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

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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.

Keywords: purification, activated carbon, ion exchange resin, poly-D-lactic acid, azeotropic polycondensation, DDGS

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

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

requires high purity of the lactide monomer prior to PLA synthesis [25]. On the other hand, direct polycondensation offers significant advantages as the polymerisation process is simpler and easier in this case. In direct polycondensation, only one step for polymer synthesis is involved, during which the lactic acid solution is heated at 130 – 140 ^{o}C. Through this process, normally low molecular weight PLA (< 5000 Da) is produced with relatively weak mechanical properties; this is due to difficulties in removing the water from the reaction mixture as the polymerisation process progresses [26]. However, Ajioka, Enomoto, Suzuki and Yamaguchi [27] successfully produced high molecular weight PLA (> 300000 Da) using a single step synthesis using organic solvent with a catalyst (tin, Sn, powder) in azeotropic condition. Azeotropic polycondensation involved refluxing of the solvent under reduced pressure to remove the condensation water that was generated during polymer synthesis. This method had been patented and used by Mitsui Toatsu Chemicals (Japan) to synthesise PLA under the commercial name LACEA [6, 23].

The aim of the study was to develop a multi-step process for the purification of D-lactic acid from a fermentation broth based on dried distiller's grains with solubles (DDGS) hydrolysate [28]. Subsequently, the purified D-lactic acid was used as monomer for PDLA synthesis by employing an azeotropic polycondensation approach. As the purification of D-lactic acid from fermentation broth hydrolysates and its polymerisation process is rarely been reported, this study provides novel information on D-lactic acid separation, employing a multiple purification step followed by single step polymerisation process.

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 ^{o}C for 10 minutes, followed by centrifugation at 17,105 *x g* for 20 minutes (4 ^{o}C). Supernatants containing D-lactic acid solutions were collected and kept at -20 ^{o}C for purification.

2.3 Resin preparation

Weak anion exchange resins (Amberlite® IRA67 and Diaion® WA30) and strong anion exchange resins (Amberlite® IRA400 and Dowex® Marathon™ MSA) were selected for this study. Before utilisation, the resins in free base form, were first converted to Cl^- form as described by Moldes et al. [29]. Resins that were purchased in Cl^- form were only washed with distilled water [11, 30]. For the acidification of the fermentation broth, a cation exchange resin, Amberlite® IRA120, a strongly acidic resin in H^+ form, was used. The resin was washed with distilled water three times to remove any contaminants. All resins were then oven dried at 50 °C overnight and stored at room temperature in closed containers before use. The properties of the ion exchange resins that were used in this study are presented in Table 1.

2.4 Screening and optimisation of anion exchange resins binding and recovery

For the screening experiments of the anion exchange resins, model lactic acid solutions were prepared using commercial lactic acid (85%, Food Chemical Codex, FCC, Sigma-Aldrich).

2.4.1 Effect of pH on lactic acid binding

The effect of pH on lactic acid binding was determined through batch experiments according to Bishai et al. [16] with slight modifications. 1 g of dried resin (Amberlite® IRA67, Diaion® WA30, Amberlite® IRA400 or Dowex® Marathon™ MSA) was mixed with 5 ml of lactic acid (50 g/l) at different initial pH conditions (2, 3, 4, 5, 6, 7 and 8) at 25 ^oC. The pH of 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]:

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

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

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

(4 - 650 mg lactic acid per 5 ml). The initial pH of the lactic acid solutions was set taking into account their optimum binding pH (obtained in Section 2.4.1) for the respective resin. The reaction took place at optimum temperature (obtained in Section 2.4.2) at 200 rpm for 8 hours. Liquid samples from each mixture were filtered and collected for further analysis.

Three different nonlinear models, the Langmuir, Freundlich and Langmuir - Freundlich models, were then fitted to the data using Origin Pro 8.0 software (OriginLab, USA) [32] using the following equations:

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

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

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

where, q is the amount of lactic acid adsorbed to the resin (mg/g), q_{max} is the maximum amount of lactic acid adsorbed to the resin (mg/g), K is the Langmuir adsorption constant, K_f is the Freundlich adsorption constant, K_{LF} is the affinity constant for adsorption, C is the amount of lactic acid (mg), n is the Freundlich adsorption constant and nLF is the Langmuir-Freundlich coefficient.

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

The resin that exhibited the highest binding capacity, q, (Amberlite® IRA67) was selected for further recovery experiments using HCl with different ionic strengths as eluent. 4 g of Amberlite® IRA67 were packed into a 100 mm (length) column (Fisher Scientific, Leicester, UK) and saturated with 20 ml of 30 g/l lactic acid solution. The resin was then washed with distilled water to remove any unbound lactic acid. Different concentrations of HCl

were used as eluent (0.05, 0.1, 0.5 and 1.0 M), which were passed down the packed column by gravity and fractions of effluents were collected (2 ml per fraction) at regular time intervals. All fractions were analysed for lactic acid concentration.

2.5 Purification of D-lactic acid from fermentation broth

2.5.1 Colour removal by activated carbon

The effect of activated carbon on the removal of the colour from the fermentation broth was determined. Powdered activated carbon was mixed with 5 ml of clarified fermentation broth at different loading concentrations (0, 1, 5, 7 and 10%, w/v), for 1.5 hours at 150 (25 $^{\circ}$C). The mixture was then separated by centrifugation at 17,105 *x g* for 10 minutes (4 $^{\circ}$C). The pellet was washed twice with distilled water and the supernatants collected for sugar and lactic acid analysis.

2.5.2 Acidification of fermentation broth by cation exchange resin

After treatment with activated carbon, the fermentation broth was subjected to Amberlite® IRA 120, H^+ resin aiming to convert sodium lactate into lactic acid [33]. 10 g of dried resin were packed into a 30 cm length Econo-glass column (i.d. 1 cm) which was first filled with distilled water and the height of the resin was fixed with a flow adaptor (i.d. 1 cm) (Biorad, California, US). Distilled water was allowed to pass through the column until the pH of effluent was around 6.5. Then, a constant flow of 3 ml/min using a peristaltic pump was applied. The fermentation broth containing sodium lactate with a pH around 5.5 was then pumped into the column at the same flow rate. When the pH of the effluent started to increase, the resin was considered saturated. Effluent fractions (4 ml each) were collected for lactic acid

and sugar analysis. Fractions containing D-lactic acid were pooled for subsequent purification. The resin was washed with distilled water to remove the remaining solution in interstitial spaces, regenerated with 1 M HCl and thoroughly rinsed with distilled water, before a new cycle [16].

2.5.3 Adsorption by anion exchange resin

Pooled supernatant fractions containing D-lactic acid were passed through an anion exchange resin, (Amberlite® IRA67) in fixed-bed column operation. 25 g of dried resin were packed into a 30 cm length Econo-glass column (i.d. 2.5 cm) which was first filled with distilled water and the upper side of resin was fixed with a flow adaptor (i.d. 2.5 cm). The system was washed with distilled water until the pH of the effluent was around 6.5. Then, the acidified broth obtained from Amberlite® IRA 120, H^+ resin was pumped into the column at 3 ml/min. D=lactic acid was recovered with 0.5 M HCl. Fractions of effluents at each stage were collected (4 ml each) for lactic acid and sugar analysis.

2.6 Azeotropic polycondensation process of PDLA

Polycondensation of PDLA was conducted as described by Ajioka et al. [27] with minor modifications. 2 g of D-lactic acid were mixed with 40 ml toluene in 100 ml reaction flask, equipped with a Dean-stark apparatus and a magnetic stirrer. In the first step of the polycondensation process, the mixture was azeotropically dehydrated at 110 ^{o}C for 3 hours to remove the free water. After removing the condensed water that was trapped in the Dean-stark apparatus, the tube was packed with molecular sieve (4 Å) and calcium chloride in layers to remove small amounts of water dissolved in the organic solvent that was produced during PLA synthesis. 0.2 g tin (ll)-2-ethylhexanoate (stannous octoate) was added to the reaction mixture

and then returned to reflux at 140 ºC for another 80 hours. The polymer produced was then recovered by filtration and vacuum dried using Bünchner funnel apparatus, followed by freeze drying (VisTis Sentry 2.0, Warminster, PA).

2.6 Analytical methods

The nitrogen content was determined using the Free Amino Nitrogen (FAN) method as described by Lie [34] with some modifications. 0.5 ml of diluted sample was mixed with 0.25 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 of KH_2PO_4 dissolved in 1 l of distilled water; pH 6.6 – 6.8) in 2 ml Eppendorf tube. The mixture was heated at 100 ºC in a thermal block (Grant, Cambridge) for exactly 16 minutes and immediately cooled in an ice bath. 1.5 ml of dilution reagent (2 g potassium iodate, KIO_3, in 616 ml distilled water and 384 ml 96% ethanol) was added and the free amino nitrogen content was measured at 570 nm. A calibration curve was constructed using glycine at different concentrations (0.25 – 2 mg/l) as standard.

Sugar and lactic acid concentrations were analysed by high performance liquid chromatography (HPLC) in an Agilent Infinity 1260 system (Agilent Technologies, USA) equipped with an Aminex HPX-87H column (Bio-rad, Hercules, CA) at a 0.6 ml/min flow rate with 5 mM H_2SO_4 as mobile phase. The temperature of the column was set at 65 ˚C and sugars and lactic acid were detected using a refractive index detector. The D-lactic acid recovery and purity were calculated using the following equations [16]:

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

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

The molecular weight and poly dispersity index (PDI) of PDLA was determined by gel permeation chromatography (GPC), using an Agilent 1100 Series chromatography system that was equipped with a refractive index RID 1200 detector (35 ^{o}C). The flow rate was set at 1.0 ml/min and the molecular size was determined using a PL gel 5µM mixed-D column (300 x 7.5 mm) and a PL gel 5µM guard column (50 x 7.5 mm). Chloroform was used as the eluent. The PDLA obtained was first dissolved in chloroform prior to analysis. The sample was analysed at room temperature using 20 µl injection volume. A calibration curve was generated using polystyrene standards with molecular weights ranging from 580 to 483,400 Da.

3. Results and Discussion

3.1 Selection of anion exchange resin

When considering ion exchange chromatography, the efficiency of the product adsorption by the resin determines the success of the purification process. Therefore, factors such as pH, temperature and lactic acid concentration were initially investigated in this study in order to select the most appropriate anion exchange resin. The effect of the ionic strength of the eluent (HCl solution) on product recovery was also investigated. Before use, the resins that existed in free base were converted to Cl^- form. The Cl^- form was selected as previous studies have shown that in this form the resins exhibit the highest adsorption capacity for lactic acid [17, 31]. Moreover, the simultaneous lactic acid recovery and resin regeneration for subsequent adsorption cycles by HCl, reduces the number of steps involved in the purification process [29]. Four different resins, categorised into weak base anion exchange (Amberlite® IRA67 and 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 ^{o}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

exchange resins is mainly attributed to pKa changes of the targeted compound as a result of the temperature change. In the case of lactic acid, as the temperature increased from 25 to 30, 40 and 50ºC, the pKa value also increased to 3.896, 3.942 and 4.028, respectively [38]. Since lactic acid adsorption to weak base anion exchange resins occurs below its pKa value, the increase in temperature from 25 to 60 ˚C did not have any significant effect.

3.1.1 Adsorption isotherms

The two resins that demonstrated the highest binding capacity, Amberlite® IRA67 and Diaion® WA30, were further tested. The adsorption isotherms were generated at 25 ºC, and are shown in

Figure 7a and 2b, respectively. Different kinetic models (Langmuir, Freundlich and Langmuir – Freundlich) were then used to fit the data, and the model parameters are presented in Table 2.

The Langmuir and Langmuir – Freundlich models fitted better the data for both resins ($R^2 > 0.9$), compared to the Freundlich model ($R^2 = 0.80$). The Langmuir – Freundlich model showed better fit than the Langmuir model for both the Amberlite® IRA67 ($R^2 = 0.94$) and the Diaion® WA30 resin ($R^2 = 0.965$). In the Langmuir model, it is assumed that the adsorption of a given adsorbate occurs as a monolayer sorption onto the surface of a resin containing a finite number of identical binding sites. The adsorbent has uniform binding sites and the adsorbate will only bind to the binding site [32, 39]. In the Freundlich model, it is assumed that the binding of the adsorbate molecules onto the adsorbent is at infinite capacity. The adsorption is not uniform and can occur in a multilayer, with the binding site that has stronger bond energy being occupied first [39]. In the present study, the adsorption capacity of the resin was specific due to the strong interactions between lactic acid and the functional groups present in the Cl^- 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

role for the detachment of lactic acid from the resin. Based on these results, 0.5 M HCl was selected to recover lactic acid in subsequent experiments.

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

3.2.1 Colour removal by activated carbon

After determining the conditions leading to maximum lactic acid adsorption and recovery using the Amberlite® IRA67 resin, the aim was to purify lactic acid from fermentation broths of *L. coryniformis* subsp. *torquens*, where DDGS hydrolysate was used as the fermentation medium. Fermentation broths usually contain besides the component of interest, residual sugars and proteins, as well as by-products of the fermentation process. In this particular case, fermented DDGS hydrolysate contained residual sugars (xylotriose, xylobiose, xylose, arabinose), organic nitrogen in the form of proteins, peptides or amino acids, polyphenols and acetic acid, all of which can contributed into the dark brown colour of the fermentation broth [41]. It is likely that a significant proportion of the dark brown colour of the medium is due to the dark colour of DDGS generated during the drum drying step in the DDGS production process. Therefore, prior to ion exchange purification, the fermented DDGS hydrolysate was initially subjected to activated carbon treatment. Figure 9 shows the effect of various activated carbon concentrations on the colour of the fermentation broth. A positive correlation can be observed as the activated carbon concentrations increased up to 5% (w/v) and the colour of the fermentation broths became notably lighter. For higher activated carbon concentrations (7 and 10%, w/v) no significant changes in colour were observed.

The effect of activated carbon concentration on the recovery of D-lactic acid, protein, oligosaccharides and monosaccharides is shown in Table 3. The recovery of D-lactic acid gradually decreased as the concentration of activated carbon increased. More specifically at 1,

5, 7 and 10% (w/v) of activated carbon, 95, 90, 88 and 85% lactic acid was recovered from the fermentation broth, respectively. A relatively small reduction in monosaccharides and proteins concentrations was overall noted (< 30% removal) when the fermentation broth was treated with activated carbon, even as high as 10% (w/v). Stone and Kozlov [42] reported that activated carbon can only adsorb low molecular weight proteins, in which is in line with protein removal data obtained in this study. On the other hand, oligosaccharides (xylotriose and xylobiose) were completely removed at 7% (w/v) activated carbon. According to Boon et al. [43], activated carbon has higher affinity for trisaccharides and disaccharides compared to monosaccharides with a capacity of 133 mg/g and 117 mg/g of activated carbon, respectively. Based on the above, 7% (w/v) activated carbon was selected as the best concentration to treat the fermentation broth to ensure a satisfactory recovery of lactic acid and at the same time the removal of oligosaccharides and partially of proteins and monosaccharides.

3.2.2 Acidification of fermentation broth by cation exchange chromatography

In lactic acid fermentations, the pH of the culture is normally controlled to prevent microbial growth inhibition and ensure an adequate growth of lactic acid bacteria [44]. NaOH is often selected as the neutralising agent because of its low cost and the fact that no gypsum is generated as a by-product which is the case when $Ca(OH)_2$ is used [45]. As a result, sodium lactate is formed in the fermentation broth. In order to recover lactic acid by anion exchange chromatography, the pH of the broth needs to be reduced to below pH 3 in order for lactic acid to be in its undissociated form rather than in the form of salt. To achieve this and taking into account that the pH of the fermentation broth used in this study was ~5.5, the fermentation broth was passed through the Amberlite® IRA120 (H^+) cation exchange resin, in order to exchange the sodium ions and release lactate ions into the solution. Figure 10a depicts the compositional profile of the solution during passing through the resin.

In cation exchange chromatography, no eluent is needed as lactic acid does not bind to Amberlite IRA® 120 (H^+). Amberlite IRA® 120 is a strong acidic cation exchange resin that contains sulfonic acid functional groups. It is assumed that the hydrogen ions (H^+) that are attached to the resin's functional group will bind to sodium lactate and convert it to lactic acid in the solution. At the same time, the cation of the lactate salt (Na^+) will be transferred to the cation exchange resin and transformed through the reaction: $P\text{-}H^+ + Na^+La^- \rightarrow P\text{-}Na^+ + H^+La^-$, where P is the polymer matrix [46]. In the first 12 ml of the fractions collected, no organic acids and sugars were detected and the pH of the collected fraction was around 6.5, indicating that the fraction contained only water (already present in the column). The pH of the solution then dropped sharply and when it reached ~2.3, the presence of D-lactic acid and other compounds such as acetic acid, xylose and arabinose were detected. The pH of eluate in the collected fraction dropped further with time, to ~1.5, indicating that sodium lactate was successfully converted to undissociated lactic acid. Fractions that had pH below 3 were then pooled together yielding a fraction with a pH of 1.67.

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

The undissociated form of D-lactic acid collected from Amberlite® IRA120 (H^+) was then subjected to Amberlite® IRA67 (Cl^-). According to Bishai et al. [16], Amberlite® IRA67 has significant commercial potential as it is a robust resin where the amines present in the functional group do not easily detach from the polymer matrix, it is easy to regenerate, and provides higher recovery of lactic acid compared to other resins. The results from this study (Figure 10b) show that during the binding stage, no lactic acid was present in the collected fraction, while other compounds such as xylose and arabinose were detected. The pH of the effluent collected during the binding stage was around 5.5 to 6.0, indicating that the organic acids present in the fermentation broth (lactic acid and acetic acid) were adsorbed to the resin.

After washing the column with water, lactic acid was desorbed with 0.5 M HCl. The first acid to be eluted was acetic acid, followed by lactic acid. Fractions that contained only lactic acid were pooled together.

Table 4 presents the recovery and purity of D-lactic acid, as well as the percentage of total sugars and protein removal after each stage of the purification process. After the fermentation broth was treated with 7% (w/v) activated carbon, a ~96% recovery of D-lactic acid recovery was observed and a 9.6% increase in D-lactic acid purity, the latter due to the complete removal of oligosaccharides and of 55% of the proteins that were initially present in the fermentation broth. During the cation exchange stage, a ~93% recovery of D-lactic acid compared to the D-lactic acid concentration in the fermentation broth was observed, and although only 1% increase in D-lactic acid purity was detected, ~21% of total sugar and ~44% of proteins were successfully removed at this stage. Cation exchange did not increase the D-lactic acid purity as its main objective was to acidify the fermentation broth. During the anion exchange step a ~80.4% recovery of D-lactic acid was observed, while 74% of the sugars were removed, leading to a cumulative sugar removal of 100%. As a consequence, the purity of D-lactic acid in the eluent was significantly increased during this step, reaching 91.8%, with the remaining components consisting most likely of small amounts of organic molecules, e.g. proteins, acetic acid and other microbial metabolites. Optical purity values for D-lactic above 92% acid are considered appropriate for the synthesis of PLA [44] and demonstrate that the proposed multi-step downstream process has significant potential for scaling up and commercialisation.

3.3 PDLA synthesis

The azeotropic dehydration polycondensation method was employed using toluene as solvent and tin (ll)-2-ethylhexanoate (stannous octoate) as catalyst during polymerisation of D-lactic acid obtained from microbial fermentation of DDGS. Tin compounds and protonic acid have been found to be the best catalysts for the direct polycondensation of high molecular weight PLA [27]. However, stannous octoate is preferable as it is approved by U. S. Food and Drug Administration (FDA) in the list of Indirect Additives used in Food Contact Substances under the Code of Federal Regulation, Title 21 (Food and Drugs). In addition to that, the removal of water is crucial in the direct polycondensation process, as its presence could initiate transesterification reactions or chain terminating reactions of the PLA produced [26, 47]. During azeotropic polycondensation, the water that was present in the feedstock (D-lactic acid) or generated during the polymerisation process was continuously distilled off from the reaction mixture through the Dean-stark trapped apparatus.

After 80 h of polymerisation, a solid yellowish crystal PDLA was produced with an average molecular weight of 3010 Da with a PDI of 4.1, categorised as low molecular weight PLA. In contrast, Kim and Woo [48] produced ten times higher molecular weight of PLLA (33000 Da) after 72 h reaction when the same polycondensation method was used. A similar finding was reported by Marques et al. [49], where 80,000 Da molecular weight of PLLA was produced after 70 h of polymer synthesis. The differences in the molecular weight produced in both cases might be due to the differences in the type of catalyst (tin (ll) chloride dihydrate) and solvent (*m*-xylene with higher than toluene by 29°C boiling point) used during the polymerisation process in those cases. Moreover, other factors such as the purity of D-lactic acid, minute amounts of carboxylic acids like acetic acid, the presence of small amounts water during the polymerisation process and the reaction temperature might also have contributed to the generation of low molecular weight PDLA [12]. Table 5**Error! Reference source not**

found. compares the molecular weight of PLA produced from this and other studies, where various synthesis methods, monomers (D- or L- lactic acid) and catalysts were employed.

This is the first work demonstrating the synthesis of D-lactic acid polymer (PDLA) employing either a direct method or a one-step polymerisation (azeotropic polycondensation) process. Pivsa-Art et al. [50] synthesised PDLA via a two-step polycondensation process, which included a melt polymerisation step followed by solid state polymerisation, and produced PLA with a molecular weight of 33300 Da. A number of interesting applications for low molecular weight PLA have been proposed in various fields, especially in the biomedical [47] and agricultural fields [51]. In the biomedical field, low molecular weight PLA is used as particles for parental controlled drug release in the human body in the form of microspheres, microcapsules, pellets or tablets. Using this approach, drugs are fabricated in a polymeric device (PLA) and the release of the drug is regulated by either diffusion through the polymer barrier or erosion of the polymer matrix [52]. PLA is preferable compared to other polymers such as polyethylene and silicon rubber, as it does not require surgical retrieval, due to its natural degradation in the body. PLA is degraded by simple non-enzymatic hydrolysis (after exposure to moisture) to its monomer (lactic acid) , which can be metabolised by the human body [47]. Drug delivery using low molecular weight PLA offers advantages over high molecular weight PLA as it has a weak retarding effect. Thus, the risk of material accumulation in tissues is reduced as PLA is relatively fast degraded to lactic acid [53].

The same mode of application (controlled release) is also being used in the agricultural field, specifically the agrochemical sector, where PLA can serve as carrier for herbicides and pesticides that are released into the soil. The advantages of using low molecular weight PLA have been demonstrated by Zhao and Wilkins [51], where delayed release of pesticides was observed in the early stages of application, which makes it desirable for sensitive targets such as seed treatment. In their study, bromacil (pesticide) was incorporated into PLA in the form

of granules and films, and the delayed release of bromacil was achieved via degradation and erosion of PLA. Since the degraded monomer (lactic acid) is safe and widely distributed in nature, the environmental problems of polymer disposal can be avoided through this approach [6, 51].

In addition, Quynh et al. [54] successfully produced PLA with high thermostability when commercial PLLA polymer (10400 Da) was crosslinked with low molecular weight PDLA (9830 Da). The stereo complex formation of PLLA and PDLA was achieved via the melt polycondensation process and increased the melting temperature of PLA from175.6 ^{o}C to 218.1 ^{o}C. This finding has widespread the application of PLA in other areas such as in the production of computer casings, automotive components and heat resistant food packaging [2].

4. Conclusions

Low molecular weight PDLA (3010 Da) was produced via single step azeotropic polycondensation process from purified D-lactic acid originated from DDGS. The developed downstream process reduced effectively the dark colour of fermentation broth, removed oligosaccharides and converted sodium lactate to undissociated lactic acid. At the end of the purification process, approximately 80.4% D-lactic acid was recovered with 91.8% purity. This study demonstrated that agricultural residues, such as DDGS, hold potential as starting materials for biopolymer production.

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683 Figure Captions

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

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

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

688 model

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

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

692 (—■—) 0.5M HCl and (—✳—) 1.0M HCl.

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

695 carbon at 25 °C for 1.5 h

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

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

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

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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
Langmuir	q_{max} (mg/g)	162.09	102.47
	K	0.0096	0.019
	R^2	0.910	0.940
Freundlich	K_f	10.88	13.379
	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]

747

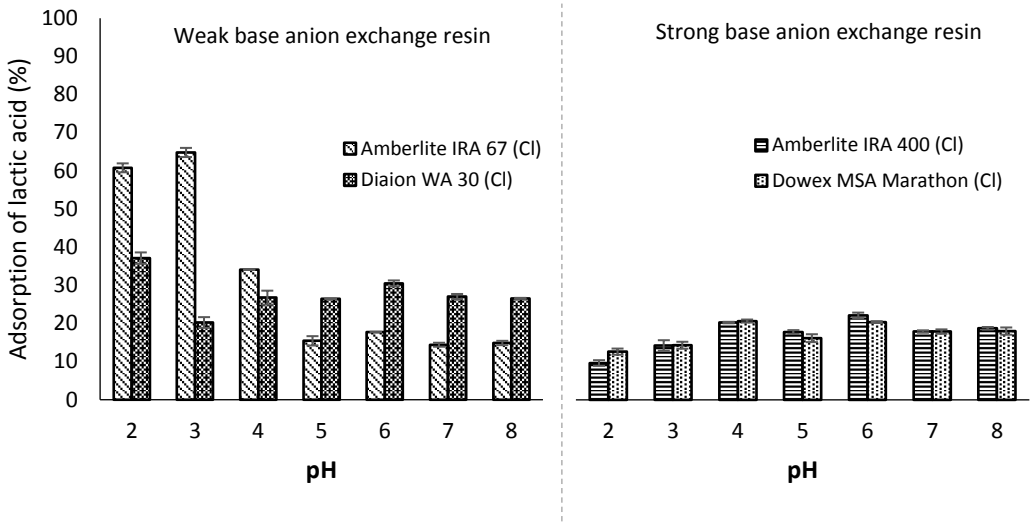
748

749

750

751

(a)


Weak base anion exchange resin
Strong base anion exchange resin
Adsorption of lactic acid (%)
Amberlite IRA 67 (Cl)
Diaion WA 30 (Cl)
Amberlite IRA 400 (Cl)
Dowex MSA Marathon (Cl)
pH
pH

(b)

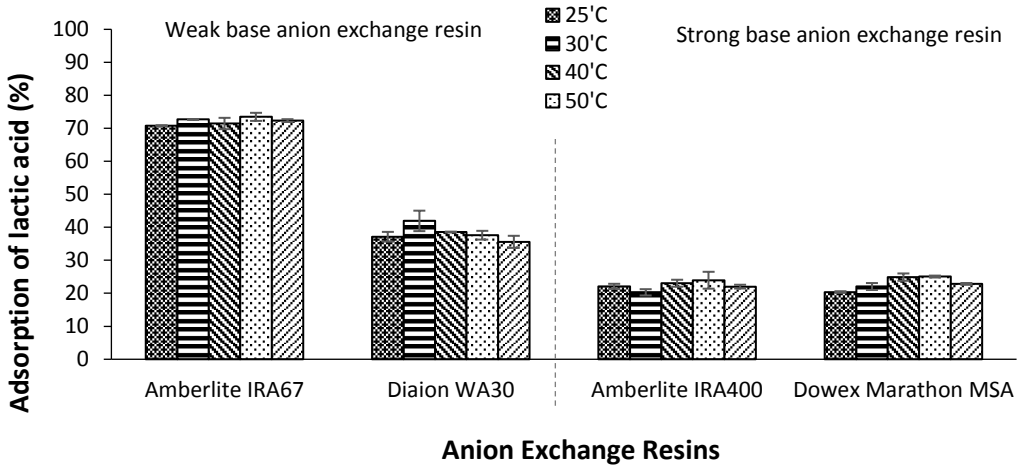
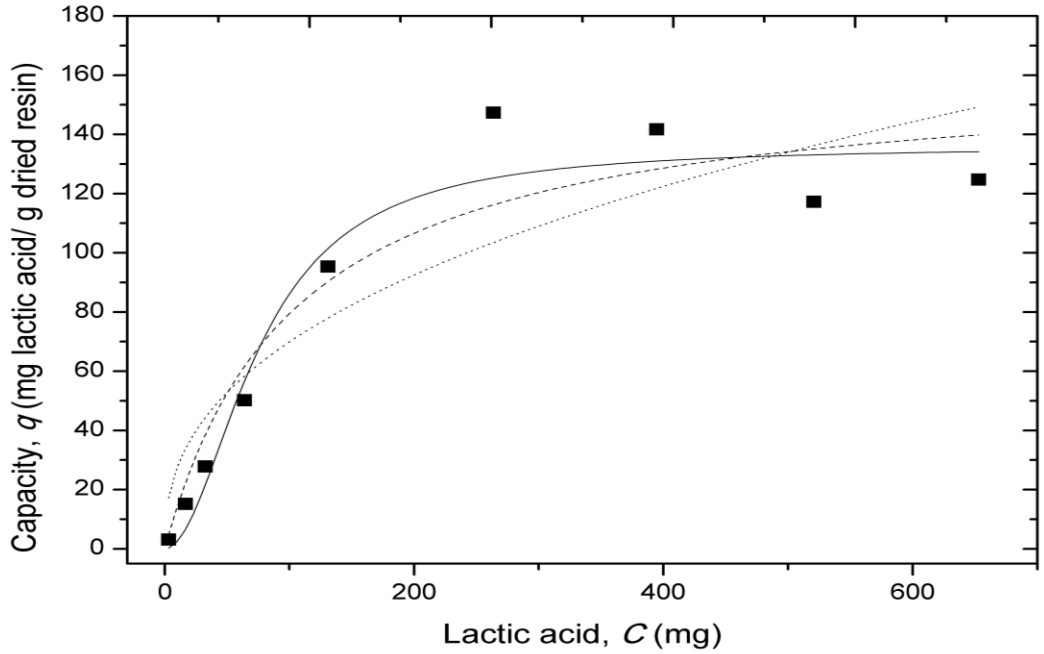

Weak base anion exchange resin
Strong base anion exchange resin
25'C
30'C
40'C
50'C
Adsorption of lactic acid (%)
Amberlite IRA67
Diaion WA30
Amberlite IRA400
Dowex Marathon MSA
Anion Exchange Resins

Figure 6

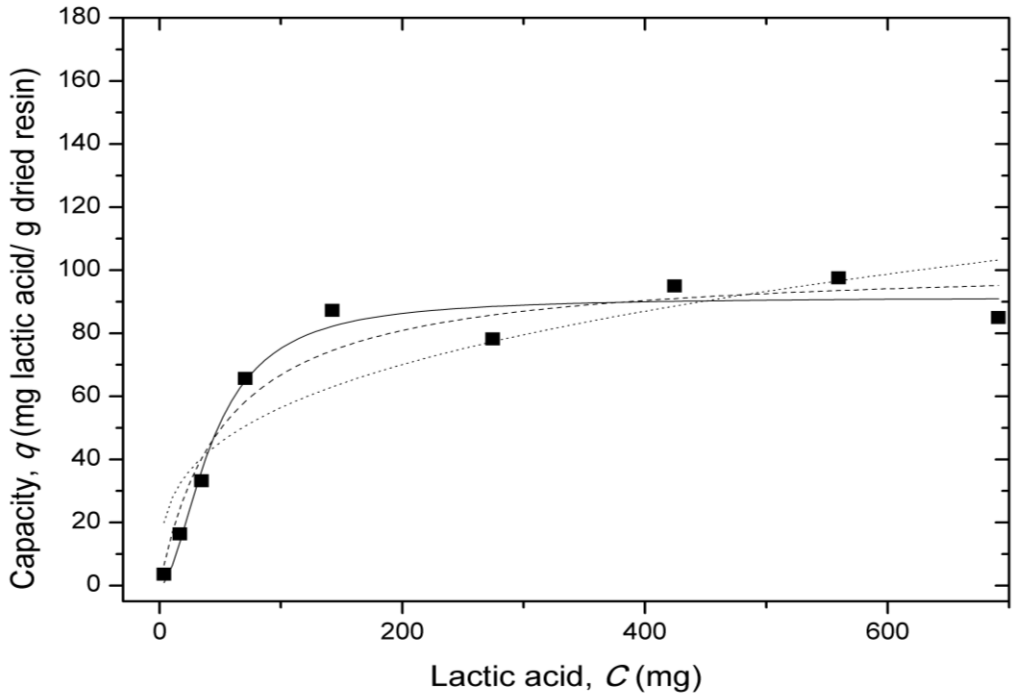
759

760 (a)



761

762 (b)



763

764 Figure 7

765

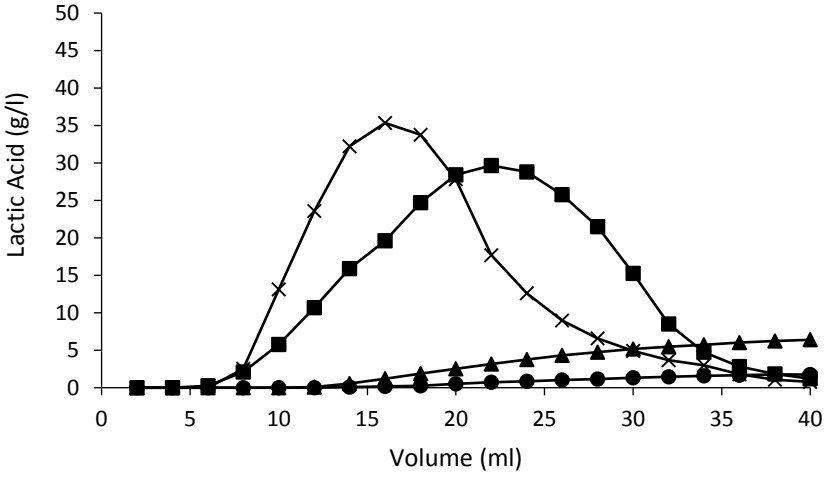


Figure 8

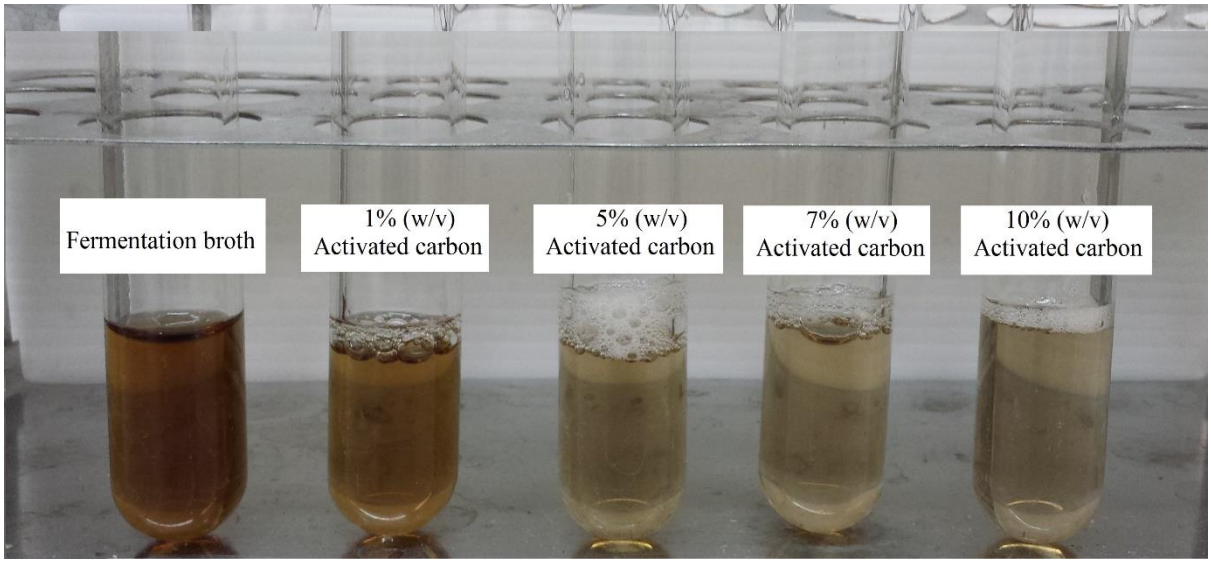
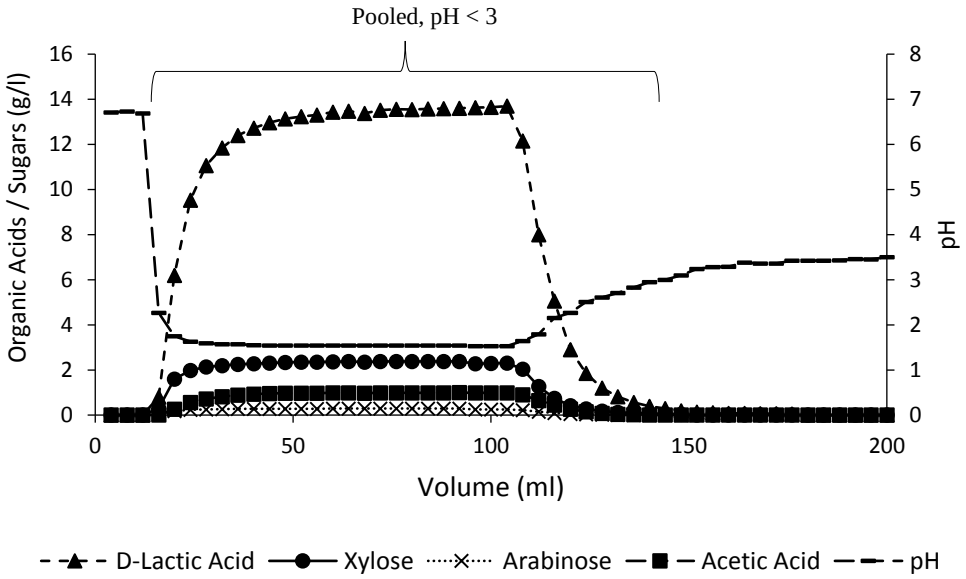

Fermentation broth
1% (w/v)
Activated carbon
5% (w/v)
Activated carbon
7% (w/v)
Activated carbon
10% (w/v)
Activated carbon

Figure 9

(a)


Pooled, pH < 3
Organic Acids / Sugars (g/l)
pH
Volume (ml)
D-Lactic Acid
Xylose
Arabinose
Acetic Acid
pH

(b)

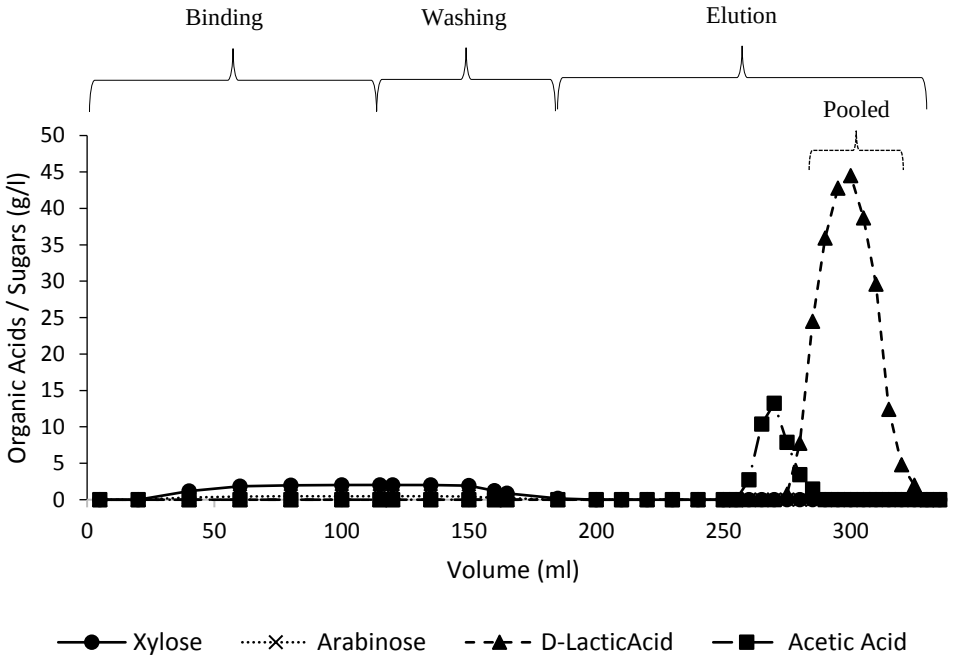

Binding
Washing
Elution
Pooled
Organic Acids / Sugars (g/l)
Volume (ml)
Xylose
Arabinose
D-LacticAcid
Acetic Acid

Figure 10