic acid production) of *L. coryniformis* in the DDGS hydrolysate as a function of  
292 time. Result shows that *L. coryniformis* grew very well in the hydrolysate, with a maximum  
293 OD<sub>600</sub> of 8.7 and a lactic acid concentration of 24.0 g/l, being obtained after 18 hours  
294 fermentation, the time point at which almost all of the available glucose was depleted. The  
295 concentration of xylobiose, xylose and arabinose remained unchanged throughout the  
296 fermentation, which is in line with the results obtained in the semi-defined media (Table 1). An  
297 increase in acetic acid production was observed after glucose depletion, reaching 2.0 g/l after 30  
298 hours fermentation. According to Yáñez, et al. [46] and Hofvendahl and Hahn-Hägerdal [47],  
299 under glucose limitation, homofermentative lactic acid bacteria tend to produce other by-  
300 products such as formic or acetic acid through alternative pyruvate catabolic pathways, whereas  
301 Slavica, et al. [39] also reported an increase in acetic acid when glucose was depleted in a MRS  
302 fermentation medium. Interestingly, between 18 and 30 hours, the concentration of lactic acid  
303 slightly decreased to 22.9 g/l, most likely due to degradation of lactic acid to acetic acid.  
304 Although lactic acid bacteria, especially lactobacilli, are classified as homofermentative or  
305 heterofermentative according to their ability to produce lactic acid through the EMP pathway,  
306 many are able to degrade lactic acid, especially if oxygen is available as an electron acceptor.  
307 The lactic acid that is initially formed from the EMP pathway can be converted to acetic acid  
308 after glucose depletion under aerobic conditions. This has been reported for *L. brevis* [48] and *L.*  
309 *plantarum* [49], although no works have shown this for with *L. coryniformis* up to now.



310 In order for lactic acid to be used as a monomer for PLA synthesis, optical purity is one  
311 of the most important factors to be considered. The optical purity of D- or L- lactic acid has to be  
312 more than 90% in order to be used for the synthesis of crystalline PLA [9, 50]. The optical purity  
313 (%) of D-lactic acid obtained with *L. coryniformis* in the DDGS hydrolysate was 99.9%  
314 indicating that the produced D-lactic acid could be potentially used for the production of  
315 crystalline poly-D-lactic acid (PDLA) if the purity levels of D-lactic acid obtained after  
316 purification of the fermentation broth are high, i.e. > 90% [50].

317

318 3.3 Simultaneous saccharification and fermentation (SSF) of *L. coryniformis* on DDGS  
319 hydrolysate

320 Figure 2 depicts the fermentation data for the SSF of *L. coryniformis* in alkaline  
321 pretreated DDGS medium. Accellerase® 1500 was used to hydrolyse DDGS during the SSF  
322 process. SSF offers several advantages compared to the sequential hydrolysis and fermentation  
323 (SHF), performed previously, as it combines enzymatic hydrolysis and fermentation in a single  
324 step process, resulting in reduced overall processing times and capital costs [51]. Moreover, SSF  
325 also reduces the potential of cellulase inhibition due to the high concentration of glucose in the  
326 hydrolysate [9]. However, compatible operating temperatures and pH for both processes  
327 (hydrolysis and fermentation) should be carefully selected to ensure high lactic acid production.  
328 Previous research works have reported that a temperature range from 37 to 40 °C and a pH  
329 around 5 is appropriate for the production of lactic acid production from biomass by lactobacilli.  
330 For example, lactic acid was produced via a SSF approach from cassava bagasse [52] and from  
331 rice bran by *L. delbrueckii* [20], and from *curcuma longa* (tumeric) biomass of by *L. paracasei*  
332 and *L. coryniformis* [53].

333 In this study, the SSF process was carried out in 100 ml bioreactor containing 33 g/l of  
334 glucose from alkaline pretreated DDGS at 37 °C, with the pH being controlled at 5 throughout  
335 the process. Since *L. coryniformis* could grow well in media with both pH 5 and 6, the former pH  
336 was selected for SSF since it was the optimum pH for cellulase activity as stated by the  
337 manufacturer. During the first 6 hours ~ 68% of the cellulose present in the alkaline pretreated  
338 DDGS (22 g/l) was converted to glucose and was not utilised by *L. coryniformis*, as the strain  
339 was still in the lag phase; as a result, low production of lactic acid (0.6 g/l) was detected during  
340 this period. *L. coryniformis* started to consume glucose after 12 hours, with the highest lactic acid  
341 and viable cell concentrations obtained after 24 hours, i.e. 28 g/l of lactic acid (99.9% optical  
342 purity of D- lactic acid) and 9.8 CFU/ml, respectively after 24 hours (Figure 2a). Unlike SHF, in  
343 SSF no reduction in the lactic acid concentration was observed after glucose depletion (24 to 48  
344 hours). This might be due to the action of the cellulose enzyme of Accellerase® 1500 that was  
345 still actively converting the remaining traces of cellulose in the DDGS to glucose, albeit in very  
346 small amounts, which was most likely rapidly consumed by *L. coryniformis*. At the end, around  
347 84.5% of the cellulose present in the pretreated DDGS was converted to lactic acid,  
348 demonstrating the efficient utilisation of DDGS during the SSF process.

349 In an attempt to increase lactic acid production, the effect of increasing the amount of  
350 pre-treated DDGS loading in the SFF process was investigated. However, increasing the  
351 substrate loading results in highly viscous suspensions, which reduces the efficiency of  
352 enzymatic hydrolysis [54]. One way to overcome this problem is via multi-step feeding or fed-  
353 batch SSF. In this approach, additional cellulosic biomass substrate is added at a particular point  
354 during the process; as a result free water is liberated, which reduces the viscosity and stiffness of  
355 the suspension [55, 56]. In this study, when the glucose concentration reached less than 0.5 g/l

356 (at 24 hours), alkaline pretreated DDGS was added at two levels, 11 g/l glucose (Figure 2b) and  
357 22 g/l glucose (Figure 2c); 34.0 g/l and 38.1 g/l of lactic acid (99.9% optical purity of D-lactic  
358 acid) were produced respectively, after 48 h of fermentation. However, a reduction in the  
359 conversion yield of glucose to lactic acid (76 % vs 70%, respectively) was observed with the  
360 higher substrate loading. This might be due to inadequate stirring at the higher solid content,  
361 which resulted in insufficient mass transfer and thus reduced the adsorption capacity of cellulase  
362 to cellulose and the efficiency of the enzymatic digestion of DDGS solids [54, 57].

363 A comparison of the fermentation characteristics obtained by employing different  
364 fermentation approaches is shown in Table 2. Overall, SSF demonstrated better fermentation  
365 characteristics compared to SHF; more specifically high D-lactic acid concentration (27.9 g/l),  
366 productivity (1.46 g/l/h), glucose conversion yield (84.5%) and D-lactic acid yield (42.3%) were  
367 observed when SSF process was employed. When DDGS solids were added using the SSF fed-  
368 batch approach, the D-lactic acid concentration increased up to ~38 g/l (when adding 22 g/l  
369 glucose from alkaline pretreated DDGS), but in this case the other fermentation characteristics  
370 decreased. On the other hand, the fermentation characteristics in the case of adding 11 g/l  
371 glucose during the SSF fed-batch approach were deemed overall optimal and demonstrate the  
372 potential of using this approach at a commercial large scale operation.

373 The feasibility of the SSF process was evaluated in a 2-L stirred tank bioreactor with  
374 1.5L working volume (Figure 3). The data obtained were very similar to those obtained for the  
375 SSF in the 100 ml bioreactor, i.e. the maximum D-lactic acid concentration was 26.4 g/l  
376 (produced after 18 hours), the glucose conversion yield was ~ 80%, the productivity was 1.47  
377 g/l/h, the lactic acid yield was ~ 40%, and the D-lactic acid optical purity was 99.9%. The key  
378 difference compared to the smaller scale SSF process was the fact that after glucose depletion

379 (18 hours), the lactic acid concentration decreased (from 26.4 g/l at 18 hours to 22.6 g/l at 30  
380 hours) and acetic acid was produced (from 1.3 g/l at 18 hours to 4.0 g/l after 30 hours). This  
381 phenomenon could be due the ability of *L. coryniformis* to convert lactic acid to acetic acid under  
382 aerobic conditions, once glucose was depleted as shown in fermentation of *L. plantarum* [49]. In  
383 order to avoid the accumulation of acetic acid in the fermentation medium, which may interfere  
384 with the purification process, it is important that the optimum time for stopping the fermentation  
385 and the optimal aeration conditions are identified.

386

#### 387 **4 Conclusions**

388 This study highlights the potential of producing D-lactic acid with high optical purity  
389 from alkaline pretreated DDGS, which is produced in large amounts by the bioethanol industry.  
390 The SSF approach using *L. coryniformis* resulted in faster and higher production of optically  
391 pure D-lactic acid (99.9%), with a higher conversion yield of glucose to lactic acid (84.5%)  
392 compared to conventional SHF (72.9%). The D-lactic production could be further enhanced by  
393 employing a fed-batch SSF process where the fermentation medium is fed with DDGS during the  
394 process, however, further work is needed to identify the operating conditions that result to  
395 increase the substrate conversion yields from 70-75 % to above 85%. The SSF process  
396 demonstrated good scalability as similar fermentation characteristics were obtained between the  
397 small (100 ml) and larger scale (2-L) fermentation vessels.

398

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402

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404

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558 Table 1: Growth parameters of *L. coryniformis* in MRS-based media

Conditions	Optical density (OD <sub>600 nm</sub> )*	Maximum specific growth rate ( $\mu_{\max}$ , h <sup>-1</sup> )	Lactic acid (g/l)	<sup>a</sup> Y <sub>Lac/S</sub> (% w/w)
Glucose (16 g/l) Uncontrolled pH	4.5 ± 0.06	0.30	12.5 ± 0.10	97.63
Glucose (16 g/l) pH 5	7.0 ± 0.50	0.36	15.7 ± 0.47	98.13
Glucose (16 g/l) pH 6	6.4 ± 0.16	0.36	15.6 ± 1.85	97.50
Glucose (20 g/l) pH 6	8.2 ± 0.04	0.38	19.7 ± 0.02	99.9
Xylose (20 g/l) pH 6	0.5 ± 0.01	-	-	-
Arabinose (20 g/l) pH 6	0.4 ± 0.17	-	-	-
Glucose and Xylose (10 g/l each) pH 6	4.5 ± 0.18	0.29	10.5 ± 0.97	52.50

559 <sup>a</sup> Y<sub>Lac/S</sub> (% w/w) = (g lactic acid produced/ g sugar consumed) x 100

560 Data based on three independent fermentation trials and are shown as mean ± std. dev.

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572 Table 2: Overall fermentation characteristics of different fermentation processes for the  
573 production of D-lactic by *L. coryniformis* cultivation

Parameter	SHF	SSF	Fed batch SSF	
	33 g/l glucose*	33 g/l glucose*	Addition of 11 g/l glucose*	Addition of 22g/l glucose*
Lactic acid production (g/l)	24.1	27.9	34.0	38.1
Lactic acid productivity (g/l/h)	1.3	1.5	0.7	0.8
Glucose conversion (%) <sup>a</sup>	72.9	84.5	76.1	70.0
Lactic acid yield (%) <sup>b</sup>	32.1	42.3	40.1	35.0

574 \*present in alkaline treated DDGS solids

575 <sup>a</sup>Lactic acid (mg) / glucose in alkaline pretreated DDGS (mg) x 100

576 <sup>b</sup>Lactic acid (mg) / pretreated DDGS (mg) x 100

577 Data based on two independent fermentation trials and are shown as mean ± std. dev.

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