# Purification and polymerisation of microbial D-lactic acid from DDGS hydrolysates fermentation

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Accepted Version

Zaini, N. A., Chatzifragkou, A. ORCID: https://orcid.org/0000-0002-9255-7871, Tverezovskiy, V. and Charalampopoulos, D. ORCID: https://orcid.org/0000-0003-1269-8402 (2019) Purification and polymerisation of microbial D-lactic acid from DDGS hydrolysates fermentation. Biochemical Engineering Journal, 150. 107265. ISSN 1369-703X doi: 10.1016/j.bej.2019.107265 Available at https://centaur.reading.ac.uk/84142/

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1 **Purification and polymerisation of microbial D-lactic acid from DDGS**

2 **hydrolysates fermentation**

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20

## Abstract

A multi-step process was developed for microbial D-lactic acid purification, followed by poly-D-lactic acid (PDLA) synthesis via azeotropic polycondensation process. Several anion exchange resins were screened for their binding capacity using model lactic acid solutions. Amberlite® IRA67 (weak base anion exchange resin) showed the highest lactic acid adsorption, with maximum adsorption capacity, $q_{max}$, of 136.11 mg lactic acid / g of resin, and was further selected to purify D-lactic acid from DDGS hydrolysates through a three-step process; (1) treatment with 7% w/v activated carbon, (2) acidification of fermentation broth (Amberlite® IRA120) and (3) adsorption of lactic acid by anion exchange (Amberlite® IRA67). At the end of the purification process, 80.4% (w/w) D-lactic acid was recovered with 91.8% (w/w) purity, indicating the effectiveness of the developed downstream process. Furthermore, a clear yellowish solid polymer with a molecular weight of 3010 Da was obtained, suitable for applications in biomedical and agricultural sectors.

2

## 1. Introduction

The world demand for lactic acid has increased significantly over the past few years, as the application of polylactic acid (PLA), offers distinctive advantages over petroleum-based polymers. PLA is a biodegradable plastic that can be used in biomedical and pharmaceutical industries as surgical suture, tissue engineering scaffolds or as drug delivery tool [1]. In the early stages of commercialisation, PLA was only produced for biomedical device applications due to its high cost [2]. Nowadays, the application of PLA has expanded to the electric and electronic industries for the production of casings and circuit boards, as well as in food industry for the production of food packaging and cutlery materials [3, 4]. Currently, the main producers of PLA are NatureWorks® LLC under the trade name Ingeo™, Cereplast, Inc. (United States), Corbion Purac (Netherlands), Toray Industries (Japan) and Zhejiang Hisun Biomaterial Co., Ltd (China) [2]. Report by IHS Markit [5], predicted that PLA will be the leading application of lactic acid by 2020. PLA offers advantages over conventional petrochemical-derived plastics being biodegradable and compostable [2], and reduces the reliance on fossils fuel for the production of plastics [6]. Moreover, PLA production has a lower environmental impact compared to conventional petroleum derived polymers, as the carbon emissions and the energy consumption are reduced by 15 to 60% and 25 to 55%, respectively [7]. The low toxicity of PLA, along with its positive environmental characteristics, has rendered it an ideal material for application in various fields, including the food, biomedical and agricultural sector [6].

The production of PLA from agricultural residues represents a promising route for production, as such biomass is available at low cost, is accessible throughout the year and does not compete with food crops. For example, corn stover [8] and rice bran [9] have been identified as potential fermentation substrates for D-lactic acid production. However, the fermentation broths derived from renewable sources contain a mixture of compounds, including a variety of

3

63  sugars and proteins, polyphenols and organic acids, and thus require an effective downstream

64  processing for the successful recovery of the targeted compound before being used as monomer

65  for polymer synthesis [10].

66      Several downstream processing techniques such as ion exchange chromatography,

67  precipitation, solvent extraction, distillation, nanofiltration, membrane extraction and

68  electrodialysis have been investigated for the recovery and purification of lactic acid from

69  fermentation broths [11-15]. Among these, adsorption by ion exchange offers a distinct

70  advantage as it is a simple and relatively cheap process that offers product specificity, which

71  leads to high purification yields [14, 16]. In organic acid separation, anion exchange resins are

72  widely used. However, no specific conclusions on the optimum conditions for lactic acid

73  binding have been drawn so far for anion exchange resins. For example, some researchers

74  reported that a solution pH above the pKa of lactic acid (pKa lactic acid, 3.86) give the highest

75  binding of lactic acid to Amberlite® IRA67 [17], Amberlite® IRA96 [16] and Amberlite®

76  IRA92 [18]. On the other hand, other studies have found that a pH below the pKa value give

77  the highest adsorption of lactic acid and other carboxylic acids to Amberlite® IRA67 [19],

78  Amberlite® IRA35 [20, 21] and Lewatit S3428 resins [22]. To promote lactic acid binding on

79  an anionic resin below the pKa value, the fermentation broth was acidified by treatment with

80  strong acid or by passing the broth through a strong acidic cation exchange resin, i.e. Duolite

81  C-464, to convert lactate salt to lactic acid [21].

82      Ring opening polymerisation and direct polycondensation are the most common

83  methods used to synthesise PLA from lactic acid. In ring opening polymerisation, PLA is

84  polymerised through a cyclic lactide intermediate. Companies such as NatureWorks® LLC

85  (United States) and Corbion N.V. (Netherlands), produce PLA through this route [4, 23, 24].

86  This protocol is of interest as it produces high molecular weight PLA. However, the procedure

87  is complicated and time consuming because it involves several polymerisation steps and

88  requires high purity of the lactide monomer prior to PLA synthesis [25]. On the other hand,

89  direct polycondensation offers significant advantages as the polymerisation process is simpler

90  and easier in this case. In direct polycondensation, only one step for polymer synthesis is

91  involved, during which the lactic acid solution is heated at $130 - 140\ ^{\circ}\text{C}$. Through this process,

92  normally low molecular weight PLA ($< 5000$ Da) is produced with relatively weak mechanical

93  properties; this is due to difficulties in removing the water from the reaction mixture as the

94  polymerisation process progresses [26]. However, Ajioka, Enomoto, Suzuki and Yamaguchi

95  [27] successfully produced high molecular weight PLA ($> 300000$ Da) using a single step

96  synthesis using organic solvent with a catalyst (tin, Sn, powder) in azeotropic condition.

97  Azeotropic polycondensation involved refluxing of the solvent under reduced pressure to

98  remove the condensation water that was generated during polymer synthesis. This method had

99  been patented and used by Mitsui Toatsu Chemicals (Japan) to synthesise PLA under the

100  commercial name LACEA [6, 23].

101       The aim of the study was to develop a multi-step process for the purification of D-lactic

102  acid from a fermentation broth based on dried distiller's grains with solubles (DDGS)

103  hydrolysate [28]. Subsequently, the purified D-lactic acid was used as monomer for PDLA

104  synthesis by employing an azeotropic polycondensation approach. As the purification of D-

105  lactic acid from fermentation broth hydrolysates and its polymerisation process is rarely been

106  reported, this study provides novel information on D-lactic acid separation, employing a

107  multiple purification step followed by single step polymerisation process.

108

## 2.    Materials and Methods

2.1    Materials

Dried Distillers Grains with Solubles (DDGS) was supplied from a bioethanol plant (Vivergo, Yorkshire, UK) and was alkaline pretreated as described by Zaini et al. [28]. Alkaline treated DDGS consisted of 52.6 g glucose, 25.0 g xylose, 10.3 g arabinose, and 0.04 g protein per 100 g of dried material. The resins (Amberlite® IRA67, Diaion® WA30, Amberlite® IRA400, Dowex® Marathon™ MSA and Amberlite® IRA120) and activated carbon used in this study were purchased from Sigma-Aldrich (US).


2.2    D-lactic acid production

D-lactic acid was produced by *L. coryniformis* subsp. *torquens* (DSM 20004) using a Simultaneous Saccharification and Fermentation (SSF) process of alkaline treated DDGS in a 2 L stirred tank bioreactor (Biostat B, Sartorious, Germany) [28]. The process was initiated by the simultaneous addition of Accellerase® 1500 (1 ml enzyme : 0.33 g cellulose) and *L. coryniformis* inoculum (starting OD of ~0.05) and was carried out for 30 hours (1.5 L fermentation medium). The SSF process was carried out at 37°C with an initial agitation speed of 250 rpm. The pH of fermentation medium was maintained at 5 with aseptic additions 5M NaOH and HCl through a peristaltic pump. The minimum dissolved oxygen (DO) level was kept at 20% by controlling automatically the stirrer speed. The culture containing the enzyme was inactivated by heat treatment at 95 °C for 10 minutes, followed by centrifugation at 17,105 *x g* for 20 minutes (4 °C). Supernatants containing D-lactic acid solutions were collected and kept at -20 °C for purification.

132    2.3    Resin preparation

133    Weak anion exchange resins (Amberlite® IRA67 and Diaion® WA30) and strong anion

134    exchange resins (Amberlite® IRA400 and Dowex® Marathon™ MSA) were selected for this

135    study. Before utilisation, the resins in free base form, were first converted to Cl⁻ form as

136    described by Moldes et al. [29]. Resins that were purchased in Cl⁻ form were only washed with

137    distilled water [11, 30]. For the acidification of the fermentation broth, a cation exchange resin,

138    Amberlite® IRA120, a strongly acidic resin in H⁺ form, was used. The resin was washed with

139    distilled water three times to remove any contaminants. All resins were then oven dried at 50

140    °C overnight and stored at room temperature in closed containers before use. The properties of

141    the ion exchange resins that were used in this study are presented in Table 1.

142

143    2.4    Screening and optimisation of anion exchange resins binding and recovery

144    For the screening experiments of the anion exchange resins, model lactic acid solutions

145    were prepared using commercial lactic acid (85%, Food Chemical Codex, FCC, Sigma-

146    Aldrich).

147

148    2.4.1    Effect of pH on lactic acid binding

149    The effect of pH on lactic acid binding was determined through batch experiments

150    according to Bishai et al. [16] with slight modifications. 1 g of dried resin (Amberlite® IRA67,

151    Diaion® WA30, Amberlite® IRA400 or Dowex® Marathon™ MSA) was mixed with 5 ml of

152    lactic acid (50 g/l) at different initial pH conditions (2, 3, 4, 5, 6, 7 and 8) at 25 °C. The pH of

153    the solutions was adjusted with 5 M NaOH. The mixtures were shaken at 200 rpm for 8 hours.

154    The liquid fractions from each mixture were collected by filtration and analysed for lactic acid

155    concentration by HPLC.

156

157    2.4.2   Effect of temperature on lactic acid binding

158    In order to investigate the effect of temperature on lactic acid binding, 1 g of each dried

159    resin (Amberlite® IRA67, Diaion® WA30, Amberlite® IRA400 or Dowex® Marathon™ MSA)

160    was mixed with 5 ml lactic acid (50 g/l), prepared at the optimum pH (obtained in Section

161    2.4.1) and incubated at temperatures of 25, 30, 40, 50 and 60 °C at 200 rpm. The mixtures were

162    shaken at 200 rpm for 8 hours. The liquid fractions from each mixture were collected by

163    filtration and analysed for lactic acid concentration by HPLC.

164    The binding capacity, $q$, and adsorption efficiency, $E$, of the resin at different pH and

165    temperatures were calculated as follows, as described by Pradhan et al. [11]:

$$q = \frac{(C_i - C_f) * V}{R} \tag{1}$$

$$E = \frac{(C_i - C_f)}{C_i} * 100 \tag{2}$$

166    where, $q$ is the amount of lactic acid adsorbed to the resin (mg/g), $E$ is the efficiency of lactic

167    acid binding (%), $C_i$ is the initial concentration of lactic acid (g/l), $C_f$ is the concentration of

168    lactic acid after being mixed with the resin (g/l), $V$ is the volume of lactic acid solution (l) and

169    $R$ is the weight of the resin (g).

170

171    2.4.3   Adsorption capacity of lactic acid by resins

172    Amberlite® IRA67 and Diaion® WA30 were selected to carry out adsorption isotherm

173    analysis in batch operation as described by Bernardo et al. [31] and John et al. [17], with slight

174    modifications. 1 g of each resin was mixed with 5 ml of lactic acid at various concentrations

175 (4 - 650 mg lactic acid per 5 ml). The initial pH of the lactic acid solutions was set taking into

176 account their optimum binding pH (obtained in Section 2.4.1) for the respective resin. The

177 reaction took place at optimum temperature (obtained in Section 2.4.2) at 200 rpm for 8 hours.

178 Liquid samples from each mixture were filtered and collected for further analysis.

179      Three different nonlinear models, the Langmuir, Freundlich and Langmuir - Freundlich

180 models, were then fitted to the data using Origin Pro 8.0 software (OriginLab, USA) [32] using

181 the following equations:

Langmuir model $$q = \frac{q_{max} * K * C}{K * C + 1} \qquad (3)$$

Freundlich model $$q = K_f * C^{\frac{1}{n}} \qquad (4)$$

Langmuir – Freundlich model $$\frac{q}{q_{max}} = \frac{K_{LF} * C^{nLF}}{1 + K_{LF} * C^{nLF}} \qquad (5)$$

182 where, $q$ is the amount of lactic acid adsorbed to the resin (mg/g), $q_{max}$ is the maximum amount

183 of lactic acid adsorbed to the resin (mg/g), $K$ is the Langmuir adsorption constant, $K_f$ is the

184 Freundlich adsorption constant, $K_{LF}$ is the affinity constant for adsorption, $C$ is the amount of

185 lactic acid (mg), $n$ is the Freundlich adsorption constant and $nLF$ is the Langmuir-Freundlich

186 coefficient.

187

188 2.4.4   Effect of ionic strength of eluent (HCl) on lactic acid recovery

189      The resin that exhibited the highest binding capacity, $q$, (Amberlite® IRA67) was

190 selected for further recovery experiments using HCl with different ionic strengths as eluent. 4

191 g of Amberlite® IRA67 were packed into a 100 mm (length) column (Fisher Scientific,

192 Leicester, UK) and saturated with 20 ml of 30 g/l lactic acid solution. The resin was then

193 washed with distilled water to remove any unbound lactic acid. Different concentrations of HCl

194 were used as eluent (0.05, 0.1, 0.5 and 1.0 M), which were passed down the packed column by

195 gravity and fractions of effluents were collected (2 ml per fraction) at regular time intervals.

196 All fractions were analysed for lactic acid concentration.

197

198 2.5 Purification of D-lactic acid from fermentation broth

199 2.5.1 Colour removal by activated carbon

200 The effect of activated carbon on the removal of the colour from the fermentation broth

201 was determined. Powdered activated carbon was mixed with 5 ml of clarified fermentation

202 broth at different loading concentrations (0, 1, 5, 7 and 10%, w/v), for 1.5 hours at 150 (25 $^{\circ}$C).

203 The mixture was then separated by centrifugation at 17,105 $x$ $g$ for 10 minutes (4 $^{\circ}$C). The pellet

204 was washed twice with distilled water and the supernatants collected for sugar and lactic acid

205 analysis.

206

207 2.5.2 Acidification of fermentation broth by cation exchange resin

208 After treatment with activated carbon, the fermentation broth was subjected to

209 Amberlite$^{\circledR}$ IRA 120, H$^{+}$ resin aiming to convert sodium lactate into lactic acid [33]. 10 g of

210 dried resin were packed into a 30 cm length Econo-glass column (i.d. 1 cm) which was first

211 filled with distilled water and the height of the resin was fixed with a flow adaptor (i.d. 1 cm)

212 (Biorad, California, US). Distilled water was allowed to pass through the column until the pH

213 of effluent was around 6.5. Then, a constant flow of 3 ml/min using a peristaltic pump was

214 applied. The fermentation broth containing sodium lactate with a pH around 5.5 was then

215 pumped into the column at the same flow rate. When the pH of the effluent started to increase,

216 the resin was considered saturated. Effluent fractions (4 ml each) were collected for lactic acid

217    and sugar analysis. Fractions containing D-lactic acid were pooled for subsequent purification.

218    The resin was washed with distilled water to remove the remaining solution in interstitial

219    spaces, regenerated with 1 M HCl and thoroughly rinsed with distilled water, before a new

220    cycle [16].

221

222    2.5.3    Adsorption by anion exchange resin

223    Pooled supernatant fractions containing D-lactic acid were passed through an anion

224    exchange resin, (Amberlite® IRA67) in fixed-bed column operation. 25 g of dried resin were

225    packed into a 30 cm length Econo-glass column (i.d. 2.5 cm) which was first filled with distilled

226    water and the upper side of resin was fixed with a flow adaptor (i.d. 2.5 cm). The system was

227    washed with distilled water until the pH of the effluent was around 6.5. Then, the acidified

228    broth obtained from Amberlite® IRA 120, $H^+$ resin was pumped into the column at 3 ml/min.

229    D=lactic acid was recovered with 0.5 M HCl. Fractions of effluents at each stage were collected

230    (4 ml each) for lactic acid and sugar analysis.

231

232    2.6    Azeotropic polycondensation process of PDLA

233    Polycondensation of PDLA was conducted as described by Ajioka et al. [27] with minor

234    modifications. 2 g of D-lactic acid were mixed with 40 ml toluene in 100 ml reaction flask,

235    equipped with a Dean-stark apparatus and a magnetic stirrer. In the first step of the

236    polycondensation process, the mixture was azeotropically dehydrated at 110 °C for 3 hours to

237    remove the free water. After removing the condensed water that was trapped in the Dean-stark

238    apparatus, the tube was packed with molecular sieve (4 Å) and calcium chloride in layers to

239    remove small amounts of water dissolved in the organic solvent that was produced during PLA

240    synthesis. 0.2 g tin (ll)-2-ethylhexanoate (stannous octoate) was added to the reaction mixture

241 and then returned to reflux at 140 °C for another 80 hours. The polymer produced was then

242 recovered by filtration and vacuum dried using Bünchner funnel apparatus, followed by freeze

243 drying (VisTis Sentry 2.0, Warminster, PA).

244

245 2.6 Analytical methods

246 The nitrogen content was determined using the Free Amino Nitrogen (FAN) method as

247 described by Lie [34] with some modifications. 0.5 ml of diluted sample was mixed with 0.25

248 ml of colour reagent (49.71 g of $Na_2HPO_4 \cdot 2H_2O$, 5 g of ninhydrin, 3 g of fructose and ~ 40 g

249 of $KH_2PO_4$ dissolved in 1 l of distilled water; pH 6.6 – 6.8) in 2 ml Eppendorf tube. The mixture

250 was heated at 100 °C in a thermal block (Grant, Cambridge) for exactly 16 minutes and

251 immediately cooled in an ice bath. 1.5 ml of dilution reagent (2 g potassium iodate, $KIO_3$, in

252 616 ml distilled water and 384 ml 96% ethanol) was added and the free amino nitrogen content

253 was measured at 570 nm. A calibration curve was constructed using glycine at different

254 concentrations (0.25 – 2 mg/l) as standard.

255 Sugar and lactic acid concentrations were analysed by high performance liquid

256 chromatography (HPLC) in an Agilent Infinity 1260 system (Agilent Technologies, USA)

257 equipped with an Aminex HPX-87H column (Bio-rad, Hercules, CA) at a 0.6 ml/min flow rate

258 with 5 mM $H_2SO_4$ as mobile phase. The temperature of the column was set at 65 °C and sugars

259 and lactic acid were detected using a refractive index detector. The D-lactic acid recovery and

260 purity were calculated using the following equations [16]:

$$Recovery\ (\%) = \frac{mg\ LA\ in\ each\ purification\ stage}{mg\ LA\ in\ fermentation\ broth} * 100 \qquad (6)$$

$$Purity\ (\%) = \frac{HPLC\ peak\ area\ of\ LA\ in\ each\ purification\ stage}{Total\ HPLC\ peak\ areas\ in\ each\ purification\ stage} * 100 \qquad (7)$$

261    The molecular weight and poly dispersity index (PDI) of PDLA was determined by gel

262    permeation chromatography (GPC), using an Agilent 1100 Series chromatography system that

263    was equipped with a refractive index RID 1200 detector (35 °C). The flow rate was set at 1.0

264    ml/min and the molecular size was determined using a PL gel 5μM mixed-D column (300 x

265    7.5 mm) and a PL gel 5μM guard column (50 x 7.5 mm). Chloroform was used as the eluent.

266    The PDLA obtained was first dissolved in chloroform prior to analysis. The sample was

267    analysed at room temperature using 20 µl injection volume. A calibration curve was generated

268    using polystyrene standards with molecular weights ranging from 580 to 483,400 Da.

269

270    **3.    Results and Discussion**

271    3.1 Selection of anion exchange resin

272    When considering ion exchange chromatography, the efficiency of the product

273    adsorption by the resin determines the success of the purification process. Therefore, factors

274    such as pH, temperature and lactic acid concentration were initially investigated in this study

275    in order to select the most appropriate anion exchange resin. The effect of the ionic strength of

276    the eluent (HCl solution) on product recovery was also investigated. Before use, the resins that

277    existed in free base were converted to Cl⁻ form. The Cl⁻ form was selected as previous studies

278    have shown that in this form the resins exhibit the highest adsorption capacity for lactic acid

279    [17, 31]. Moreover, the simultaneous lactic acid recovery and resin regeneration for subsequent

280    adsorption cycles by HCl, reduces the number of steps involved in the purification process

281    [29]. Four different resins, categorised into weak base anion exchange (Amberlite® IRA67 and

282    Diaion® WA30) and strong base anion exchange (Amberlite® IRA400 and Dowex®

283    Marathon™ MSA) were tested against different initial pH values, ranging from 2 to 8, as shown

284    in Figure 6a.

285    Among the tested resins, the weak base anion exchange resins showed capability for

286    lactic acid binding at low pH, with the highest adsorption being 65% with Amberlite® IRA67

287    at pH 3, which corresponded to a maximum binding capacity ($q_{max}$) of 155 mg lactic acid/g of

288    resin. For strong base anion exchange resins, less than 22% of lactic acid was adsorbed by both

289    Amberlite® IRA400 and Dowex® Marathon™ MSA, with the highest binding at pH 6 and pH

290    4, respectively, suggesting that the pH did not influence the adsorption of lactic acid to the

291    strong base anion exchange resins. On the other hand, the adsorption of weak base anion

292    exchange resins was strongly influenced by the pH of the feed solution. In the case of

293    Amberlite® IRA67 and Diaion® WA30, the best pH for lactic acid adsorption was below its

294    pKa (3.86), where lactic acid exists in its undissociated form [21, 22, 35]. This can be

295    associated with the charge of the tertiary amine (the functional group in both resins) which is

296    cross-linked to the polymeric matrix (acrylic or styrene) in Amberlite® IRA67 and Diaion®

297    WA30. It is assumed that the lone pair electron of the nitrogen atom in the tertiary amine is

298    likely to hydrogen bond to lactic acid through the chloride ion [20, 36]. This mechanism is also

299    supported by works from Yousuf et al. [35], Kislik [13] and Kulprathipanja and Oroskar [20],

300    who reported that possible interactions between amine-based extractants and carboxylic acids

301    are through hydrogen bonding, acid-base interaction, hydrophobic interaction, ion-ion pair

302    formation or solvation.

303    Subsequently, the effect of temperature (25 to 60 °C) on lactic acid adsorption was

304    investigated at pH values that were previously shown to give the highest lactic acid binding for

305    each resin (Figure 6b). No differences were observed between the different temperatures, for

306    each resin. As shown previously, the highest lactic acid adsorption (~73%) was exhibited by

307    Amberlite® IRA67. According to Niazi and Brown [37], the effect of temperature on ion

14

308  exchange resins is mainly attributed to pKa changes of the targeted compound as a result of the

309  temperature change. In the case of lactic acid, as the temperature increased from 25 to 30, 40

310  and 50ºC, the pKa value also increased to 3.896, 3.942 and 4.028, respectively [38]. Since

311  lactic acid adsorption to weak base anion exchange resins occurs below its pKa value, the

312  increase in temperature from 25 to 60 ºC did not have any significant effect.

313  3.1.1 Adsorption isotherms

314      The two resins that demonstrated the highest binding capacity, Amberlite® IRA67 and

315  Diaion® WA30, were further tested. The adsorption isotherms were generated at 25 ºC, and are

316  shown in

317  Figure 7a and 2b, respectively. Different kinetic models (Langmuir, Freundlich and Langmuir

318  – Freundlich) were then used to fit the data, and the model parameters are presented in Table

319  2.

320      The Langmuir and Langmuir – Freundlich models fitted better the data for both resins

321  ($R^2 > 0.9$), compared to the Freundlich model ($R^2 = 0.80$). The Langmuir – Freundlich model

322  showed better fit than the Langmuir model for both the Amberlite® IRA67 ($R^2 = 0.94$) and the

323  Diaion® WA30 resin ($R^2 = 0.965$). In the Langmuir model, it is assumed that the adsorption of

324  a given adsorbate occurs as a monolayer sorption onto the surface of a resin containing a finite

325  number of identical binding sites. The adsorbent has uniform binding sites and the adsorbate

326  will only bind to the binding site [32, 39]. In the Freundlich model, it is assumed that the

327  binding of the adsorbate molecules onto the adsorbent is at infinite capacity. The adsorption is

328  not uniform and can occur in a multilayer, with the binding site that has stronger bond energy

329  being occupied first [39]. In the present study, the adsorption capacity of the resin was specific

330  due to the strong interactions between lactic acid and the functional groups present in the Cl⁻

331  form of the resin. As a result, the Freundlich model did not fit as well as the other models the

kinetic data. According to Sala et al. [32], the Langmuir-Freundlich model is the simple generalisation of both isotherms, modelling the adsorption cooperativity of the two different binding mechanisms. This level of cooperativity can be determined from the $nLF$ value, where, when $nLF > 1$, a positive cooperativity is indicated. When $0 < nLF < 1$, a negative cooperativity in binding process is indicated, whereas when $nFL$ value $= 1$, it is assumed that the adsorption is purely independent, and no interaction takes place between absorbents. In this study, the $nFL$ values for both resins were $> 1$, with 1.96 for Amberlite$^®$ IRA67and 1.83 for Diaion$^®$ WA30, indicating a positive cooperativity of the two binding mechanisms.

The maximum binding capacity of a resin ($q_{max}$) can also be predicted from the Langmuir and Langmuir–Freundlich models, however it cannot be obtained from the Freundlich model as this model assumes that lactic acid binding to the resin is unlimited. The highest $q_{max}$ values for Amberlite$^®$ IRA67 were 162.09 and 136.11 mg/g resin based on the Langmuir and Langmuir–Freundlich models, respectively. This value, however, was lower than the $q_{max}$ value reported by Garrett et al. [40], i.e. 203 mg/g of resin, as predicted by the Langmuir model.


3.1.2 Effect of HCl strength on the recovery of lactic acid

The strength of the eluent on the recovery of lactic acid plays a key role for ensuring that all of the lactic acid that is bound to the resin is detached. In this study, HCl was used as an eluent to recover lactic acid from Amberlite$^®$ IRA67, which was deemed from the previous work to be the most suitable resin. Figure 8 depicts the elution profiles of lactic acid at different HCl concentrations. At 0.05 M and 0.1 M HCl, only 5.1 and 22.1% lactic acid was recovered from Amberlite$^®$ IRA67, respectively. However, at 0.5 and 1.0 M, ~ 96% and 100% recovery of lactic acid was achieved, respectively, indicating that the strength of the eluent plays critical

356 role for the detachment of lactic acid from the resin. Based on these results, 0.5 M HCl was

357 selected to recover lactic acid in subsequent experiments.

358

359 3.2 Separation and purification of D-lactic acid from fermentation broth

360 3.2.1 Colour removal by activated carbon

361 After determining the conditions leading to maximum lactic acid adsorption and

362 recovery using the Amberlite® IRA67 resin, the aim was to purify lactic acid from fermentation

363 broths of *L. coryniformis* subsp. *torquens*, where DDGS hydrolysate was used as the

364 fermentation medium. Fermentation broths usually contain besides the component of interest,

365 residual sugars and proteins, as well as by-products of the fermentation process. In this

366 particular case, fermented DDGS hydrolysate contained residual sugars (xylotriose, xylobiose,

367 xylose, arabinose), organic nitrogen in the form of proteins, peptides or amino acids,

368 polyphenols and acetic acid, all of which can contributed into the dark brown colour of the

369 fermentation broth [41]. It is likely that a significant proportion of the dark brown colour of the

370 medium is due to the dark colour of DDGS generated during the drum drying step in the DDGS

371 production process. Therefore, prior to ion exchange purification, the fermented DDGS

372 hydrolysate was initially subjected to activated carbon treatment. Figure 9 shows the effect of

373 various activated carbon concentrations on the colour of the fermentation broth. A positive

374 correlation can be observed as the activated carbon concentrations increased up to 5% (w/v)

375 and the colour of the fermentation broths became notably lighter. For higher activated carbon

376 concentrations (7 and 10%, w/v) no significant changes in colour were observed.

377 The effect of activated carbon concentration on the recovery of D-lactic acid, protein,

378 oligosaccharides and monosaccharides is shown in Table 3. The recovery of D-lactic acid

379 gradually decreased as the concentration of activated carbon increased. More specifically at 1,

380  5, 7 and 10% (w/v) of activated carbon, 95, 90, 88 and 85% lactic acid was recovered from the

381  fermentation broth, respectively. A relatively small reduction in monosaccharides and proteins

382  concentrations was overall noted ($< 30\%$ removal) when the fermentation broth was treated

383  with activated carbon, even as high as 10% (w/v). Stone and Kozlov [42] reported that activated

384  carbon can only adsorb low molecular weight proteins, in which is in line with protein removal

385  data obtained in this study. On the other hand, oligosaccharides (xylotriose and xylobiose) were

386  completely removed at 7% (w/v) activated carbon. According to Boon et al. [43], activated

387  carbon has higher affinity for trisaccharides and disaccharides compared to monosaccharides

388  with a capacity of 133 mg/g and 117 mg/g of activated carbon, respectively. Based on the

389  above, 7% (w/v) activated carbon was selected as the best concentration to treat the

390  fermentation broth to ensure a satisfactory recovery of lactic acid and at the same time the

391  removal of oligosaccharides and partially of proteins and monosaccharides.

392

393  3.2.2 Acidification of fermentation broth by cation exchange chromatography

394       In lactic acid fermentations, the pH of the culture is normally controlled to prevent

395  microbial growth inhibition and ensure an adequate growth of lactic acid bacteria [44]. NaOH

396  is often selected as the neutralising agent because of its low cost and the fact that no gypsum

397  is generated as a by-product which is the case when $Ca(OH)_2$ is used [45]. As a result, sodium

398  lactate is formed in the fermentation broth. In order to recover lactic acid by anion exchange

399  chromatography, the pH of the broth needs to be reduced to below pH 3 in order for lactic acid

400  to be in its undissociated form rather than in the form of salt. To achieve this and taking into

401  account that the pH of the fermentation broth used in this study was ~5.5, the fermentation

402  broth was passed through the Amberlite® IRA120 (H⁺) cation exchange resin, in order to

403  exchange the sodium ions and release lactate ions into the solution. Figure 10a depicts the

404  compositional profile of the solution during passing through the resin.

18

405        In cation exchange chromatography, no eluent is needed as lactic acid does not bind to

406        Amberlite IRA® 120 (H+). Amberlite IRA® 120 is a strong acidic cation exchange resin that

407        contains sulfonic acid functional groups. It is assumed that the hydrogen ions (H+) that are

408        attached to the resin's functional group will bind to sodium lactate and convert it to lactic acid

409        in the solution. At the same time, the cation of the lactate salt (Na+) will be transferred to the

410        cation exchange resin and transformed through the reaction: $P-H^+ + Na^+La^- \rightarrow P-Na^+ + H^+La^-$,

411        where P is the polymer matrix [46]. In the first 12 ml of the fractions collected, no organic

412        acids and sugars were detected and the pH of the collected fraction was around 6.5, indicating

413        that the fraction contained only water (already present in the column). The pH of the solution

414        then dropped sharply and when it reached ~2.3, the presence of D-lactic acid and other

415        compounds such as acetic acid, xylose and arabinose were detected. The pH of eluate in the

416        collected fraction dropped further with time, to ~1.5, indicating that sodium lactate was

417        successfully converted to undissociated lactic acid. Fractions that had pH below 3 were then

418        pooled together yielding a fraction with a pH of 1.67.

419

420        3.2.3 Recovery of D-lactic acid by anion exchange chromatography resin

421        The undissociated form of D-lactic acid collected from Amberlite® IRA120 (H+) was

422        then subjected to Amberlite® IRA67 (Cl-). According to Bishai et al. [16], Amberlite® IRA67

423        has significant commercial potential as it is a robust resin where the amines present in the

424        functional group do not easily detach from the polymer matrix, it is easy to regenerate, and

425        provides higher recovery of lactic acid compared to other resins. The results from this study

426        (Figure 10b) show that during the binding stage, no lactic acid was present in the collected

427        fraction, while other compounds such as xylose and arabinose were detected. The pH of the

428        effluent collected during the binding stage was around 5.5 to 6.0, indicating that the organic

429        acids present in the fermentation broth (lactic acid and acetic acid) were adsorbed to the resin.

19

430    After washing the column with water, lactic acid was desorbed with 0.5 M HCl. The first acid

431    to be eluted was acetic acid, followed by lactic acid. Fractions that contained only lactic acid

432    were pooled together.

433    Table 4 presents the recovery and purity of D-lactic acid, as well as the percentage of

434    total sugars and protein removal after each stage of the purification process. After the

435    fermentation broth was treated with 7% (w/v) activated carbon, a ~96% recovery of D-lactic

436    acid recovery was observed and a 9.6% increase in D-lactic acid purity, the latter due to the

437    complete removal of oligosaccharides and of 55% of the proteins that were initially present in

438    the fermentation broth. During the cation exchange stage, a ~93% recovery of D-lactic acid

439    compared to the D-lactic acid concentration in the fermentation broth was observed, and

440    although only 1% increase in D-lactic acid purity was detected, ~21% of total sugar and ~44%

441    of proteins were successfully removed at this stage. Cation exchange did not increase the D-

442    lactic acid purity as its main objective was to acidify the fermentation broth. During the anion

443    exchange step a ~80.4% recovery of D-lactic acid was observed, while 74% of the sugars were

444    removed, leading to a cumulative sugar removal of 100%. As a consequence, the purity of D-

445    lactic acid in the eluent was significantly increased during this step, reaching 91.8%, with the

446    remaining components consisting most likely of small amounts of organic molecules, e.g.

447    proteins, acetic acid and other microbial metabolites. Optical purity values for D-lactic above

448    92% acid are considered appropriate for the synthesis of PLA [44] and demonstrate that the

449    proposed multi-step downstream process has significant potential for scaling up and

450    commercialisation.

451

452    3.3    PDLA synthesis

453        The azeotropic dehydration polycondensation method was employed using toluene as

454    solvent and tin (ll)-2-ethylhexanoate (stannous octoate) as catalyst during polymerisation of D-

455    lactic acid obtained from microbial fermentation of DDGS. Tin compounds and protonic acid

456    have been found to be the best catalysts for the direct polycondensation of high molecular

457    weight PLA [27]. However, stannous octoate is preferable as it is approved by U. S. Food and

458    Drug Administration (FDA) in the list of Indirect Additives used in Food Contact Substances

459    under the Code of Federal Regulation, Title 21 (Food and Drugs). In addition to that, the

460    removal of water is crucial in the direct polycondensation process, as its presence could initiate

461    transesterification reactions or chain terminating reactions of the PLA produced [26, 47].

462    During azeotropic polycondensation, the water that was present in the feedstock (D-lactic acid)

463    or generated during the polymerisation process was continuously distilled off from the reaction

464    mixture through the Dean-stark trapped apparatus.

465        After 80 h of polymerisation, a solid yellowish crystal PDLA was produced with an

466    average molecular weight of 3010 Da with a PDI of 4.1, categorised as low molecular weight

467    PLA. In contrast, Kim and Woo [48] produced ten times higher molecular weight of PLLA

468    (33000 Da) after 72 h reaction when the same polycondensation method was used. A similar

469    finding was reported by Marques et al. [49], where 80,000 Da molecular weight of PLLA was

470    produced after 70 h of polymer synthesis. The differences in the molecular weight produced in

471    both cases might be due to the differences in the type of catalyst (tin (ll) chloride dihydrate)

472    and solvent (*m*-xylene with higher than toluene by 29°C boiling point) used during the

473    polymerisation process in those cases. Moreover, other factors such as the purity of D-lactic

474    acid, minute amounts of carboxylic acids like acetic acid, the presence of small amounts water

475    during the polymerisation process and the reaction temperature might also have contributed to

476    the generation of low molecular weight PDLA [12]. Table 5**Error! Reference source not**

477 **found.** compares the molecular weight of PLA produced from this and other studies, where

478 various synthesis methods, monomers (D- or L- lactic acid) and catalysts were employed.

479       This is the first work demonstrating the synthesis of D-lactic acid polymer (PDLA)

480 employing either a direct method or a one-step polymerisation (azeotropic polycondensation)

481 process. Pivsa-Art et al. [50] synthesised PDLA via a two-step polycondensation process,

482 which included a melt polymerisation step followed by solid state polymerisation, and

483 produced PLA with a molecular weight of 33300 Da. A number of interesting applications for

484 low molecular weight PLA have been proposed in various fields, especially in the biomedical

485 [47] and agricultural fields [51]. In the biomedical field, low molecular weight PLA is used as

486 particles for parental controlled drug release in the human body in the form of microspheres,

487 microcapsules, pellets or tablets. Using this approach, drugs are fabricated in a polymeric

488 device (PLA) and the release of the drug is regulated by either diffusion through the polymer

489 barrier or erosion of the polymer matrix [52]. PLA is preferable compared to other polymers

490 such as polyethylene and silicon rubber, as it does not require surgical retrieval, due to its

491 natural degradation in the body. PLA is degraded by simple non-enzymatic hydrolysis (after

492 exposure to moisture) to its monomer (lactic acid) , which can be metabolised by the human

493 body [47]. Drug delivery using low molecular weight PLA offers advantages over high

494 molecular weight PLA as it has a weak retarding effect. Thus, the risk of material accumulation

495 in tissues is reduced as PLA is relatively fast degraded to lactic acid [53].

496       The same mode of application (controlled release) is also being used in the agricultural

497 field, specifically the agrochemical sector, where PLA can serve as carrier for herbicides and

498 pesticides that are released into the soil. The advantages of using low molecular weight PLA

499 have been demonstrated by Zhao and Wilkins [51], where delayed release of pesticides was

500 observed in the early stages of application, which makes it desirable for sensitive targets such

501 as seed treatment. In their study, bromacil (pesticide) was incorporated into PLA in the form

502 of granules and films, and the delayed release of bromacil was achieved via degradation and

503 erosion of PLA. Since the degraded monomer (lactic acid) is safe and widely distributed in

504 nature, the environmental problems of polymer disposal can be avoided through this approach

505 [6, 51].

506   In addition, Quynh et al. [54] successfully produced PLA with high thermostability

507 when commercial PLLA polymer (10400 Da) was crosslinked with low molecular weight

508 PDLA (9830 Da). The stereo complex formation of PLLA and PDLA was achieved via the

509 melt polycondensation process and increased the melting temperature of PLA from175.6 $^{\circ}$C to

510 218.1 $^{\circ}$C. This finding has widespread the application of PLA in other areas such as in the

511 production of computer casings, automotive components and heat resistant food packaging [2].

512

513 **4. Conclusions**

514   Low molecular weight PDLA (3010 Da) was produced via single step azeotropic

515 polycondensation process from purified D-lactic acid originated from DDGS. The developed

516 downstream process reduced effectively the dark colour of fermentation broth, removed

517 oligosaccharides and converted sodium lactate to undissociated lactic acid. At the end of the

518 purification process, approximately 80.4% D-lactic acid was recovered with 91.8% purity. This

519 study demonstrated that agricultural residues, such as DDGS, hold potential as starting

520 materials for biopolymer production.

521

526

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681

682

Figure Captions

Figure 1: Effect of (a) pH and (b) temperature on lactic acid binding by four anionic resins

Figure 2: Adsorption isotherms of lactic acid to (a) Amberlite® IRA67 and (b) Diaion®

WA30 at 25°C; - - - Langmuir model, …… Freundlich model, ___ Langmuir-Freundlich

model

Figure 3: Elution profiles of lactic acid from the Amberlite® IRA67 resin (saturated with 20 ml

of 30 g/l lactic acid) at different concentrations of HCl at 25°C; (━●━) 0.05M, (━▲━) 0.1M HCl,

(━■━) 0.5M HCl and (✳) 1.0M HCl.

Figure 4: Samples of fermentation broth treated with different concentrations of activated

carbon at 25 °C for 1.5 h

Figure 5: Elution profile of fermentation broth on (a) cation exchange chromatography with

Amberlite IRA120 (H$^+$) resin and (b) adsorption and elution profile of fermentation broth

during anion exchange chromatography with Amberlite® IRA67 (Cl$^-$) resin

706     Table 1: Properties of ion exchange resins (information provided by Sigma-Aldrich, US)

| Ion exchange type | Resin | Strength | Particle size (µm) | Matrix | Active functional group | pH |
|---|---|---|---|---|---|---|
| Anion | Amberlite® IRA67 | Weak basicity | 500-750 | Acrylic (gel) | Tertiary amine | 0 - 7 |
| Anion | Diaion® WA30 | Weak basicity | 680 | Styrene-divinylbenzene (highly porous) | Tertiary amine | 0 - 9 |
| Anion | Amberlite® IRA400 | Strong basicity | 600-750 | Styrene-divinylbenzene (gel) | Quaternary ammonium | 0 - 14 |
| Anion | Dowex® Marathon™ MSA | Strong basicity | 640 | Styrene-divinylbenzene (macroporous) | Quaternary ammonium | 0 - 14 |
| Cation | Amberlite® IRA120 | Strong acidity | 620-830 | Styrene-divinylbenzene (gel) | Sulfonic acid | 0 - 14 |

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719    Table 2: Langmuir, Freundlich and Langmuir – Freundlich isotherm parameters describing
720    the adsorption of lactic acid to Amberlite® IRA67 and Diaion® WA30

| Model | Parameter | Amberlite® IRA67 | Diaion® WA30 |
|---|---|---|---|
|  | $q_{max}$ (mg/g) | 162.09 | 102.47 |
| Langmuir | $K$ | 0.0096 | 0.019 |
|  | $R^2$ | 0.910 | 0.940 |
|  | $K_f$ | 10.88 | 13.379 |
| Freundlich | $n$ | 2.475 | 3.200 |
|  | $R^2$ | 0.801 | 0.800 |
| Langmuir-Freundlich | $q_{max}$ (mg/g) | 136.11 | 91.51 |
|  | $K_{LF}$ | 0.0002 | 0.001 |
|  | $nLF$ | 1.96 | 1.83 |
|  | $R^2$ | 0.940 | 0.965 |

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733 Table 3: Recovery of D-lactic acid, free amino nitrogen, oligosaccharides and
734 monosaccharides after treatment with activated carbon at different concentrations

| Activated carbon (%, w/v) | *Recovery (%) | | | | | |
|---|---|---|---|---|---|---|
| | D-lactic acid | Free amino nitrogen | Oligosaccharides | | Monosaccharides | |
| | | | Xylotriose | Xylobiose | Xylose | Arabinose |
| 0 | 100 ± 0.0 | 100 ± 2.5 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 | 100 ± 0.0 |
| 1 | 95 ± 0.4 | 94.0 ± 0.2 | 77.4 ± 0.5 | 87.6 ± 0.0 | 90.4 ± 0.9 | 83.4 ± 1.6 |
| 5 | 90 ± 0.3 | 83.6 ± 1.3 | 0 | 42.8 ± 1.0 | 85.7 ± 0.6 | 79.9 ± 0.9 |
| 7 | 88 ± 3.4 | 83.0 ± 1.2 | 0 | 0 | 82.2 ± 3.0 | 76.7 ± 2.7 |
| 10 | 85 ± 1.9 | 80.2 ± 2.8 | 0 | 0 | 72.7 ± 1.7 | 70.0 ± 1.4 |

735 *Initial concentration in the fermentation broth: D- lactic acid, 25.9 g/l; xylotriose, 1.0 g/l; xylobiose,
736 3.8 g/l; xylose, 4.8 g/l; arabinose, 0.5 g/l and free amino nitrogen, 152.8 mg/l

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743 Table 4: Recovery and purity of D-lactic acid from the fermentation broth during the different downstream processing stages

| Purification stages | Volume (ml) | D-lactic Acid | | | Total Oligosaccharides | | Total Monosaccharides | | Free amino nitrogen | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | g/l | Recovery (%) | Purity* (%) | g/l | Cumulative removal (%) | g/l | Cumulative removal (%) | mg/l | Cumulative removal (%) |
| Fermentation broth | 50 | 25.9 | 100 | 54.6 | 4.5 | 0 | 5.3 | 0 | 152.8 | 0 |
| 7% Activated carbon | 92 | 13.5 | 95.9 | 64.2 | 0 | 100 | 2.7 | 4.5 | 37.8 | 55 |
| Cation exchange chromatography (Amberlite® IRA120, H⁺) | 132 | 9.1 | 92.5 | 65.2 | 0 | 100 | 1.7 | 26.2 | 0.6 | 99 |
| Anion exchange chromatography (Amberlite® IRA67, Cl⁻) | 29 | 36.0 | 80.4 | 91.8 | 0 | 100 | 0 | 100 | 0.4 | 99 |

744 *The purity (%) of lactic acid in the eluate was determined according to Equation

745 Table 5: Comparisons of molecular weight of PLA produced form this and other studies,
746 using different synthesis methods, catalysts and monomers were.

| Method | Lactic acid form | Catalyst | Polymer | Molecular weight (Da) | Reference |
|---|---|---|---|---|---|
| Azeotropic polycondensation | D | Tin (ll)-2-ethylhexanoate, $SnC_{16}H_{30}O_4$ | PDLA | 3010 | This study |
| | L | Tin (ll) chloride dihydrate, $SnCl_2.2H_2O$ | PLLA | 33000 | [48] |
| | L | Tin (ll) chloride dihydrate, $SnCl_2.2H_2O$ | PLLA | 80000 | [49] |
| Direct polycondensation | L | - | PLLA | 90000 | [55] |
| | L | Antimony trioxide, $Sb_2O_3$ | PLLA | 67000 | [56] |
| Melt polymerisation | DL | Tin (ll) chloride, $SnCl_2$ | PDLLA | 4100 | [57] |
| 1.Solid state polymerisation (SSP) 2.Melt polymerisation | L | Creatinine, CR | PLLA | 120000 | [58] |
| 1. Melt polymerisation 2. Solid state polymerisation (SSP) | D | 2-Naphthalenesulfonic acid (2-NSA) | PDLA | 33300 | [50] |
| 1.Ring opening polymerisation (ROP) 2.Chain extension | L | 1.Tin (ll)-2-ethylhexanoate, $C_{16}H_{30}O_4Sn$ 2.Hexamethylene diisocyanate, HDI | PLLA | 203000 | [59] |
| Enzymatic polymerisation | DL | - | PDLLA | 2400 | [60] |
| | L | - | PLLA | 4500 | [61] |

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(a)



(b)



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753                                    Figure 6

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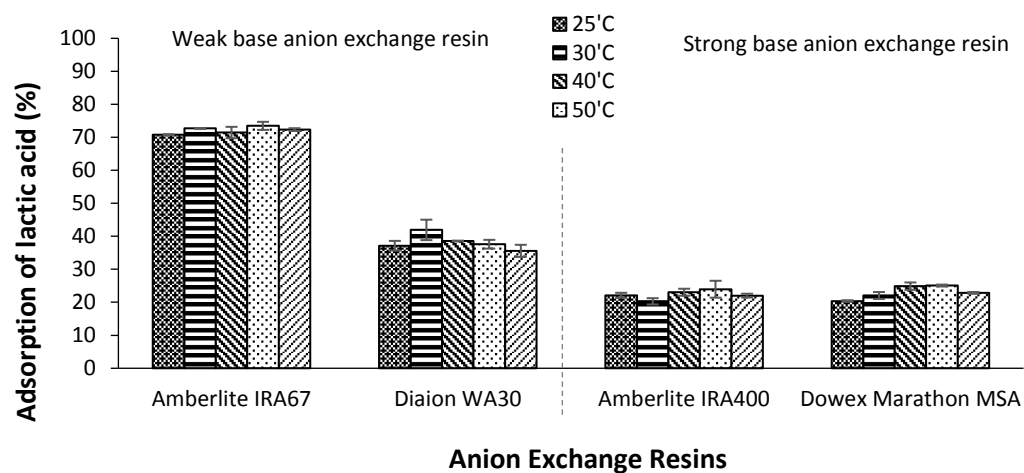
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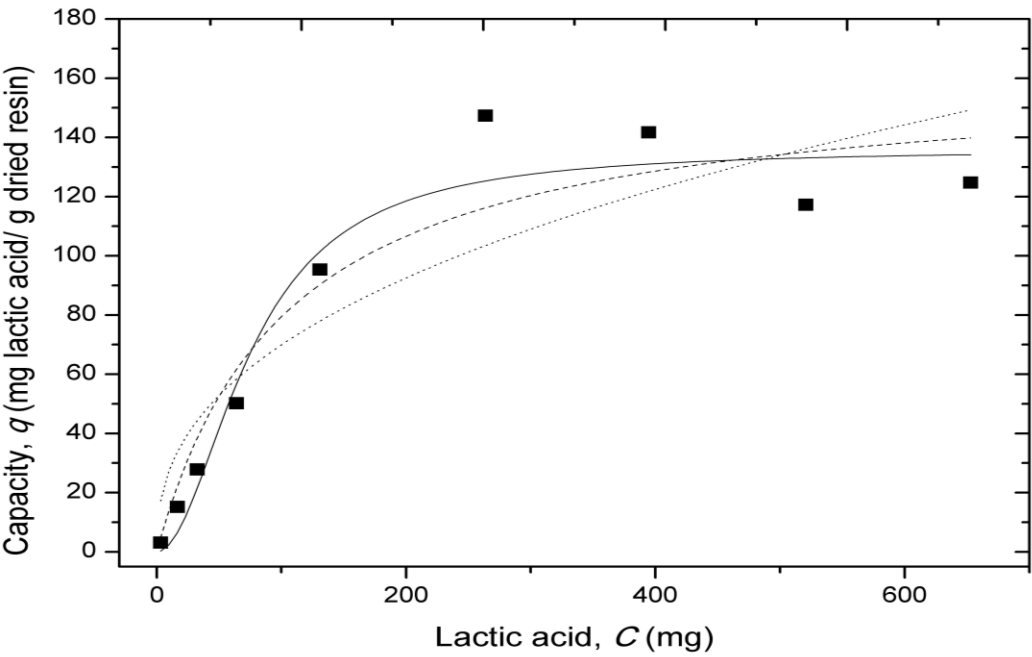
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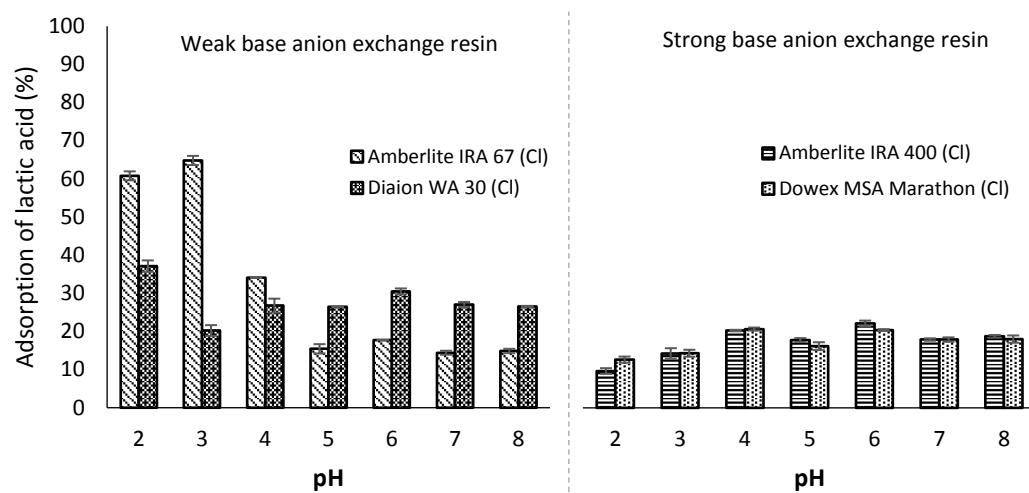
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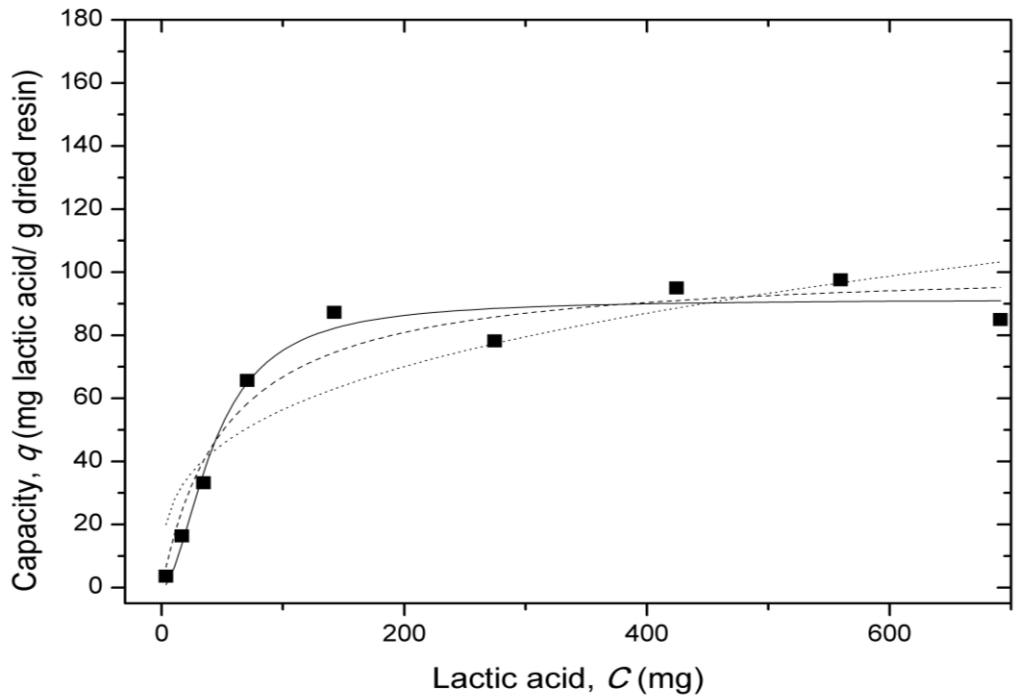760          (a)



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762          (b)



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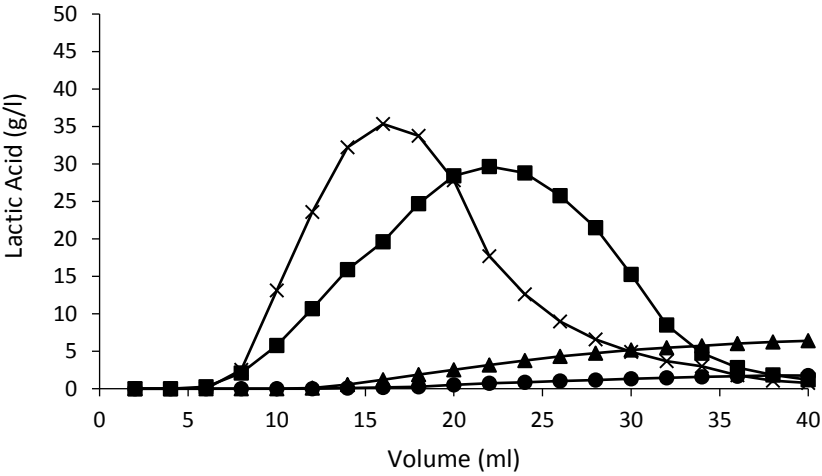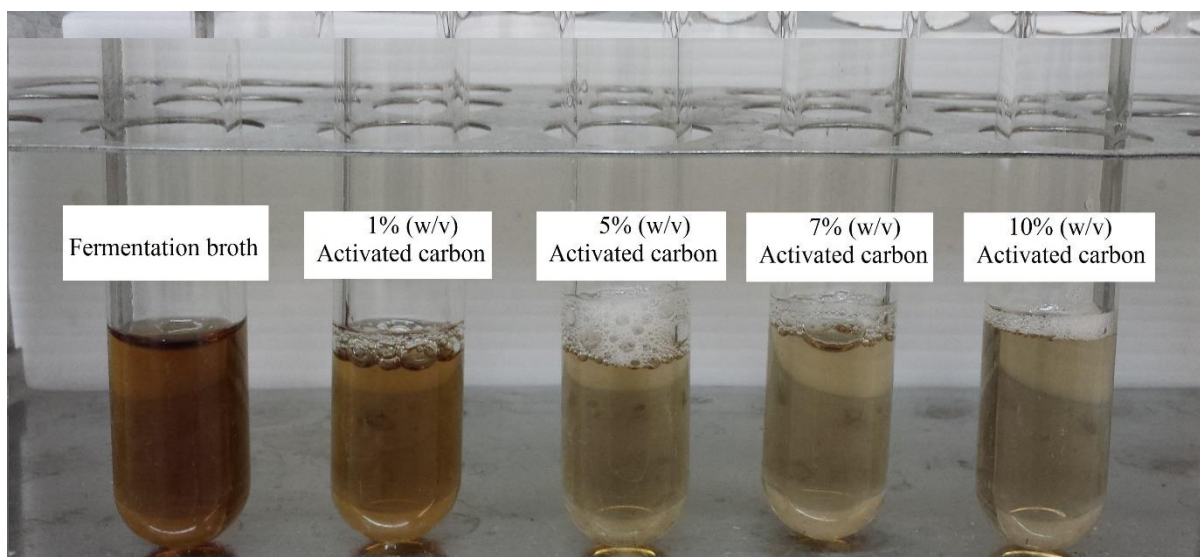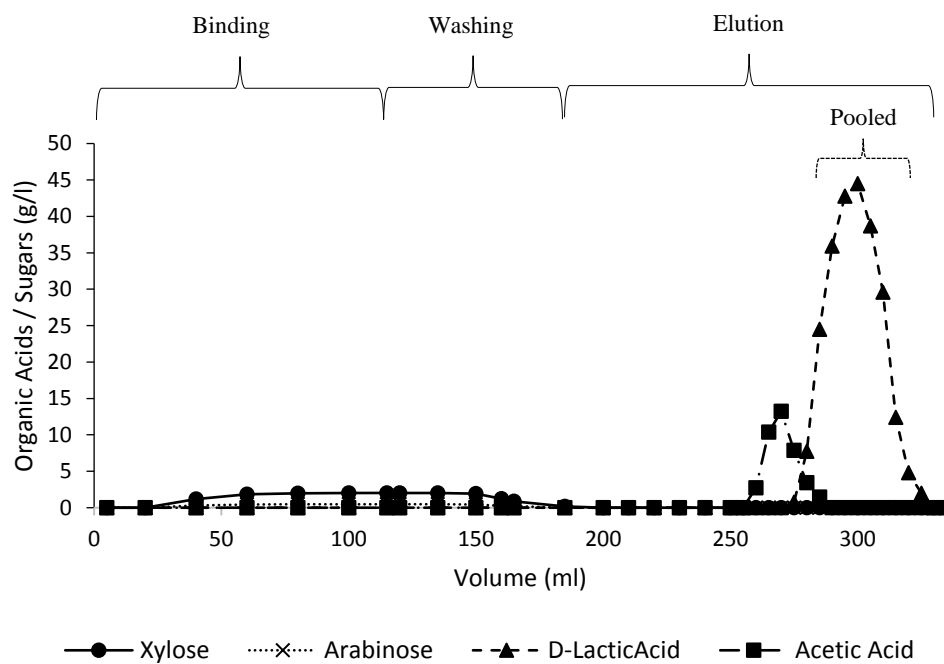764                                        Figure 7

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Figure 8

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Figure 9

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804                                    Figure 10

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