รายงานวิจัยฉบับสมบูรณ์

โครงการ

การผลิตกรดแลคติกโดยกระบวนการหมักแบบแลกเปลี่ยนไอออน การกู้คืนและการบำบัดเพื่อให้บริสุทธิ์บางส่วน

โดย ดร.มัลลิกา บุญมี

วันที่ 14 พฤษภาคม 2553

สัญญาเลขที่ MRG5180253

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ผู้วิจัย ดร.มัลลิกา บุญมี

สังกัด ภาควิชาเทคโนโลยีชีวภาพ คณะเทคโนโลยี
มหาวิทยาลัยขอนแก่น

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ MRG5180253

ชื่อโครงการ การผลิตกรดแลคติกโดยกระบวนการหมักแบบแลกเปลี่ยนไอออน การกู้คืนและการบำบัดเพื่อให้บริสุทธิ์บางส่วน

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บทคัดย่อ

ในการใช้เรซินแลกเปลี่ยนไอออนชนิดประจุลบ Amberlite®IRA-67 เพื่อแยกแลคเตทไอออนออกจากอาหารเลี้ยงเชื้อทันทีที่ผลิตขึ้นระหว่างกระบวนการผลิตกรดแลคติกจากกลูโคสโดย *Lactococcus lactis* NZ 133 (ATCC 11454) เป็นการลดการยับยั้งการเจริญและการผลิตกรดโดยแลคเตทไอออน ซึ่งส่งผลให้การผลิตกรดแลคติกมีผลได้และอัตราการผลิตที่สูงขึ้นเมื่อใช้กลูโคสความเข้มข้น 111 156 และ 212 กรัมต่อลิตร ทั้งนี้การผลิตกรดแลคติกมีอัตราการผลิตสูงสุดเมื่อใช้กลูโคสเริ่มต้น 156 กรัมต่อลิตรและมีการเติมเรซิน 310 กรัมในปริมาตรเพาะเลี้ยง 500 มิลลิลิตร โดยมีอัตราการผลิต 5.23 กรัมต่อชั่วโมงหรือเทียบเท่า 10.7 กรัมต่อลิตรต่อชั่วโมง แลคเตทไอออนที่จับกับเรซินถูกกู้คืนกลับมาในรูปของกรดแลคติกโดยการชะเรซินที่บรรจุในคอลัมน์แก้วด้วยกรดไฮโดรคลอริก 1 โมล่าร์ที่ไหลด้วยอัตราเร็ว 0.1 ของปริมาตรเบด (Bed Volume หรือ BV) ต่อนาทีซึ่งเป็นสภาวะที่ใช้ปริมาตรสารชะน้อยที่สุด การฟื้นสภาพเรซินเพื่อนำกลับมาใช้ใหม่ทำโดยใช้สารละลายโซเดียมไฮดรอกไซด์ 1 โมล่าร์ไหลผ่านเรซินที่ชะแลคเตทออกแล้วด้วยความเร็ว 1 BV ต่อนาที จากนั้นสารละลายจากการชะและน้ำหมักที่มีกรดแลคติกอยู่จะถูกทำให้บริสุทธิ์ขึ้นบางส่วนด้วยวิธีการสกัดด้วยของเหลว โดยสารที่ใช้สกัดคือ Trioctylamine (TOA) ละลายใน octanol ที่ความเข้มข้น TOA ใกล้เคียงกับความเข้มข้นกรดแลคติก โดยใช้สัดส่วน 1 ต่อ 1 โดยปริมาตร การสกัดใช้เวลา 2 ชั่วโมงที่ 30 องศาเซลเซียสโดยมีการผสมอย่างสม่ำเสมอ ซึ่งการสกัดด้วยของเหลวนี้สามารถทำให้สารละลายกรดแลคติกบริสุทธิ์ขึ้นได้บางส่วนเนื่องจากโปรตีนซึ่งเป็นองค์ประกอบในอาหารเพาะเลี้ยงจุลินทรีย์ไม่ละลายเข้าไปในวัฏภาคสารอินทรีย์พร้อมกับกรดแลคติก ทั้งนี้กระบวนการโดยรวมสามารถกู้คืนกรดแลคติกได้สูงสุดประมาณ 70 เปอร์เซ็นต์ โดยมีการสูญเสียกรดในขั้นตอนการล้างก่อนการชะประมาณ 11 เปอร์เซ็นต์และอีก 18 เปอร์เซ็นต์ในขั้นตอนการสกัด

คำหลัก กรดแลคติก *Lactococcus lactis* เรซินแลกเปลี่ยนประจุ การสกัดด้วยของเหลว

Abstract

Project Code MRG5180253

Project Title Lactic acid production by ion exchange fermentation, its recovery and partial purification scheme

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Abstract

Anion exchange resin, Amberlite® IRA-67, was incorporated into lactic acid fermentation from glucose using *Lactococcus lactis* NZ133 (ATCC 11454) in order to remove lactate ions as they were produced. Reduction in lactate inhibition improved lactic acid production in term of yield and productivity when the initial glucose concentrations were 111, 156 and 212 gl^{-1}. The highest lactic acid productivity was 5.23 gh^{-1} or equivalent to approximately 10.7 g $l^{-1}h^{-1}$ when using 156 g l^{-1} glucose with addition of 310 g resin in 500-ml working volume. The lactic acid from lactate-bound resin was recovered by elution through a glass column with 1 M HCl flowing at 0.1 $BVmin^{-1}$, which were the conditions that minimal eluant volume was used. The resin was regenerated for reuse with 1 M NaOH flowing at 0.1 $BVmin^{-1}$. The eluant and the fermentation broth containing lactic acid was partially purified by liquid-liquid extraction using 1:1 trioctylamine (TOA) in octanol as the extractant, at a similar TOA concentration to lactic acid in the eluant. The extraction with gentle mixing was carried out for 2 hours at 30°C. The liquid extraction can partially purify the lactic acid as the protein, which was the component of culture medium, was not simultaneously migrated into the organic phase along with lactic acid. Combining all the steps, the overall process showed lactic acid recovery of up to 70% with approximately 11% loss in washing of resin prior to elution and 18% in extraction step.

Keywords Lactic acid, *Lactococcus lactis*, ion exchange resin, liquid extraction

1. Executive Summary

At the present, lactic acid is widely used in various applications especially as a monomer or copolymer for biodegradable plastic production, or polylactic acid (PLA), in addition to its uses in food, chemical and pharmaceutical industries. Lactic acid can be produced by chemical synthesis or biological fermentation by lactic acid bacteria, which is mainly used for lactic acid production. However, lactic acid production by lactic acid bacteria has limitation on growth and product formation kinetics as they are subjected to the inhibition by lactate ions produced during fermentation. Therefore, lactate ion removal during fermentation is one of the alternatives for improvement in cell's growth and productivity of lactic acid. Moreover, separation of lactate ions would be coupled with fermentation process as in situ extractive fermentation. In this study, a plan for lactic acid production by batch fermentation with lactate removal using anion exchange resin Amberlite® IRA-67 (ion exchange fermentation), lactic acid recovery from the resin and partial purification of lactic acid by liquid-liquid extraction were investigated.

When cultivating *Lactococcus lactis* NZ133 (ATCC 11454) using batch fermentations of 500-ml working volume at 111.4, 155.8 and 211.9 gl^{-1} initial glucose, lactic acid concentration of 76.4, 102.1 and 135.2 gl^{-1} were obtained respectively within 18, 82 and 101 h. The lactic acid yields were 0.99, 0.80 and 0.80 gg^{-1} with the productivities of 4.21, 1.24 and 1.34 $gl^{-1}h^{-1}$, respectively. When using the batch fermentation with addition of ion exchange resin, the fermentation times were faster than those of the conventional batch fermentation by up to 5.4 times. Furthermore, lactate separation during the fermentation as the result of resin addition enhanced lactic acid yield and productivity. The highest lactic acid yield of 1.09 gg^{-1} was observed when using 111.4 gl^{-1} glucose with addition of 300 g resin. The highest lactic acid productivity was 5.23 gh^{-1} when using 155.8 gl^{-1} with addition of 300 g resin. This value was equivalent to approximately 10.7 $gl^{-1}h^{-1}$, an 8.6 times increase as compared with that of the conventional batch.

Separate experiments on post-fermentation resin treatment were carried out using the lactate-bound resin obtained from the fermentation step. The studies

involved with the treatment included washing, elution and regeneration. In washing step, comparison between cleaning with water and methanol was investigated. Lesser lactic acid loss by 1.1 folds was resulted when methanol was used as wash solution. However, water was chosen over methanol due to its availability and more importantly the economy of the process.

Elution study to determine the concentration and the flow rate of HCl to be used in eluting lactate ions from the lactate-bound resin was carried out in the 100-ml packed column. The 3^2 factorial design was carried out with HCl concentrations of 1, 2 and 4 M and the flow rates of 0.1, 0.5 and 1.0 $mlmin^{-1}$. The conditions that resulted in the eluted lactic acid of 4.3 ± 0.6 g with the minimal volume of 300 ± 60 ml were 1 M HCl with the flow rate of 0.1 $BVmin^{-1}$ or 10 $mlmin^{-1}$.

Regeneration of the resin to hydroxyl form for reusing in the fermentation step employed NaOH as the regenerant. Similar 3^2 factorial design was investigated with 1, 2 and 4 M NaOH at 0.1, 0.5 and 1.0 $mlmin^{-1}$. Minimal regenerant volume of 323 ± 101 ml was resulted with the use of of 1 M NaOH at 0.1 $BVmin^{-1}$ or 10 $mlmin^{-1}$.

Following the elution of the lactate-bound resin, the eluant containing lactic acid was to be further partially purified using liquid-liquid extraction. The initial investigation into the extraction system for lactic acid was carried out in order to select the extractant/diluent system and other basic operating conditions using aqueous solution of 100 gl^{-1} lactic acid. Trioctylamine (TOA) and octanol were chosen as the extractant and diluent respectively at the volume ratio of organic to aqueous phases of 1:1 at 30 °C for 2 h. With the selected conditions, 81.8% extraction of lactic acid from aqueous solution was achieved. The studies on the effect of concentration and volume ratio indicated that the extraction of lactic acid by reactive extraction was based on equal molar stoichiometric ratio between lactic acid and TOA. Furthermore, temperature did not show to exert significant effect on the extraction performance.

The conditions were applied to extract 100 gl^{-1} lactic acid in M17 broth prepared to imitate the fermentation broth. With extra components in the lactic acid solution, the volume ratio of the organic and aqueous phase changed such that the organic phase gained extra volume and the phase appeared turbid dark-yellow colour. Pretreating the lactic-containing M17 broth with cation exchange resin, Amberlite® IRA-98, eliminated the volume change problem although slight drop in

lactate concentration was observed. Extraction of the pretreated broth resulted in 83.0% extraction.

The extractions of eluant containing approximately 0.2 M lactic acid, with and without resin treatment were 71.9% and 74.2% respectively when using 0.2 M TOA in octanol as the extractant at 1:1 organic to aqueous phase volume ratio. In all extractions with protein-containing solution, the results showed the remaining protein components in the aqueous phase indicated that protein was not transferred or diffused to the organic phase together with lactic acid-extractant complex.

In the final part of the study, selected conditions from separated studies of fermentation, ion exchange resin elution and liquid extraction were carried out continuously in order to determine the overall production of lactic acid using the proposed production and recovery process. The ion exchange fermentation was carried out in 1-liter glass fermenter with 500-ml working volume using modified M17 broth with 168.2 gl^{-1} initial glucose with the addition of 311.5 g resin. Total lactate of 77.9 g was produced with the resin-bound lactate of 46.7 g and lactate in fermentation broth of 31.2 g. After the overall recovery and partial purification steps, lactic acid recovery of up to 70% could be achieved with approximately 11% loss in washing of resin prior to elution and 18% in extraction step. Moreover, the liquid-liquid extraction using TOA in octanol could partially purify lactic acid as the protein, which was the component in M17 broth, was not simultaneously migrated into the organic phase along with lactic acid.

2. Objectives

1) Comparing lactic acid production capabilities between conventional batch fermentation and batch fermentation with lactate removal using anion exchange resin in the cultivations with high sugar contents
2) Study the primary protocol for lactate recovery and partial purification

3. Materials and Methods

3.1. Microorganism for lactic acid production

Lactic acid was produced by Lactococcus lactis NZ133 (ATCC 11454) which were obtained from Cooperative Research Centre (CRC) for Food Industry Innovation, University of New South Wales, Australia. The bacteria were stored in 10% skim milk solution at –20 °C

3.2. Media for lactic acid production

3.2.1. M17 broth

M17 broth of 1 litre composed of M17 broth powder (CM0817, Oxoid, England), 37.25 g, dissolved in 950 ml distilled water and mixed with 50 ml of 10% (w/v) glucose solution. The 10% glucose solution was prepared by dissolving 10 g of glucose in 100 ml distilled water. Preparations of M17 broth and glucose solutions were separated. They were mixed when cooled after sterilization at 121 °C for 15 min.

M17 agar was prepared from M17 broth with addition of 20 g agar to 1 litre of M17 broth solution.

3.2.2. M17 modified broth

M17 modified broth (Boonmee, 2003) of 1 litre composed of 14 g Biopeptone (RM021, Himedia, India), 7 g Protose ME (RM003, Himedia, India), 3.5 g yeast extract (XI00801000, Scharlau, Spain), 0.35 g MgSO4.7H2O, 0.7 g ascorbic acid and 40 g glucose. This proportion of the components was designated 1 M M17. When using concentration of glucose over 40 g/l, the proportion of Biopeptone, Protose ME and yeast extract were increased according to the increase in glucose concentration. Preparation of M17 broth components and glucose solutions were separated. They were mixed after sterilization at 121 °C for 15 min.

3.3. Solutions and reagents

3.3.1. General solutions and solutions for analysis

All the solutions were prepared using distilled water unless stated otherwise.

5 M sodium hydroxide (NaOH) solution

Preparation of 5 M NaOH solution was done by dissolving 200 g NaOH (482, Ajax, New Zealand) in water and volume of the solution was adjusted to 1 litre.

0.85% sodium chloride (NaCl) solution

One litre of 0.85% NaCl composed of 8.5 g NaCl.

0.1 M ammonium dihydrogen orthophosphate $(NH_4)H_2PO_4$ solution

One litre of 0.1 M $(NH_4)H_2PO_4$ composed of 11.50 g $(NH_4)H_2PO_4$. The pH of the solution was adjusted to 2.5 using concentrated phosphoric acid solution. Approximately 3 ml of the acid was used per 3 litres of the solution to reach the required pH level.

80% acetonitrile solution

One litre of 80% acetonitrile composed of 800 ml HPLC grade acetonitrile (LD00204000, Scharlau, Spain).

Lowry solutions for protein assay

Solutions involved in protein assay by Lowry's method include 1% (w/v) copper sulphate ($CuSO_4.5H_2O$) (solution A), 2% (w/v) sodium potassium tartrate ($KNaC_4H_4O_6.4H_2O$) (solution B), 0.2 M sodium hydroxide (NaOH) (solution C) and 4% (w/v) sodium carbonate ($NaCO_3$) (solution D). All solutions were stored at room temperature.

Solution E was prepared by mixing 49 ml of solution C and 49 ml of solution D. Then 1 ml of solution A and 1 ml of solution B were added to the mixture. Solution E must be freshly prepared for each assay.

Solution F was prepared by diluting folin-ciocalteu's phenol reagent (190583Q, BDH, England) at 1:1 ratio with distilled water.

3.3.2. Solutions for washing, elution and regeneration of ion exchange resin

100% methanol solution

Methanol was commercial grade and supplied in 10.0 litre container.

1, 2 and 4 M hydrochloric acid (HCl)

The 1, 2 and 4 M HCl solutions were prepared by diluting 87.7, 175.4 and 350.9 ml of 37% HCl (20252.420, BDH, England) in filtered water respectively and the volume of the solution was adjusted to 1 litre.

1, 2 and 4 M sodium hydroxide (NaOH)

Preparation of 1, 2 and 4 M NaOH solutions were done by dissolving 40, 80 and 160 g of NaOH (482, Ajax, New Zealand) in filtered water and volume of the solution was adjusted to 1 litre.

0.05 M silver nitrate ($AgNO_3$)

One litre of 0.05 M $AgNO_3$ composed of 8.49 g $AgNO_3$ (102333J, BDH, England) in distilled water. The solution was kept at 4 ˚C and away from light.

3.3.3. Solutions for liquid-liquid extraction

Lactic acid solution

Lactic acid solution (101384Q, BDH, England) was diluted in distilled water or M17 modified broth to reach the final concentration of approximately 100 gl-1 or 1.1 M. The pH level of the solution was then adjusted to 2.0 using NaOH solution.

Octylamine solution

Octylamine (8.06917.0251, MERCK, Germany), a primary amine with 8 carbons chain length, was diluted in octanol (163386.1611, Panreac, EU), methyl isobutyl ketone (MIBK) (8.20820.2500, MERCK, Germany) or oleyl alcohol (8.20923, MERCK, Germany) to the final concentration of 1.1 M.

Dioctylamine solution

Dioctylamine (D201146, Aldrich, Japan), a secondary amine with 8 carbons chain length, was diluted in octanol, MIBK or oleyl alcohol to the final concentration of 1.1 M.

Trioctylamine solution

Trioctylamine (8.08649.0500, MERCK, Germany), a tertiary amine with 8 carbons chain length, was diluted in octanol, MIBK or oleyl alcohol to the final concentration of 0.6, 1.1 and 2.0 M.

Tridodecylamine solution

Tridodecylamine (8.21160.0250, MERCK, Germany), a tertiary amine with 12 carbons chain length, was diluted in octanol, MIBK or oleyl alcohol to the final concentration of 1.1 M.

3.4. Lactic acid fermentation processes

3.4.1. Inoculum preparation

Lactococcus lactis NZ133 was streaked on M17 agar (section 3.2.1) and incubated at 37 °C for 24-48 h. A few single colonies were then dispersed into 20 ml of M17 broth (section 3.2.1) and further incubated at 37 °C for 16 h.

3.4.2. Lactic acid production by batch fermentation

The addition of 11.5 ml inoculum was performed to 500 ml M17 modified broth (section 3.2.2) in a 1-litre glass bioreactor placed in the temperature-controlled water bath (N1-2RC, BIOER, Japan) at 37 °C placed above a magnetic stirring plate. The components in the bioreactor were mixed using magnetic bar at sufficient stirring rate to ensure homogeneity. The pH level was controlled at 6.5 by automatic addition of 5 M NaOH through peristaltic pump (SJ-1211, ATTO, Japan). The fermentation broth was taken out in a regular interval for analysis of cell growth, glucose consumption and lactic acid production.

3.4.3. Lactic acid production by fermentation with addition of ion exchange resin

A weak anion exchange resin Amberlite® IRA-67 (Rohm and Hass, France) from previous study (Boonmee, 2003) was chosen to be used in this study.

The fermentation was started by adding 11.5 ml of inoculum into 500 ml M17 broth (section 3.2.2) in the 1-litre glass bioreactor at 37 °C. The bioreactor setting was similar to that described in section 3.4.2. The pH level was maintained at approximately pH 6.5 by manual addition of IRA-67 anion exchange resin when the

pH level was lower than 6.45. When the pre-determined amount of IRA-67 resin was added, pH control was carried out by 5 M NaOH as described in section 3.4.2. The rate of stirrer was manually adjusted during the experiment to ensure thorough mixing.

Before the sample broth was taken for analysis, the magnetic stirrer was switched off for approximately 1 min to allow the resin to settle. The sample broth was pipetted carefully without taking along the resin. The magnetic stirrer was then switched on. The resin was separated from the fermentation broth at the end of fermentation for washing step. The sample broth was analyzed for cell growth, glucose consumption and lactic acid production. Whenever applicable, the remaining fermented broth was kept to be used in liquid-liquid extraction step.

3.5. Methods involved in ion exchange resin operation

3.5.1. Preparation of anion exchange resin

The anion exchange resin in OH- form was soaked in filtered water for 24 h before decantation by vacuum filtration. The moist resin was aseptically transferred into the sterile beaker for use in the experiment.

3.5.2. Washing of resin

At the end of fermentation, the resin was separated from fermentation broth by sieve filtration. The resin of approximately 300 ml was transferred into the beaker and washed with 250 ml predetermined volume of filtered water or methanol. The resin in the beaker was manually stirred for 1-2 min before the removal of wash liquid and analysis of lactic acid loss. The resin was then rewashed with the same volume of the wash liquid. Washing step was terminated with the visual appearance of clear wash liquid.

3.5.3. Resin elution

Approximately 100 ml of washed resin was transferred into a small column of 2.5 cm in diameter and 30.0 cm in length. The HCl solution of various concentrations was fed from the top of the column by peristaltic pump (7518-00, Easy-load, Taiwan) at various flow rates in a downward direction. Approximately 20-ml fractions of the outlet stream from column were collected in test tubes as

samples, until the fading yellow colour of the stream was observed. The total elution time was then recorded.

Then, the HCl solution was replaced by filtered water in order to wash HCl from resin bed. Washing step by water is completed when the pH level of the outlet water stream from the column was between 6.0-7.0 based on detection with pH strip (1.09535.0001, MERCK, Germany).

Calculation for eluted lactic acid volume of each sample fraction was carried out and each fraction was analyzed for lactic acid concentration. Total lactic acid (in grams) and total volume at the end of elution were calculated using the following formulas:

Total lactic acid (g) = $\left([HA]_1 * V_1\right) + \left([HA]_2 * V_2\right) + ... + \left([HA]_n * V_n\right)$

Total volume (ml) = $V_1 + V_2 + ... + V_n$

Where

$[HA]_i$ = lactic acid concentration in fraction $_i$ of the outlet stream from column (gl^{-1})

V_i = volumes of fraction *i* (ml)

n = fraction number

The selected elution conditions were used in the large column of 5.0 cm diameter and 43.0 cm long, when bulk resin was to be eluted prior to regeneration.

3.5.4. Resin regeneration

The regeneration step immediately followed the elution step in order to regenerate the resin for new cycles. Various concentrations of NaOH solutions were fed into the small column (section 3.5.3) at various flow rates, also in a downward direction. The progress of regeneration step was monitored by manual addition of $AgNO_3$ solution to the samples taken from the outlet stream of the column which was also collected in approximately 20-ml fractions. White precipitate of AgCl indicated the presence of Cl^- in the sample, while the brownish-green precipitate (Ag_2O) indicated that the complete removal of Cl^-. The regeneration step ended when the white precipitate could not be detected visually. The regeneration time was recorded and volume of each fraction was measured.

After the regeneration step was completed, the resin was washed by filtered water until all NaOH was removed. It was indicated by the pH level of exiting wash

water. The final measured pH value from the outlet should be in the range of 6.0-7.0 using a pH strip (1.09535.0001, MERCK, Germany).

The selected regeneration conditions were used in the large column (section 3.5.3) and the regenerated resin was used in the new cycles.

3.6. Liquid-liquid extraction

Lactic acid solution, lactic acid in M17 modified broth without glucose or the eluted solution from the resin (section 3.5.3) was extracted by the extractants section 3.3.3). The extraction process was carried out in a 15-ml capped polypropylene tube with varied organic (extractant) to aqueous phase ratios according to the experimental plan to reach the final volume of 10 ml. The two phases were mixed gently using Tube Tumbler Rotator Mixer (SBS550-2, Select Bio Products, USA) at rotational speed of 20 rpm. The setting was placed in an incubator with the temperature controlled according to the experimental plan. The sample from aqueous phase was taken by using 1- or 3-ml syringes for analysis of lactic acid concentration. Lactic acid in the organic phase was determined by mass balance. The results were used in calculation of the distribution coefficient (K_D) and the extraction percentage (% extraction) using the following formulas:

$$K_D = \frac{HA_{aq,init} - HA_{aq,final}}{HA_{aq,final}}$$

$$\% \text{ extraction} = \frac{[HA]_{aq,init} - [HA]_{aq,final}}{[HA]_{aq,init}} * 100$$

Where

$HA_{aq,init}$ = initial amount of lactic acid in aqueous phase (g)

$HA_{aq,final}$ = amount of lactic acid in aqueous phase at equilibrium (g)

$[HA]_{aq,init}$ = initial lactic acid concentration in aqueous phase (gl^{-1})

$[HA]_{aq,final}$ = lactic acid concentration in aqueous phase at equilibrium (gl^{-1})

3.7. Methods for Analysis

3.7.1. Cell growth analysis

Cell density (OD_{620})

The sample taken from the bioreactor was centrifuged at 8,161 x g* for 3 min. The known volume of supernatant was discarded and replaced by equal volume of 0.85% NaCl. Cell pellet was resuspended and the optical density (OD) was determined at 620 nm using spectrophotometer (V-1100D, Mapada, China). The 0.85% NaCl was used as the blank and to set zero reading.

Dried cell weight (DCW)

The 5-ml sample from the bioreactor was centrifuged at 10,000 rpm (202M, Sigma, Germany) for 3 min. Then the known volume of supernatant was discarded and replaced by equal volume of 0.85% NaCl. Cell resuspension was done in order to wash the cell. Cell suspension was centrifuged again at 10,000 rpm (202M, Sigma, Germany) for 3 min. Cell wash was done twice. After the final centrifugation, supernatant was discarded and small amount of water was added. Cells were resuspended in a centrifuge tube before being transferred to the pre-weighed test tube. The cell was dried in a hot air oven at 105 °C for 16 h. The test tube containing dried cell was taken out from the oven and immediately transferred to a desiccator. The tube was left to cool down until the room temperature was reached prior to dried cell weight determination. .

Cell viability

The sample from the bioreactor was serially diluted 107-108 folds. Then 2-3 dilutions were selected to be spread on M17 agar plate (section 3.2.1) using l00 µl of the cell suspension. The plates were incubated at 37 °C for 24 h. The plates with 30-300 colonies were counted for the number of colonies. Cell viability ($CFUml^{-1}$) was calculated using the following formula:

$$\text{Cell viability} = \frac{number\ of\ colonies\ on\ plate}{volume\ of\ cell\ suspension\ (ml)} * dilution\ level$$

* Using 10,000 rpm (16M, Labnet, USA)

3.7.2. Glucose concentration

The sample of 10 µl was pipetted into a test tube. One millilitre of glucose liquicolor (10121, Human, Germany) solution was added and the mixture was incubated at room temperature for 10 min. The optical density was then determined at 500 nm (OD_{500}). Glucose concentration can be read from the standard curve constructed using glucose standard solutions in range of 0-3 gl^{-1}. Water was used in place of sample as the control in the analysis.

3.7.3. Protein concentration

Lowry's method was applied in protein determination. The sample of 0.5 ml was taken into the test tube and 2.5 ml of solution E (section 3.3.1) was added. They were mixed by vortex mixer and left at room temperature for 10 min. Then, 0.25 ml of solution F (section 3.3.1) was added, mixed and settled for 30 min. The optical density was then determined at 750 nm (OD_{750}). The values were compared with standard curve constructed using 0.05-0.3 gl^{-1} BSA protein solution (P5369, SIGMA, Germany). Water was used in the control set for the assay by replacing the sample in the analysis.

3.7.4. Lactic acid concentration

Lactic acid concentration was analyzed using High Performance Liquid Chromatography or HPLC (LC-20A, Shimadzu, Japan). Control of the conditions, display and analysis of chromatogram were provided through LC Solution program (version 1.22 SP1, Shimadzu, Japan). The peak of lactic acid appeared during the retention time of 5.6-5.8 min. The concentration of the acid was determined from the chromatogram peak area in comparison with standard curve constructed using 0.0625, 0.125, 0.25, 0.5 and 1.0 gl^{-1} lactic acid. Column and conditions used in the analysis were summarised in Table 1.

Table 1 Column and conditions for HPLC analysis of samples

Column for analysis	Inersil® HPLC column ODS-3V 5 µm 4.6 x 250 mm
Sample volume	10 µl
Column temperature	40 °C
Column pressure	75-77 kg/cm^3

Column maximal pressure	200 kg/cm^3
Mobile phase/liquid phase	0.1 M $(NH_4)H_2PO_4$ at pH 2.5
Flow rate	1.0 ml min^{-1}
Detector	Refractive Index Detector (RID)

3.7.5. Statistical analysis

The results from elution and regeneration steps were analyzed to determine the factor that affected the elution and regeneration processes. Analysis of variance (ANOVA) and interpretation of the results were carried out using Design-Expert® (version 7.0.0, Stat-Ease, England).

4. Results and Discussions

4.1. Lactic acid production by batch fermentation and batch fermentation with ion exchange resin addition

The aims of this experiment were to investigate and to compare lactic acid productivities between conventional batch fermentation and fermentation with ion exchange resin addition at high initial glucose concentrations. Three levels of resin addition were investigated for each glucose concentration.

4.1.1. Control batch fermentation for lactic acid production by *Lactococcus lactis* NZ133

The batch fermentation using 100, 150 and 200 gl^{-1} initial glucose† were carried out as the control sets for ion exchange fermentation at the same glucose level. With 90.9 gl^{-1} initial glucose concentration, *L. lactis* NZ133 consumed glucose completely within 18 h (Figure 1). The final dried cell concentration was 5.93 gl^{-1} and the viable cell was 1.96x10^{10} CFUml^{-1}. The cell productivity and the cell yield of 0.33 gl^{-1}h^{-1} and 0.06 gg^{-1} were obtained respectively. The cell concentration stopped increasing when lactate concentration accumulated to 47.9 gl^{-1}. The final lactic acid

† Actual values were 90.9, 159.8 and 229.9 gl^{-1}.

concentration produced was 76.4 gl^{-1}. The lactic acid yield and productivities were calculated to be 0.99 gg^{-1} and 4.21 $gl^{-1}h^{-1}$ (volumetric) or 2.38 gh^{-1} (total) respectively.

When using 159.8 gl^{-1} initial glucose concentration, the fermentation was completed in 82 h which was significantly longer than 90.9 gl^{-1} initial glucose concentration. The cell trend was similar to that of 90.9 gl^{-1} glucose (Figure 2). The final cell was 5.54 gl^{-1} and the viable cell was $0.92x10^{10}$ $CFUml^{-1}$ which were similar also to 90.9 gl-1 initial glucose. However, the cell productivity and the cell yield decreased to 0.07 $gl^{-1}h^{-1}$ and 0.04 gg^{-1} respectively. The final lactic acid concentration produced was 102.1 gl^{-1} which corresponded to the yield of 0.80 gg^{-1}. The lactic acid productivities were calculated to be 1.24 $gl^{-1}h^{-1}$ (volumetric) or 0.77 gh^{-1} (total).

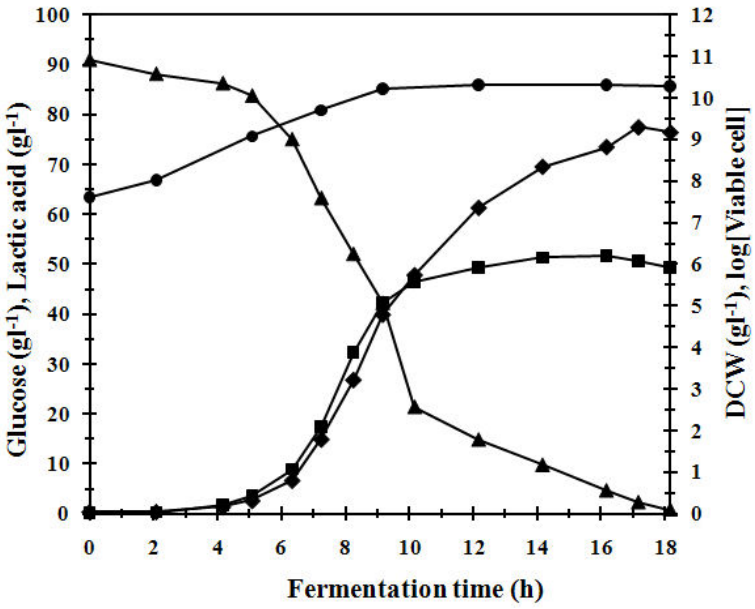


Figure 1 **Fermentation profile when cultivating *L. lactis* NZ133 in M17 modified broth with 90.9 gl^{-1} initial glucose concentration: (◆) lactic acid, (▲) glucose, (■) DCW and (●) viable cell**

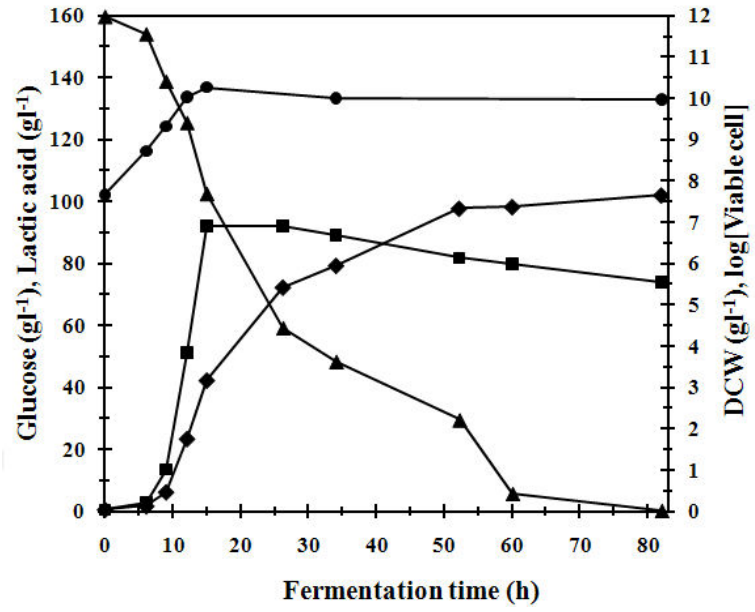


Figure 2 **Fermentation profile when cultivating *L. lactis* NZ133 in M17 modified broth with 159.8 gl^{-1} initial glucose concentration: (◆) lactic acid, (▲) glucose, (■) DCW and (●) viable cell**

Both lactic acid yield and productivity decreased when increasing initial glucose from 90.9 gl^{-1} to 159.8 gl^{-1}.

The batch fermentation using 229.9 gl^{-1} glucose was the experiment with the highest glucose level. Figure 3 shows that *L. lactis* NZ133 could metabolise glucose completely in 101 h. The dried cell concentration and viable cell decreased to the lower levels in comparison with 90.9 gl^{-1} and 159.8 gl^{-1} initial glucose. The cell and viable cell at the end of cultivation were 4.96 gl^{-1} and $0.50x10^{10}$ $CFUml^{-1}$ respectively. Moreover, the cell productivity decreased to 0.04 $gl^{-1}h^{-1}$ and cell yield to 0.02 gg^{-1}. The production of cell began to slow down when lactate concentration in the broth was 65.8 gl^{-1} but lactate was still produced until the end of cultivation but at a much slower rate after approximately 72 h. Lactic acid concentration at the end of the cultivation was 135.2 gl^{-1}. The lactic acid yield and productivities were 0.80 gg^{-1} and 1.34 $gl^{-1}h^{-1}$ (volumetric) or 0.89 gh^{-1} (total) respectively.

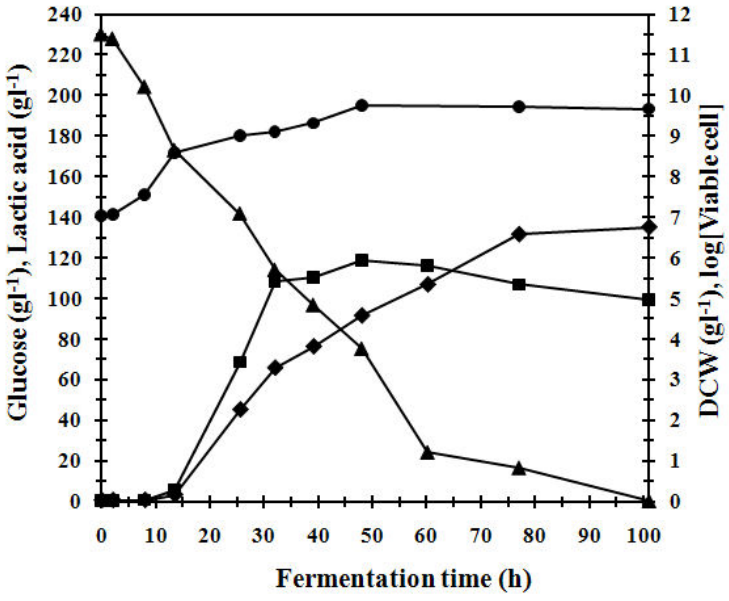


Figure 3 Fermentation profile when cultivating *L. lactis* NZ133 in M17 modified broth with 229.9 gl^{-1} initial glucose concentration: (◆) lactic acid, (▲) glucose, (■) DCW and (●) viable cell

4.1.2. Batch fermentation with ion exchange resin addition for lactic acid production by *L. lactis* NZ133

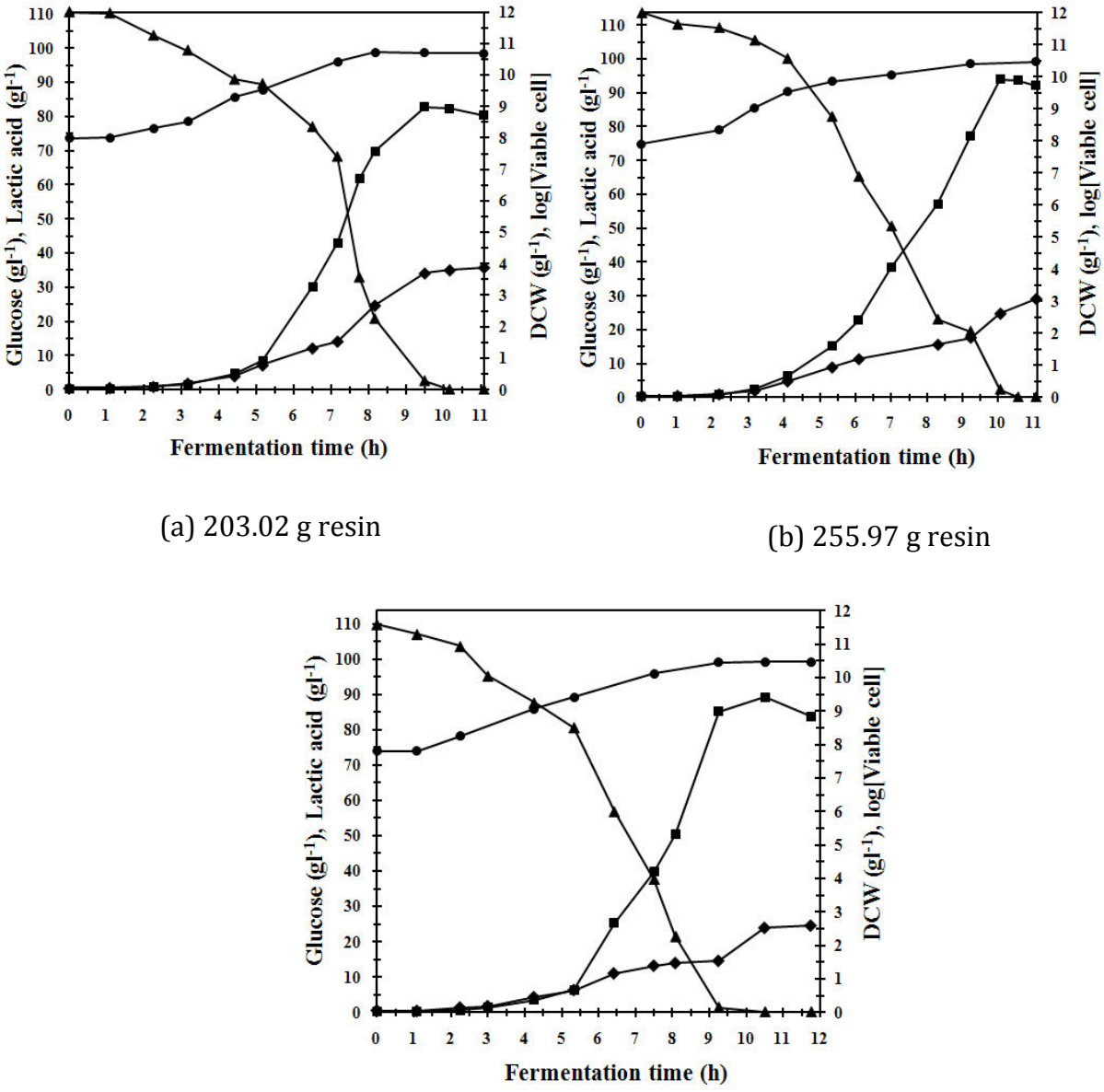
In lactic acid production by conventional batch fermentation at high sugar concentration, there were several limitations which included long fermentation time, lactate inhibition on growth effect and decreased lactic acid yield. Fermentation with addition of ion exchange technique employed ion exchange resin to remove lactate and control pH during fermentation time. The fermentations with 200, 250 and 300 g (plan) anion exchange resin addition were carried out using the media with 100, 150 and 200 gl^{-1} (plan) initial glucose.

Batch fermentation with 111.4 gl^{-1} (average) initial glucose and ion exchange resin addition

The anion exchange IRA-67 resin was used in batch fermentations of *L. lactis* NZ133. The cultivation procedures followed those described in section 3.4.3. The resin of 203.02, 255.97 or 309.03 g was progressively added to the fermentation to control the pH level at 6.5. The pH control was later switched to alkali addition after all the specified amount of resin had been added. The fermentation profile for 203.02 g resin addition is shown in Figure 4(a).

The fermentation was completed within 11 h which was 7 h shorter than the batch with 90.9 gl^{-1} initial glucose. The viable cell also increased to $4.80x10^{10}$ $CFUml^{-1}$ in comparison with to $1.96x10^{10}$ $CFUml^{-1}$ of the batch fermentation alone. The final dried cell concentration, yield and productivity were 8.71 gl^{-1}, 0.09 gg^{-1} and 0.79 $gl^{-1}h^{-1}$ which were higher than those of batch fermentation at the same glucose level. The final lactate concentration in the fermenter was 35.7 gl^{-1}.

The results when increasing the resin added to 255.97 g showed that *L. lactis* NZ133 could utilize glucose completely within 11 h (Figure 4(b)) as with 203.02 g resin addition. The viable cell at the end of cultivation was $2.90x10^{10}$ $CFUml^{-1}$. The final cell concentration was 9.73 gl^{-1}, cell yield was 0.09 gg^{-1} and productivity was 0.88 $gl^{-1}h^{-1}$, which were increased when compared with 203.02 g resin addition at the same initial glucose. The lactate concentration in the fermentation broth was 29.1 gl^{-1} which was less than the application of 203.02 g resin.



(a) 203.02 g resin

(b) 255.97 g resin

(c) 309.03 g resin

Figure 4 Fermentation profile of batch fermentation with addition of ion exchange resin by *L. lactis* NZ133 with initial glucose concentration of 111.4 gl^{-1} (average) : (◆) lactic acid, (▲) glucose, (■) DCW and (●) viable cell

The enhancement of resin addition to 309.03 g resulted in the similar fermentation time as using 203.02 and 255.97 g resin addition at 12 h. The viable cell at the end of fermentation was 2.90x10^{10} CFUml^{-1}. The final cell concentration, yield and productivity slightly decreased from 255.97 g resin addition to 8.81 gl^{-1}, 0.08 gg^{-1}

and 0.80 $gl^{-1}h^{-1}$. The final lactate concentration in the fermenter of 24.5 gl^{-1} was obtained (Figure 4(c)).

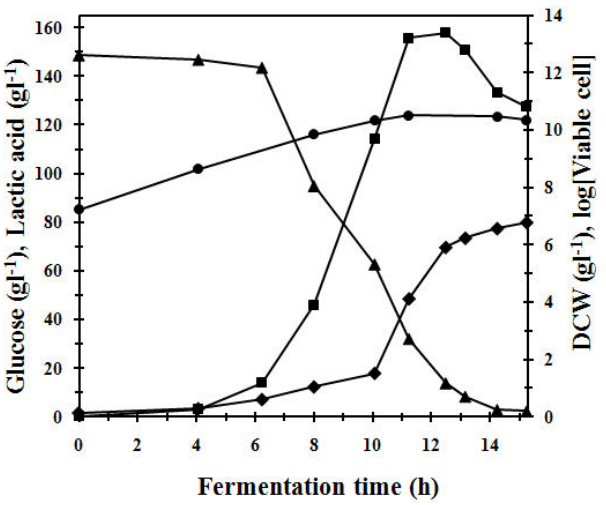
Batch fermentation with 155.8 gl^{-1} (average) initial glucose and ion exchange resin addition

Similar experiments to that described in section 3.4.3 were carried out but with a medium containing higher glucose concentration of 150 gl^{-1}‡. When 203.78 g IRA-67 resin was added into the fermenter, the final cell concentration was 10.81 gl^{-1} with the viable cell of $2.20x10^{10}$ $CFUml^{-1}$. The fermentation completed within 15 h (Figure 5(a)) which was 5 times shorter than the conventional batch at the same glucose concentration. The cell yield and productivity were 0.07 gg^{-1} and 0.72 $gl^{-1}h^{-1}$ respectively. At the end of cultivation, the final lactate concentration in the fermenter reached 79.9 gl^{-1}.

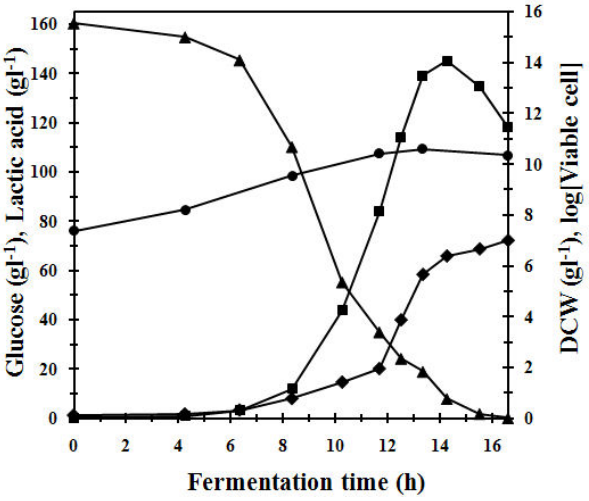
When 264.32 g IRA-67 resin was added into the fermenter, *L. lactis* NZ133 metabolised glucose completely within 16 h and achieved 11.46 gl^{-1} of final dried cell concentration (Figure 5(b)). The cell yield was 0.07 gg^{-1} and productivity was 0.72 $gl^{-1}h^{-1}$ with the viable cell of $2.30x10^{10}$ $CFUml^{-1}$. The result indicated the same cell growth as with an addition of 203.78 g resin. The final lactate concentration in the fermenter was 72.1 gl^{-1} which was slightly less than the previous experiment with 203.78 g resin.

When the resin addition was increased to 309.60 g, the fermentation completed within 15 h with 11.24 gl^{-1} of cell produced (Figure 5(c)). The viable cell at the end of fermentation was $2.46x10^{10}$ $CFUml^{-1}$. The cell yield was calculated to be 0.07 gg^{-1} and cell productivity was 0.75 $gl^{-1}h^{-1}$. The final lactate concentration in the fermentation broth was 65.5 gl^{-1}. The final lactate concentration in fermentation broth with 155.8 gl^{-1} (average) glucose was clearly higher than when using 111.4 gl^{-1} (average) glucose in all levels of resin addition.

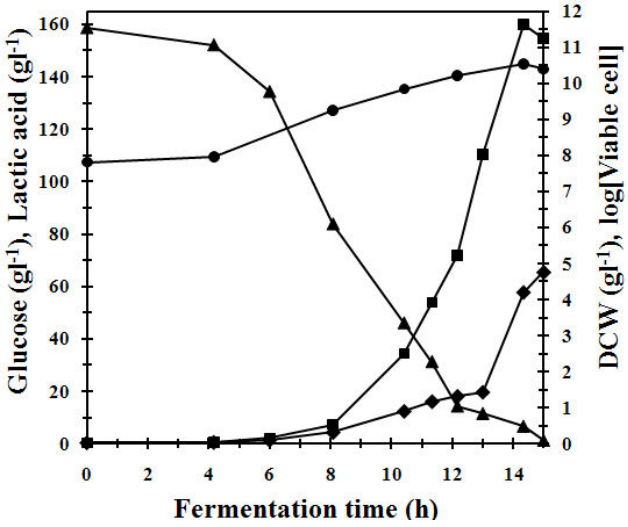
‡ Actual values were 148.5, 160.4 and 158.5 gl^{-1}.



(a) 203.78 g resin



(b) 264.32 g resin



(c) 309.60 g resin

Figure 5 **Fermentation profile of batch fermentation with addition of ion exchange resin by *L. lactis* NZ133 with initial glucose concentration of 155.8 gl^{-1} (average) : (◆) lactic acid, (▲) glucose, (■) DCW and (●) viable cell**

Batch fermentation with 211.9 gl^{-1} (average) initial glucose and ion exchange resin addition

When the fermentation with addition of ion exchange resin at the highest initial glucose concentration in this study (200 gl^{-1})§ was investigated, the result showed that when 205.72 g IRA-67 resin was added into the fermenter, the cell and the viable cell concentration were 9.47 gl^{-1} and 1.00x10^{10} $CFUml^{-1}$ respectively at the final fermentation time of 29 h (Figure 6(a)). The cell yield was 0.04 gg^{-1} and productivity was 0.33 $gl^{-1}h^{-1}$. The final lactate concentration in the fermentation broth was 115.2 gl^{-1} which was higher than when using 111.4 gl^{-1} and 155.8 gl^{-1} initial glucose at the same level of resin addition.

When the fermentation with an addition of 257.20 g IRA-67 resin into the fermenter was carried out (Figure 6(b)), *L. lactis* NZ133 could use glucose completely within 30 h and produced the final cell concentration of 9.46 gl^{-1} with the viable cell of 0.36x10^{10} $CFUml^{-1}$. The cell yield was 0.04 gg^{-1} and productivity was 0.32 $gl^{-1}h^{-1}$. The final lactate concentration in the fermenter was 94.1 gl^{-1}.

When increasing the resin addition to 306.18 g into the fermenter, glucose was used completely in 29 h (Figure 6(c)). The cell concentration of 9.37 gl^{-1} was obtained with the cell viability of 2.70x1010 $CFUml^{-1}$. The cell yield was 0.05 gg^{-1} and cell productivity was 0.32 $gl^{-1}h^{-1}$. The final lactate concentration remained in the fermentation broth was 88.9 gl^{-1}. Lactate concentration in fermentation broth at all levels of resin addition using 211.9 gl^{-1} initial glucose was higher than when using 111.4 gl^{-1} and 155.8 gl^{-1} initial glucose.

4.1.3. Determination of total lactic acid produced, lactic acid yield and productivity

When using the fermentation with addition of IRA-67 anion exchange resin, the produced lactic acid in the form of lactate ions was separated into two fractions, namely, resin-bound lactate and lactate in the fermentation broth. The remaining lactate in the broth was determined by HPLC whereas the lactate adsorbed to the

§ Actual values were 217.3, 211.7 and 206.7 gl^{-1}.

resin could be determined from resin capacity[**], which was estimated to be 0.15 $g_{lactate}\ g^{-1}_{resin,\ wet}$. Total lactic acid estimated from the sum of both fractions represented the closet estimation of the actual value.

[**] See section 7.1

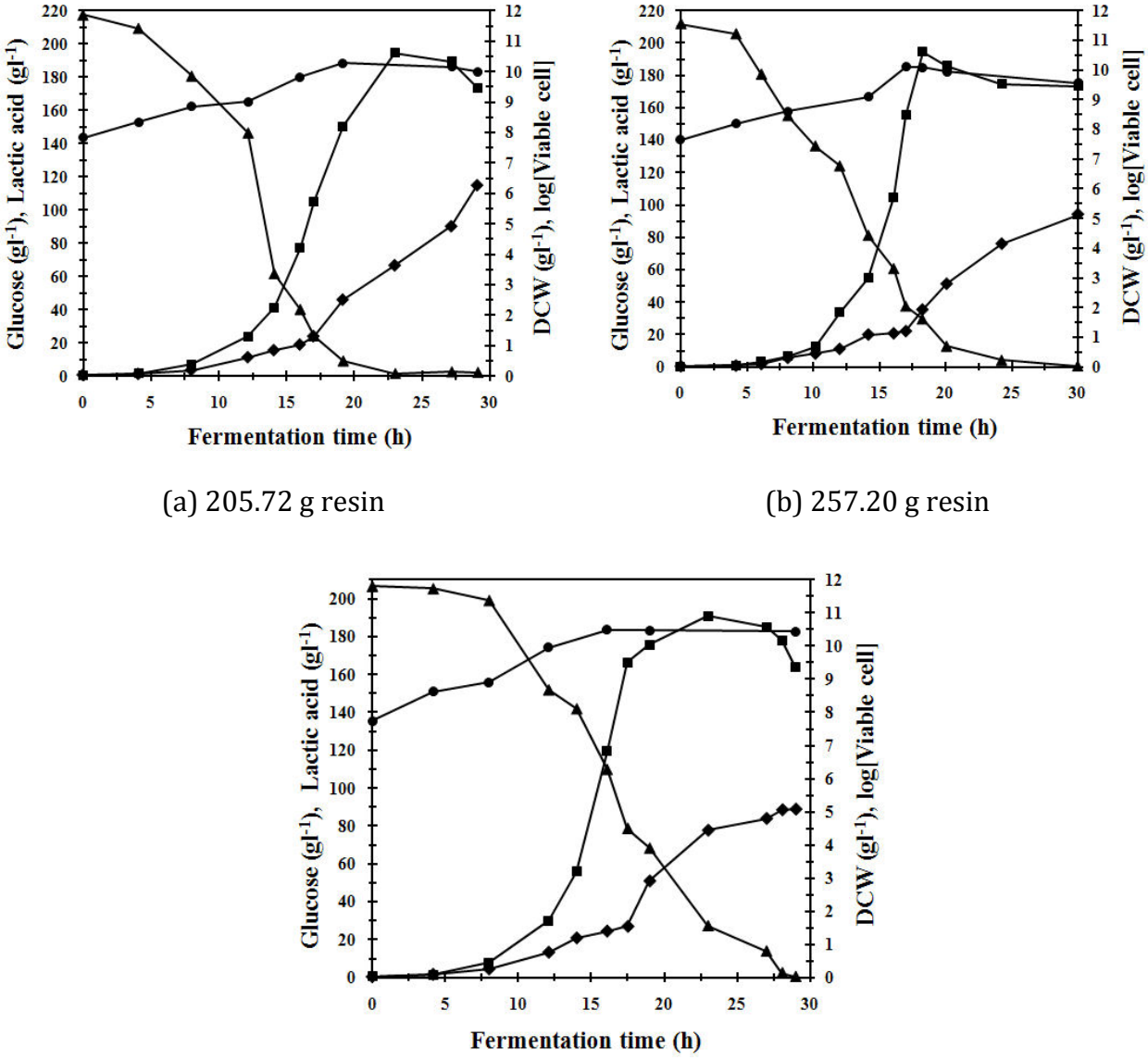


Figure 6 **Fermentation profile of batch fermentation with addition of ion exchange resin by *L. lactis* NZ133 with initial glucose concentration of 211.9 gl^{-1} (average): (◆) lactic acid, (▲) glucose, (■) DCW and (●) viable cell**

After determination of produced lactic acid, the corresponding yield and productivity could be calculated. The results were shown in Table 2. The fermentation with addition of 203.02, 255.97 and 309.03 g resin for 111.4 gl^{-1} initial glucose resulted in the lactic acid yields of 0.90, 0.96 and 1.09 gg^{-1} and total productivities of 4.30, 4.72 and 4.93 gh^{-1} respectively. When using 155.8 gl^{-1} initial glucose, lactic acid yields and productivities were 0.99, 0.96 and 1.02 gg^{-1} and 4.65,

4.53, 5.23 gh^{-1} with 203.78, 264.32 and 309.60 g resin addition respectively which was higher than the batch at the same level of glucose. Similar trend for 211.9 gl^{-1} initial glucose resulted in the corresponding lactic acid yields of 0.84, 0.83 and 0.90 gg^{-1} with total productivities of 3.06, 2.65 and 3.09 gh^{-1} with addition of 205.72, 257.20 and 306.18 g resin respectively.

It was indicated that the batch fermentation with 111.4, 155.8 and 211.9 gl^{-1} initial glucose and resin addition at all levels resulted in the increased lactic acid productivity in comparison with batch fermentation alone at the same initial glucose concentration. However, the fermentation with 211.9 gl^{-1} initial glucose and resin addition resulted in the lower productivity level than 111.4 and 155.8 gl^{-1} initial glucose with resin addition at all 3 levels. The lactic acid yields of 111.4 gl^{-1} (average) initial glucose batches were not different from that of the batch fermentation alone. Whereas increasing the initial glucose to 155.8 gl^{-1} and 211.9 gl^{-1} with every levels of resin addition, the yields were higher than those of the batches at the same glucose concentration.

Table 2 Parameters involved in lactic acid production by the batch fermentation with ion exchange resin

Batch fermentation	Resin addition (g)	Lactate in the broth (g)	Lactate-bound resin* (g)	Total lactate (g)	Yield (gg^{-1})	Total productivity (gh^{-1})
111.4 gl^{-1} glucose	**0.00**	43.3	-	43.3	0.99	2.38
	203.02	17.3	30.5	47.8	0.90	4.30
	255.97	13.8	38.4	52.1	0.96	4.72
	309.03	11.6	46.4	57.9	1.09	4.93
155.8 gl^{-1} glucose	**0.00**	62.9	-	62.9	0.80	0.77
	203.78	40.3	30.6	70.9	0.99	4.65
	264.32	35.6	39.65	75.2	0.96	4.53
	309.60	32.0	46.4	78.5	1.02	5.23
211.9 gl^{-1} glucose	**0.00**	90.6	-	89.9	0.80	0.89
	205.72	58.0	30.9	88.9	0.84	3.06

	257.20	46.3	38.6	84.9	0.83	2.65
	306.18	43.8	45.9	89.7	0.90	3.09

* Expected lactate adsorbed in resin based on resin capacity of 0.15 g_{lactic} acid /g_{resin} (See Table 10 page 60).

4.1.4. Discussion on lactic acid production by fermentation with ion exchange addition

The results obtained in the present study showed the decreasing trend in lactic acid concentration and productivity for cultivation of L. lactis NZ133 in batch fermentation when the initial glucose was increased from 90.9 gl^{-1} to 229.9 gl^{-1}. With higher levels of initial glucose at 159.8 gl^{-1} and 229.9 gl^{-1}, lactic acid production and productivities clearly decreased with the increasing trend in lactate accumulated in the fermentation broth. When lactate concentration reached to approximately 50 gl^{-1}, which was the critical concentration for growth of *L. lactis* NZ133, the cell could not grow further. The result agreed with that reported by Boonmee et al. (2003) that the lactate concentration of 47.1 gl^{-1} inhibited the cell growth of *L. lactis* NZ133. The value was lower than those of *L. lactis* and *L. lactis* subsp. *cremoris* whose critical lactate concentration values of 60 gl^{-1} and 70 gl^{-1} were reported (Bergère, 1968; Bibal et al., 1988; Bibal et al., 1989). The production of lactic acid in an absence of cell growth was a characteristic of lactic acid bacteria, which was named uncoupling acid production (Desjardins et al., 1990).

When using the fermentation with addition of IRA-67 anion exchange resin, the fermentation time was shorter than that of the conventional batch at the same glucose concentration. This was the result of resin addition that removed lactate ions from the fermentation broth which in turn mitigated product inhibition on growth (González et al., 2000; Jianglong et al., 1994; Srivastava et al., 1992). The improvement in cell concentration and glucose conversion to lactic acid were thus achieved for all investigated range of initial glucose concentrations in comparison with the batch fermentation alone.

However, increasing initial glucose from 111.4 gl^{-1} to 211.9 gl^{-1} did not significantly increase the dried cell weight concentration from approximately 9 gl^{-1} with 111.4 gl^{-1} initial glucose to 11 gl^{-1} with 211.9 gl^{-1} initial glucose (fermentation with resin addition). When initial glucose (average) increased to 155.8 gl^{-1} and

211.9 gl^{-1}, lactate concentration was accumulated in range of 65-79 gl^{-1} and 88-115 gl^{-1} respectively, which were the levels that indicated inhibition on growth. Therefore, the cell could not grow further when compared with 111.4 gl^{-1} glucose, where the accumulated lactate in the broth was in the range of 25-36 gl^{-1}.

Considering lactic acid production, lactic acid yields increased when resin was added as compared with the conventional batch fermentation especially in cultivations with 155.8 gl^{-1} and 211.9 gl^{-1} initial glucose. This was because more lactate was produced than those in the conventional batches at the same glucose concentrations. In the batch fermentation alone, the cell need maintenance when cultivated in the medium with high substrate concentration thus glucose conversion to lactic acid had to be reduced and used for the other activities of cell as adaptation to osmotic pressure situation.

When using 211.9 gl^{-1} initial glucose with ion exchange resin addition, the lactic acid productivities were lower than those in 111.4 gl^{-1} and 155.8 gl^{-1} initial glucose with ion exchange resin addition. Lower productivities were mainly due to a longer fermentation time especially during uncoupling product formation period, which was still observed even when lactate was removed by the resin. Lactic acid production by *L. lactis* NZ133, as in other lactic acid bacteria, was divided into two periods i.e. growth and non-growth associated production (Luedeking and Piret, 1959). During the growth associated production, the cell at 211.9 gl^{-1} initial glucose could experience higher level of substrate inhibition than the other lower initial glucose system which was reflected by a longer cultivation time during this period. For non-growth associated production period, the fermentation at 211.9 gl^{-1} initial glucose took longer time to consume glucose completely as evident from the beginning of this growth cessation period.

4.2. Recovery of lactic acid from anion exchange resin and resin regeneration

The resin saturated with lactate ions after the fermentation process could be reused for new cycles. The resin was washed in order to clean the resin prior to the separation of lactate from resin by elution step. The resin after the elution step could be regenerated for using in new fermentation batches.

4.2.1. Anion exchange resin washing

The washing step was carried out prior to elution in order to remove the cell and other debris attached to the lactate-bound resins. The investigation aimed to follow the lactic acid loss when using water and methanol as washing solution.

The results of water and methanol washing of the approximately 300 ml resin are shown in Table 3. Visual observation indicated the presence of clear wash water after 13 cycles of washing times based on washing volume of 250 ml in each cycle. Large lactic acid loss was observed in the first 3 wash volumes. Washing by water resulted in total lactic acid loss of 5.51 g, which was slightly higher than 4.81 g loss from washing by methanol. Although the lactic acid loss in washing by methanol was 12.7% lesser than that from washing by water, water may be selected as the solution for washing step to decrease the operating cost on an industrial point of view (Rojan et al., 2008). Therefore the water was chosen as a washing solution in the process instead.

Table 3 Lactic acid loss during washing step by water and methanol

Fraction number	Lactate in washed water (gl^{-1})		Lactate loss in washing (g)	
	water	methanol	water	methanol
1	10.35 ± 0.12	9.57 ± 1.05	2.59 ± 0.03	2.41 ± 0.28
2	4.90 ± 3.38	3.54 ± 1.03	1.23 ± 0.86	0.88 ± 0.25
3	1.55 ± 0.13	1.38 ± 0.48	0.39 ± 0.03	0.34 ± 0.12
4	0.76 ± 0.18	0.67 ± 0.22	0.19 ± 0.05	0.17 ± 0.05
5	0.57 ± 0.16	0.38 ± 0.13	0.14 ± 0.04	0.10 ± 0.03
6	0.52 ± 0.12	0.30 ± 0.06	0.13 ± 0.03	0.07 ± 0.02
7	0.50 ± 0.15	0.27 ± 0.02	0.12 ± 0.04	0.07 ± 0.01
8	0.47 ± 0.14	0.23 ± 0.01	0.12 ± 0.03	0.06 ± 0.00

9	0.69 ± 0.14	0.82 ± 0.78	0.17 ± 0.03	0.21 ± 0.20
10	0.44 ± 0.08	0.62 ± 0.64	0.11 ± 0.02	0.16 ± 0.16
11	0.45 ± 0.28	0.66 ± 0.24	0.11 ± 0.07	0.17 ± 0.06
12	0.35 ± 0.08	0.33 ± 0.02	0.09 ± 0.02	0.08 ± 0.00
13	0.44 ± 0.23	0.39 ± 0.28	0.11 ± 0.06	0.10 ± 0.07

4.2.2. Anion exchange resin elution

In elution step, hydrochloric acid (HCl) was used as an eluant for elution of lactate from lactate-bound resin. Lactate ions bound to the resin were replaced by chloride ions (Cl^-) resulting in lactic acid as a final product. In the elution study, the flow rate of HCl and HCl concentration were investigated for their influences on lactic acid separation from IRA-67 resin.

The experiment followed general factorial design of two factors, each with 3 levels. Lactate-bound resin of approximately 100 ml was transferred into a small column size of 2.5 cm in diameter and 30.0 cm in length. Result from one of the condition, the flow rate of 0.1 $BVmin^{-1}$ and 1 M HCl concentration, is shown in Figure 7 indicated that lactate ion was not separated from resin in the first few fraction volumes, as no lactate could be detected in the outlet stream. The lactic acid concentration then increased and reached maximum concentration before starting to decrease. Accumulated lactic acid from the elution step calculated from the concentration of each fraction showed a rapid accumulation following the increase in lactic acid concentration. The accumulation rate decreased when lactic acid concentration in outlet stream started to drop and reach stationary phase when there was almost no change in lactic acid concentration in the outlet stream. The shown elution profile was similar in all conditions used in this experiment. Total amount of lactic acid separated from resin, time and volume of HCl used in elution are summarised in Table 4.

Table 4 illustrated the effects of increasing the flow rate and HCl concentration, the eluted amount of lactic acid was similar in all conditions. An amount of total lactic acid was in range of approximately 4-5 g. The flow rate had significant effect to elution time and volume of HCl. When the flow rates were increased from 0.1 $BVmin^{-1}$ to 1.0 $BVmin^{-1}$, elution time decreased while used HCl volume increased. Whereas when the HCl concentration was increased from 1 M to 4 M HCl, elution time and HCl volume were not different at a particular flow rate.

In addition, visual observation of the outlet stream showed that during the first few fractions, the outlet liquid appeared colourless. The yellow colour became more intense in the latter fractions. The yellowness colour of eluate was then becoming paler towards the end of the elution.

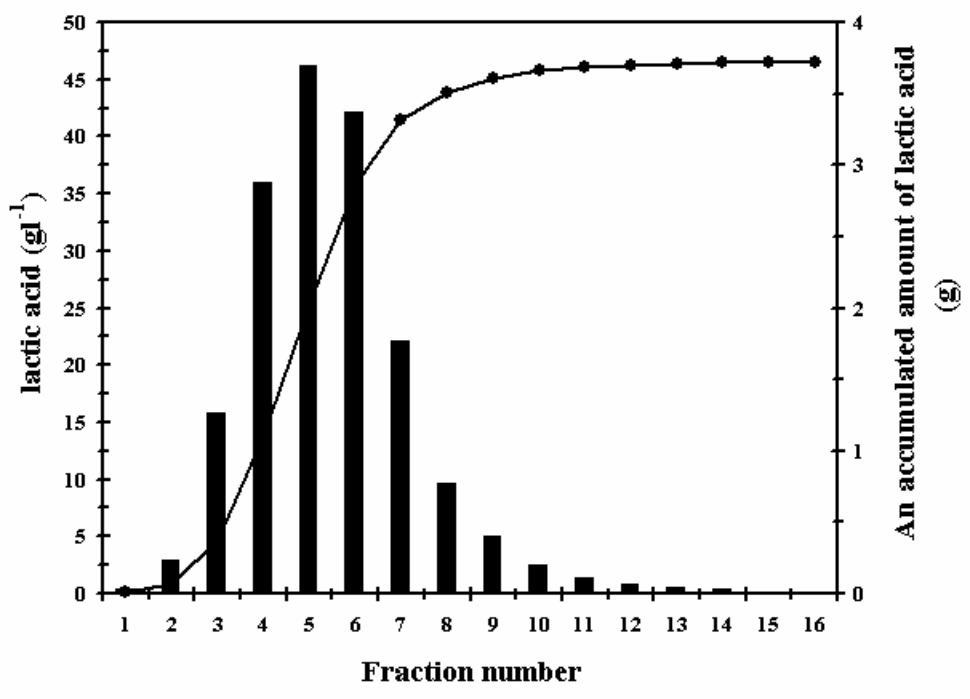


Figure 7 **Profile of lactic acid concentration (bar graph) and accumulated lactic acid (line graph) at the flow rate of 0.1 BVmin^{-1} and 1 M HCl concentration in elution step**

Table 4 **Effect of flow rate and concentration of HCl on lactic acid elution (resin volume = 100 ml)**

		B1 (1 M HCl)	B2 (2 M HCl)	B3 (4 M HCl)
A1 (flow rate 0.1 BVmin^{-1})	**Lactic acid (g)**	4.29 ± 0.59	4.54 ± 0.73	4.07 ± 0.51
	Time* (min)	30.0 ± 6.0	28.6 ± 6.6	27.3 ± 3.3
	Volume (ml)	300 ± 60	286 ± 66	273 ± 33
A2 (flow rate 0.5 BVmin^{-1})	**Lactic acid (g)**	3.77 ± 0.35	4.08 ± 0.57	4.24 ± 0.66
	Time* (min)	8.2 ± 1.4	8.6 ± 1.8	9.2 ± 1.2
	Volume (ml)	409 ± 69	432 ± 92	458 ± 58
A3 (flow rate 1.0 BVmin^{-1})	**Lactic acid (g)**	4.05 ± 1.15	4.63 ± 1.49	4.63 ± 1.54

* Reported times were calculated from flow rate multiplied by accumulated fraction volume up until fraction that contained less than 0.9 g/l lactic acid. Time was not considered as a response in this study as it was dependent on flow rate.

	Time* (min)	6.4 ± 0.8	6.2 ± 1.2	6.5 ± 1.4
	Volume (ml)	638 ± 80	624 ± 121	653 ± 144

Note: Lactic acid = total lactic acid recovered from IRA-67 resin (g)
Time = total time used for elution (min)
Volume = total volume of eluant (ml)

When considering the suitable condition based on maximum total lactic acid eluted from the resin and minimum HCl volume, the analysis showed that using 2 M HCl at the flow rate of 0.1 $BVmin^{-1}$ was the best condition for all the conditions tested for lactic acid elution in this experiment. The conditions were selected by the program as the most suitable conditions to be used in lactate elution.

However, when the desirability was considered (Section 7.2, Table 24 page 75), it was found that the desirability of 0.663 when using the flow rate of 0.1 $BVmin^{-1}$ and 2 M HCl was similar to 0.602 when using 1 M HCl at the flow rate of 0.1 $BVmin^{-1}$. Furthermore, when calculating the mole of HCl used in both cases, HCl used when eluted with 1 M HCl at 0.1 $BVmin^{-1}$ was 52.6% lesser than that used when eluted with 2 M HCl at 0.1 $BVmin^{-1}$. Therefore, the flow rate of 0.1 $BVmin^{-1}$ of 1 M HCl was chosen for the further experiment.

4.2.3. Anion exchange resin regeneration

After lactate elution from IRA-67 resin, regeneration of the chloride ion-bound resin was operated prior to resin reuse. Sodium hydroxide (NaOH) was used as the regenerant. After chloride ions (Cl^-) were replaced by hydroxide ions (OH^-), the hydroxide-bound resin could be reused in the fermentation to adsorb lactate ions.

The experiment followed 3^2 factorial design. Table 5 showed that the flow rate and NaOH concentration had significant effect to regeneration time and volume of NaOH. When the flow rates were increased from 0.1 $BVmin^{-1}$ to 1.0 $BVmin^{-1}$, regeneration time decreased while used NaOH volume increased. When considering the NaOH concentration increased from 1 M to 4 M NaOH, regeneration time and NaOH volume decreased.

Completion of the regeneration step was monitored by adding a few drop of $AgNO_3$ solution into the outlet stream from the column in each fraction volume.

White precipitate (AgCl) was presented when the Cl^- from chloride ion-bound resin was replaced with the OH- from NaOH solution. The regeneration process was operated until brownish-green precipitate (Ag_2O) occurred and no white precipitate (AgCl) could be detected. It indicated that all the Cl^- had been removed completely and resin was fully absorbed with the OH^- and was ready to be reused in the fermentation process.

Table 5 **Effect of flow rate and NaOH concentration on the regeneration time and regenerant volume in regeneration step (resin volume = 100 ml)**

		B1 (1 M NaOH)	B2 (2 M NaOH)	B3 (4 M NaOH)
A1 (flow rate 0.1 BVmin^{-1})	**Time (min)**	48.8 ± 4.2	48.4 ± 2.5	47.0 ± 4.2
	Volume (ml)	323 ± 101	366 ± 44	316 ± 34
A2 (flow rate 0.5 BVmin^{-1})	**Time (min)**	14.8 ± 2.6	10.8 ± 1.3	8.1 ± 1.0
	Volume (ml)	620 ± 96	500 ± 73	331 ± 59
A3 (flow rate 1.0 BVmin^{-1})	**Time (min)**	12.0 ± 0.5	11.2 ± 1.9	9.5 ± 1.1
	Volume (ml)	953 ± 48	749 ± 37	536 ± 94

Note: Time = total actual time used for regeneration (min)
Volume = total volume of regenerant (ml)

ANOVA-Analysis of Variance

Two main factors which included flow rate and NaOH concentration significantly affected the regeneration time and NaOH volume at 95% confident level (Section 7.2, Table 25–26, page 78). Moreover, the interaction of the both factors also significantly affected ($p \leq 0.05$) the NaOH volume used in regeneration.

When analysing for the suitable regeneration condition based on minimum volume of NaOH, the analysis by the program suggested that using 4 M NaOH at the flow rate of 0.1 BVmin^{-1} was the desirable condition.

However, when the desirability was considered (Section 7.2, Table 27, page 80), the desirability of 0.877 when using 4 M NaOH at the flow rate of 0.1 BVmin^{-1} was similar to 0.869 when using 1 M NaOH at the flow rate of 0.1 BVmin^{-1}. Furthermore, when calculating the mole of NaOH used in both cases, NaOH used when regenerated with 1 M NaOH at 0.1 BVmin^{-1} was 25.2% lesser than that used when regenerated with 4 M NaOH at 0.1 BVmin^{-1}. Therefore, the flow rate of 0.1 BVmin^{-1} of 1 M NaOH was chosen for further experiment.

4.2.4. Discussion on lactic acid elution and resin regeneration

After the fermentation process, the lactate-bound resin was transferred into the column for recovery of lactic acid and regeneration of resin. Lactate-bound resin was washed before elution. In washing step, it was found that washing the resin by methanol resulted in lesser loss of lactic acid than washing by water. The reason for lesser loss of lactic acid when using methanol could be seen and explained through some studies on lactic acid elution. Rojan et al. (2008) had reported in the lactic acid recovery using Amberlite® IRA-67 that higher lactic acid recovery was resulted when using water in comparison with methanol.

Methanol might promote lactic acid adsorption on the resin and that the elution was affected by dielectric constant of solvent. Cao et al. (2002) suggested that methanol, an organic solvent with dielectric constant (k) of 32.6 at 25 °C, decreased the elution effect of H_2SO_4 (k of 100.0 at 25 °C) in a study on lactic acid recovery using Amberlite® IRA-400. Since H_2SO_4 was claimed to be the most effective eluant in that study, it could be implied that water, also with high dielectric constant of 78.5 at 25 °C was a better eluant of lactic acid (k of 22.0 at 16 °C) than methanol.

In the other word, methanol was poorer eluant than water and less lactic acid was eluted or loss when passing methanol through the lactate-bound resin.

In elution step, visual observation of the colour of the outlet stream was used to determine the end point of the elution. The yellow colour of the eluted stream (outlet/lactic acid) was the colour from the component of fermentation medium such as yeast extract, peptone and meat extract. The validity of this monitoring technique could be confirmed by ANOVA procedure of the elution data when amount of lactic acid was the response. As the analysis suggested that model cannot be used to predict the response and the overall mean was a better predictor, monitoring the end of elution by observing the yellowness of the outlet stream could be applied.

4.3. Liquid-liquid extraction

The liquid-liquid extraction was studied in two main parts. One part was the investigation of factors that affected the extraction performance to recover 1.1 M or approximately 100 gl^{-1} lactic acid (aqueous) solution. High lactic acid (100 gl^{-1}) was used in the study as it was expected at the beginning of the study that the eluate from ion exchange resin and the fermentation broth might contain high concentration of

lactic acid when using high initial glucose concentration in the fermentation. In another part, the extraction study using M17 modified broth containing lactic acid as representative of fermentation broth was carried out using the conditions selected from the experiment with aqueous solution. The protein and coloured molecules as possible impurities in the broth and in the eluate from elution step were also investigated for their effect on partial purification by liquid-liquid extraction.

4.3.1. Extraction of lactic acid from lactic acid aqueous solution

Initial selection of diluents

In order to select the diluents to use in further extraction studies, octanol, MIBK and oleyl alcohol were used as the solvent or diluent for DOA, TOA and TDDA. After extraction of 1.1 M (~ 100 gl^{-1}) lactic acid for 30 min at the organic to aqueous volume ratio of 1:1, the highest lactic acid extraction in term of percentage was resulted when octanol was used as the diluent. Using MIBK and oleyl alcohol as the diluents resulted in similar extraction trend. MIBK offered better extraction performance to the extractant except when DOA was the extractant where the use of all 3 diluents provided similarly high extraction percentage. However, MIBK offered superior extraction performance to tertiary amines as compared to oleyl alcohol (Table 6). Therefore, octanol and MIBK were chosen for further study.

Kinetics of lactic acid extraction from aqueous solution by amine extractants

When high concentration of aqueous lactic acid was extracted for 12 h at the organic to aqueous volume ratio of 1:1, the extraction of 1.1 M lactic acid from lactic aqueous solution was completed within approximately 2 h (Figure 8). After that the distribution coefficients were relatively constant until the end of the experiment, which indicated no further extraction of lactic acid to the organic phase.

Table 6 Extraction percentage of lactic acid in various extractant/diluent systems

Diluents/Extractants	**% Extraction**		
	DOA (secondary amine:C8)	TOA (tertiary amine:C8)	TDDA (tertiary amine:C12)

Octanol	93.9 ± 0.3	86.8 ± 3.5	69.6 ± 2.0
MIBK	93.7 ± 2.4	84.1 ± 3.5	65.1 ± 1.0
Oleyl alcohol	93.9 ± 2.9	78.6 ± 2.1	61.4 ± 2.7

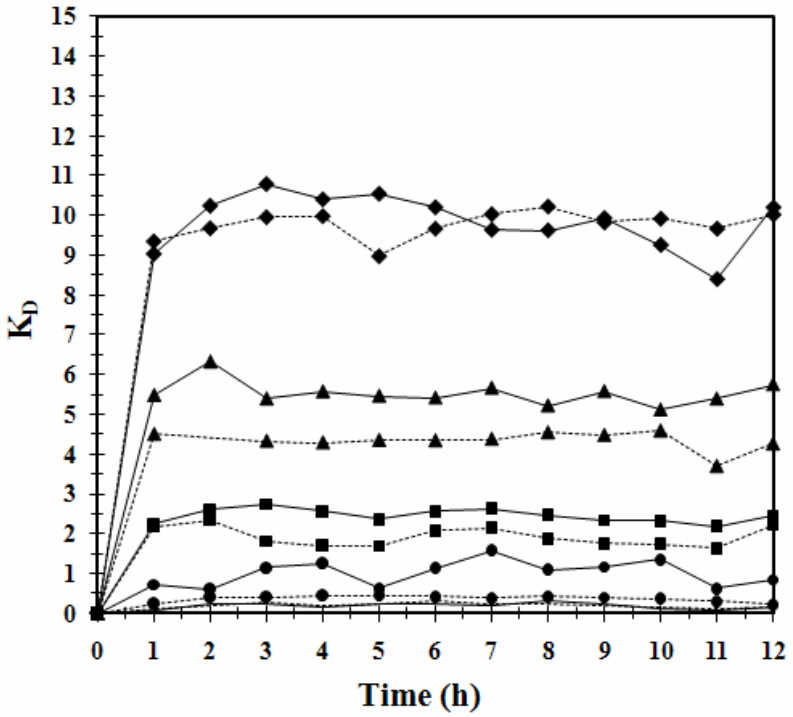


Figure 8 **Profile of distribution coefficient (K_D) when extracting 1.1 M lactic acid aqueous solution over 12 h using various extractants in octanol (solid lines) and MIBK (broken lines) : (—) no extractant, (●) OA, (◆) DOA, (▲) TOA and (■) TDDA**

The highest lactic acid extraction in term of distribution coefficient (K_D) was obtained when using DOA as the extractant. The highest K_D values were 10.1 and 9.8 when diluted in octanol and MIBK respectively. The K_D values of lactic acid extraction using TOA as the extractant were lesser from 5.5 in octanol to 4.4 in MIBK. When using TDDA in octanol and MIBK for lactic acid extraction, the lower K_D values of 2.5 and 1.9 were resulted respectively. The lowest K_D values of 1.0 and 0.4 were obtained when OA was used as the extractant and diluted in octanol and MIBK respectively in extractant-diluent system. Therefore, The K_D values of various extractants were decreased in the order of DOA > TOA > TDDA > OA. Moreover, the result showed that when the extractants were diluted in octanol, the K_D values were slightly higher than when diluted in MIBK. Lactic acid extraction using only diluent was also investigated. It resulted in the lowest extraction with the K_D value of 0.2 in both of diluents. The results indicated that the lactic acid extraction using the extractant-diluent system was better than pure diluent.

Although the extraction of lactic acid using DOA as the extractant gave the highest K_D values, it was not chosen. The main reason was that DOA, which is a secondary amine, tended to react irreversibly with carboxylic acid, making the stripping of solvent difficult (Hong et al., 1999). Secondary amines are also reported as subjected to amide formation upon regeneration by distillation (Uslu and Ìnci, 2007). TOA was selected instead as the extractant for lactic acid extraction with the use of octanol as diluent.

Effect of extractant concentrations and organic : aqueous volume ratio on extraction performance

Further study using 3 concentrations of TOA in octanol at 3 ratios of organic (extractant/diluent) to aqueous (lactic acid) phases showed that higher lactic acid extraction in term of extraction percentage were resulted when increasing the ratio of organic to aqueous phases at every initial TOA concentrations (Figure 9). In the organic to aqueous volume ratio of 1:4, the extraction percentage increased from 7.6% to 50.1% with increasing TOA concentration from 0.6 M to 2.0 M, which was the opposite when the ratio was 4:1. At the organic to aqueous phase volume ratio of 4:1, the extraction percentage slightly decreased from 89.9% to 88.7% when TOA concentration increased from 0.6 M to 1.1 M and clearly decreased from 88.7% to 80.5% when increasing TOA concentration from 1.1 M to 2.0 M.

Whereas, lactic acid extraction at the organic to aqueous volume ratio of 1:1 was enhanced from 56.4% to 82.8% with increasing TOA concentration from 0.6 M to 1.1 M. However, when TOA concentration increased from 1.1 M to 2.0 M, the lower extraction percentage of 74.5% was obtained (Figure 9).

Although the use of 1.1 M TOA at 1:1 organic to aqueous volume ratio resulted in 82.8% lactic acid extraction which was slightly lower than the highest value of 89.9% at the ratio 4:1, the condition was chosen for reducing the operating cost. The volume ratio of 1:1 used approximately 37.5% less organic phase volume than that used in the 4:1 ratio while only 7.9% extraction was compromised.

The selected condition of the organic to aqueous volume ratio was verified for the extraction ability again in finer scale of TOA concentrations from 0.6 to 2.0 M. Similar trend to the previous result was obtained. When increasing concentration of TOA from 0.6 M to 1.1 M, extraction percentage was clearly increased from 42.5% to

81.8% while decreasing extraction percentage from 81.8% to 75.2% was observed when concentration of TOA increased to 2.0 M (Figure 10).

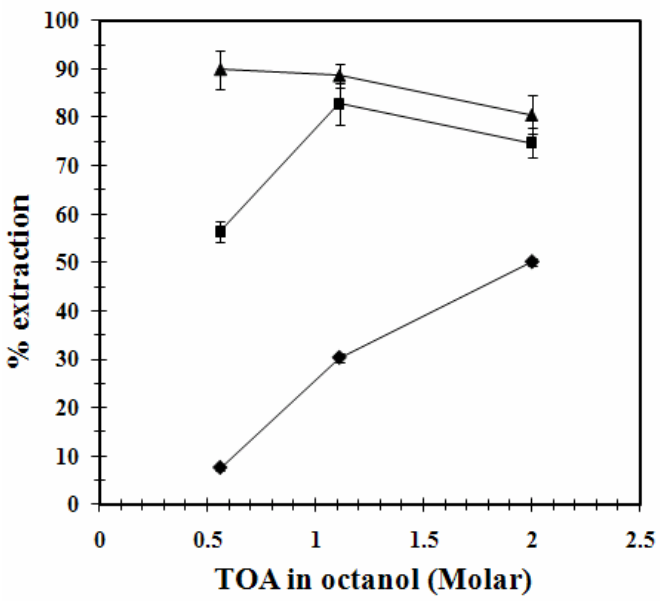


Figure 9 **Extraction percentage of 1.1 M lactic acid aqueous solution when using various concentrations of TOA in octanol at different volume ratios of organic phase to aqueous phase; (◆) 1:4, (■) 1:1 and (▲) 4:1**

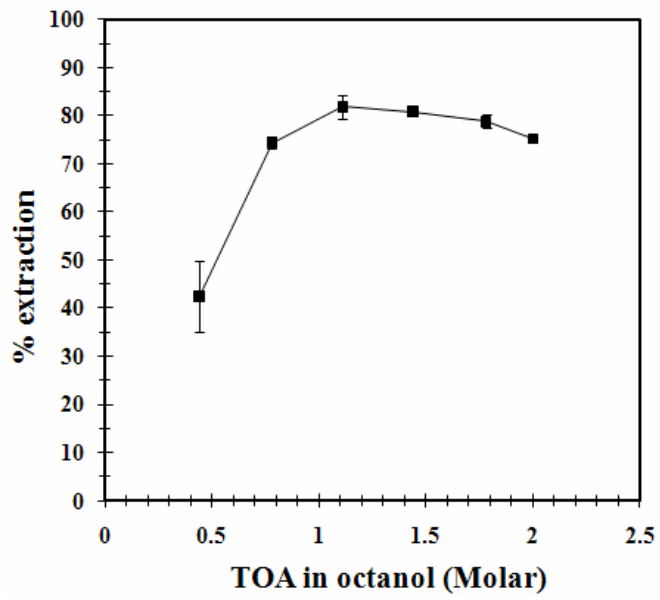


Figure 10 **Extraction percentage of 1.1 M lactic acid aqueous solution when using various concentrations of TOA in at 1:1 volume ratio of organic phase to aqueous phase**

Effect of temperature level on extraction performance

One important factor affecting the distribution coefficient (K_D) in liquid-liquid extraction is temperature level (Kahya et al., 2001). When the extraction was investigated for its ability to extract 1.1 M lactic acid aqueous solution

with 1.1 M TOA in octanol using equal volume ratio of organic to aqueous phases at various temperature levels of 20, 30 and 40 °C for 2 h. The result is presented in Table 7. The extraction percentage slightly decreased from 85.7% to 84% with increase in temperatures from 20 °C to 30 °C and further decreased to 82.7% when the temperature increased to 40 °C. The results indicated that the range of temperatures used in this study had no large effect in extraction ability. It also implied that the extraction could be carried out at room temperature with the variation in extraction percentage of no more than 3% between the temperature levels of 20, 30 and 40 °C.

4.3.2. Extraction of lactic acid from M17 modified broth containing lactic acid

Lactic acid extraction in M17 modified broth was carried out as the model for lactic acid extraction from fermentation broth after fermentation process for lactic acid recovery. In this experiment, lactic acid extraction was investigated when extracting 1.1 M lactic acid concentration dissolved in 1 M M17 modified broth (section 3.2.2) without glucose, as the fermentation broth was not expected to have remaining glucose after the fermentation process. The protein concentration in M17 modified broth was also determined and used as the indicator for partial purification by liquid-liquid extraction.

Table 7 Extraction percentage of 1.1 M lactic acid aqueous solution when using 1.1 M TOA in octanol at 1:1 volume ratio of organic phase to aqueous phase at various temperature levels

Temperature (°C)	% extraction
20	85.6 ± 11.9
30	84.0 ± 1.5

40	82.7 ± 3.5

Kinetics of lactic acid extraction from M17 modified broth containing lactic acid

The extraction of lactic acid from M17 modified broth containing lactic acid was completed within approximately 2 h (Figure 11) as indicated by constant K_D until the end of experiment. This was similar to lactic acid extraction from lactic acid aqueous solution. It could imply that organic extraction for 2 h was sufficient.

DOA showed the highest distribution coefficient (K_D) of 48.1 and 49.7 when used as the extractant and diluted in octanol and MIBK respectively. When using TOA as the extractant for lactic acid extraction, the K_D value was 20.7 when octanol was the diluent and slightly decreased to 17.7 when mixed in MIBK. The lower K_D values of 7.3 and 6.8 were obtained when extracting using TDDA in octanol and MIBK respectively. Using OA as the extractant in octanol resulted in the low KD value of 6.1 while using OA in MIBK gave the lowest K_D value of 1.6. Consequently, the K_D values of various extractants were decreased in the following order: DOA > TOA > TDDA > OA. The result was also similar to extraction of lactic acid in aqueous solution. In fact, the implementation of pure diluents for lactic acid extraction only resulted in K_D value of 0.6 and 0.3 in octanol and MIBK respectively. Such result confirmed that lactic acid extraction using the extractant dissolved in diluent was superior than using the diluent alone.

Although the extraction of lactic acid using DOA as the extractant gave the highest K_D values, it was not chosen. The reason was similar to the above section where lactic acid was extracted from aqueous solution. Therefore, TOA was selected as the extractant for lactic acid extraction with the use of octanol as diluent.

The extraction of 1.1 M lactic acid in M17 modified broth resulted in the distinctive variation of organic to aqueous volume ratio at the end of the extraction. The organic phase volume increased from 5 ml to approximately 8 ml. While the aqueous phase decreased from 5 ml to approximately 2 ml. The organic phase also appeared turbid dark-yellow colour. Changing of volume ratio after the extraction affected the calculations of distribution coefficient (K_D) and extraction percentage if the calculation was based on concentration. The K_D values reported in this part and section 4.3.1 were calculated based on mass balance. This calculation resulted in the

KD values that were much larger than those reported in the study using aqueous solution of lactic acid, when there was no volume change. However, the trend of the extraction results should not be affected. The possible cause of volume change after the extraction was initially investigated with initial hypothesis that M17 modified broth could contain molecules such as protein and some colour molecules that might be the cause of volume change problem. Therefore, the attempt to remove such molecules from M17 broth was carried out.

Removal of molecules other than lactate from M17 modified broth containing lactic acid (1.1 M) was carried out by adding 20% (w/w) IRA-98 cation exchange resin and stirred for approximately 2 h. It was found that the colour of M17 broth with and without resin treatment was not different, observing by optical density (OD) analysis at 570 nm. Therefore, IRA-98 resin might not able to remove the colour molecule from M17 broth. When both resin-treated and non-treated M17 modified broth containing lactic acid (1.1 M) were extracted using 1.1 M TOA in octanol, the volume of organic phase did not change when using the resin-treated broth while the opposite was observed when using the non-treated resin. Although the coloured molecules may not be removed, the cation exchange resin might remove some other molecules which were cationic in property and were able to be extracted also by the extractant used. Therefore, the volume change in organic phase may simply cause by high concentration of species extractable by the extractant.

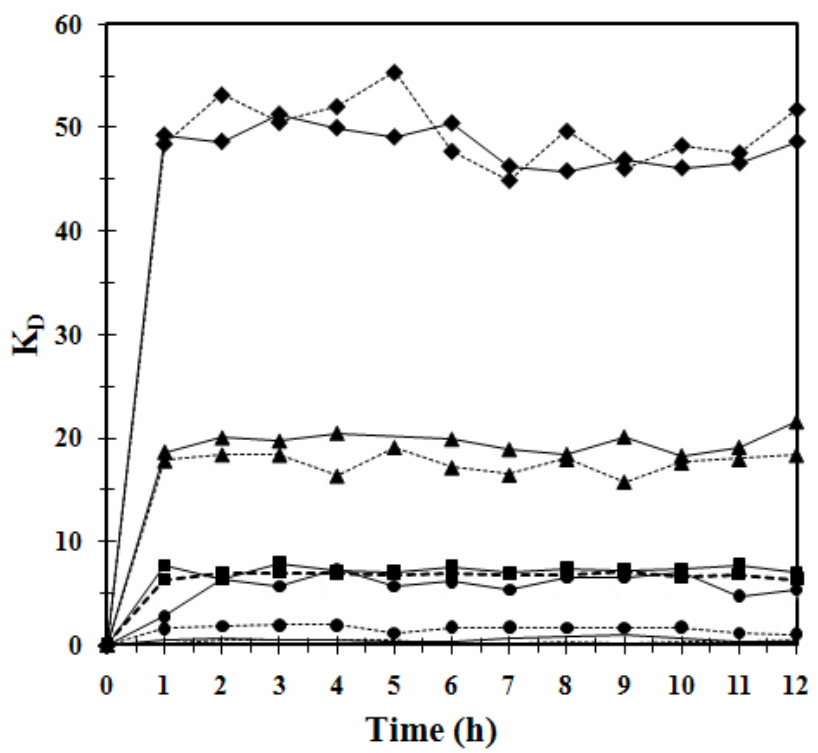


Figure 11 Profile of distribution coefficient (KD) when extracting 1.1 M lactic acid in M17 modified broth over 12 h using various extractants in octanol (solid lines) and MIBK (broken lines): (—) no extractant, (●) OA, (◆) DOA, (▲) TOA and (■) TDDA

In order to prove the above hypothesis, the extractions of lactic acid from M17 modified broth containing 0.2 M and 1.1 M initial lactic acid using 0.2 M and 1.1 M TOA in octanol respectively as the extractant phase were compared. It was found that the volume ratio at the end of extraction changed when extracting lactic acid from M17 broth containing 1.1 M lactic acid while there was no visible volume change when extracting from M17 broth containing 0.2 M lactic acid. Therefore, higher organic contents in M17 broth when using higher lactic acid concentration was likely the cause of volume change in organic phase.

From the above results, it was decided that IRA-98 resin would be used to treat the broth containing high lactic acid concentration in order to alleviate the volume change problem.

Treatment of lactic acid solutions with IRA-98 cation exchange resin on extraction performance

When M17 modified broth containing 1.1 M lactic acid was treated with IRA-98 cation exchange resin, lactic acid was decreased to 0.8 M. Further extraction of the resin-treated broth with 1.1 M TOA in octanol at 1:1 organic to aqueous phase

volume ratio, 30 °C for 2 h (selected conditions from section 4.3.1 page 40 and 42 and section 4.3.2 page 43) resulted in 83.0% extraction percentage.

In this experiment, protein as another main component in M17 modified broth was monitored before and after the extraction. The protein concentration was determined in the aqueous phase. The initial protein concentration in M17 modified broth was 15.4 gl^{-1} and the concentration after treatment with IRA-98 resin was 13.6 gl-1. When lactic acid in the resin-treated broth was extracted, the similar level of protein concentration of 13.2 gl^{-1} remained (Table 8). Such result indicated that protein was not extracted into the organic phase.

When the extraction of 1.1 M lactic acid in resin-treated M17 modified broth and in aqueous solution was compared, the similar extraction percentage was resulted. The extraction percentages were 83.6% and 81.2 % when extracting 1.1 M lactic acid in M17 broth and in aqueous solution respectively.

Table 8 Lactic acid and protein concentrations when extracting 1.1 M lactic acid in M17 modified broth with IRA-98 resin treatment

	Lactic acid (gl^{-1})	Protein (gl^{-1})
M17 broth before treatment with resin	96.2	15.4
M17 broth after treatment with resin	75.7	13.6
Resin-treated M17 broth after extraction with TOA/octanol	12.9	13.2

The extraction of lactic acid in the eluate which was the outlet stream from the column in elution step (section 4.2.2) was also carried out as it contained also major fraction of lactic acid. The extractions of lactic acid from the eluate with and without resin treatment were investigated. The initial lactic acid in the eluate was 0.2 M. When treating eluate with IRA-98 resin, a small decrease in lactic acid concentration was observed. The results of the extraction of the eluate with and without resin treatment were 71.9% and 74.2% respectively when using 0.2 M TOA in octanol as the extractant at 1:1 organic to aqueous phase volume ratio (Table 9).

The initial protein concentration in the eluate was 1.1 gl^{-1}, which was relatively unchanged after treating with IRA-98 resin. When lactic acid in the resin-treated and non-treated eluates were extracted, the protein concentration in the solution after the extraction was 1.0 gl^{-1}. It indicated that protein was not removed into the organic phase and lactic acid was the major species that had been extracted.

Table 9 Lactic acid and protein concentrations when extracting 0.2 M lactic acid in the eluate with and without IRA-98 resin treatment

	Lactic acid (gl^{-1})	Protein (gl^{-1})
Eluate before extraction	19.86	1.08
Eluate after extraction with TOA/octanol	5.13	1.04

Resin-treated eluate before extraction	17.85	1.01
Resin-treated eluate after extraction with TOA/octanol	5.02	0.99

4.3.3. Discussion on liquid-liquid extraction of lactic acid

When extracting lactic acid from aqueous solution, it was found that using extractant-diluent system enhanced the extraction performance in term of distribution coefficient that the coefficient was higher than when using pure diluent. Similar results by Keshav et al. (2008) showed that application of the conventional diluent for propionic acid, did not yield good extraction result in comparison with TOA in various diluents, which provided the higher K_D values. The probable reason was the high affinity of acid for water that mitigated K_D values. The physical extraction with conventional solvents was not an efficient method for recovery of carboxylic acid. In order to increase selectivity and yield of acid in liquid extraction, a combination of diluent with an extractant should be used as it could chemically form complex with acid (Kailas and Pangarkar, 2006; Tamada and King, 1990; Uslu, 2007; Uslu and Ìnci, 2007).

In the extraction of lactic acid using the extractant-diluent system, DOA gave the highest K_D value as evident from the report by Kawano et al. (1983) that showed Amberlite® LA-2, a secondary amine, to give higher extraction efficiency than using TOA, a tertiary amine, in hexane and benzene for the extraction of acetic acid and propionic acid. Furthermore, the report by Ricker (1978) indicated that the extraction of acetic acid by Amberlite® LA-2 in methyl isobutyl ketone (MIBK) was superior to Alamine® 336 (tertiary amine) in diisobutyl ketone. However, tertiary amine has been widely used in liquid extraction because secondary amines tend to react irreversibly with carboxylic acid, making the stripping of solvent difficult (Hong et al., 1999). Secondary amines are also reported as subjected to amide formation upon regeneration by distillation (Uslu and Ìnci, 2007). When the carboxylic acid and secondary amine was formed, the ammonium salts thermally dehydrated to form the corresponding amide (Figure 12). This was also compared to primary amines which were miscible in water and hence deterred from the application in aqueous extraction. According to the observation made in this study, the volume of organic phase slightly decreased after the extraction which might suggest dissolution of OA into the aqueous phase.

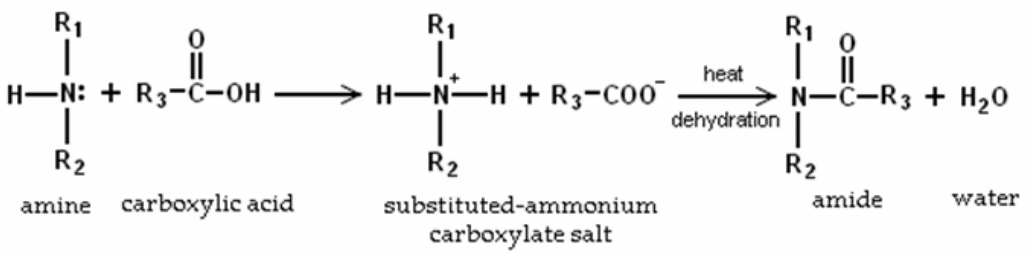


Figure 12 The structure of carboxylic acid-secondary amine complex to amide formation.

The chain length might also affect the extraction. TOA with 8 carbon atoms gave better extraction efficiency than 12 carbon atoms of TDDA which were also a tertiary amine. Similar study by Matsumoto et al. (2003) also supported this result. They had concluded that extractability of extractant increased with increasing alkyl chain length and tri-n-octylamine (TOA) enabled the best extractability. However, further increase in chain length resulted in a decrease in extractability.

Extraction using various concentrations of TOA in octanol at various ratios of organic to aqueous phases showed that higher extraction percentages were resulted when increasing the ratio of organic to aqueous phases at every TOA concentrations used. The results agreed with Kahya et al. (2001) who reported that the value of K_D increased with increasing Alamine® 336 concentrations from 15% to 50% in oleyl alcohol at volume ratio of organic to aqueous phases of 1:4 to 1:1. Another study reported that when lactic acid was extracted with 50% TOA in octanol and paraffin liquid, the extraction percentage increased with increasing organic to aqueous phase ratio from 1:1 to 4:1 while it did not improve further at higher phase ratio (Choudhury and Swaminathan, 1998).

At the organic to aqueous volume ratio of 1:4, volume of organic phase became the limitation to the extraction. Therefore, increasing extractant concentration resulted in higher extraction efficiency as more extractant was available. The opposite situation in case of 4:1 ratio could be due to the presence of more diluents. At the ratio 4:1, organic or extractant phase was no longer the limit such that enough or excess extractant was available at every extractant concentrations. Since the extraction of lactic acid depended upon the formation of lactic acid/amine complex (Figure 13) and the presence of diluents provided the opportunity of extractant to form the complexes (Keshav et al., 2008). The extraction

occurred better at lower extractant (TOA) concentration as more diluent was presented.

At the ratio of 1:1, previous explanations could be applied to both changes in the extraction percentage trends. The increasing trend when TOA concentration was below 1.1 M, which was equivalent to concentration of lactic acid, was due to increasing extractant availability. Whereas, less diluent was the cause for the decrease in extraction percentage when TOA concentration exceeded 1.1 M. The implementation of diluents in the extraction process is an important factor for improvement of acid extraction. Since most of extractants are viscous, they are usually dissolved in diluents to improve their physical properties. Diluent provided general solvation and affected the extraction power of the extractant by providing specific interaction. Furthermore, diluent prevent third phase formation that builds up due to the association of carboxylic acid and extractant (Keshav et al., 2008). According to the report by Yang et al. (1991) that also described that the higher concentrations of Alamine® 336 increased the viscosity of the organic phase, which was not favorable for extraction. Moreover, for extractions with high concentration of amine in diluent, a third emulsion phase could be observed at the interface between the aqueous and organic phases. The result was also indicated that lactic acid-TOA complex was combined with the ratio of 1:1. Formation of a 1:1 lactic acid-amine complex is common (Kailas et al., 2004) with the general structure shown in Figure 13. As observed from the structure, acid interacted directly with the amine to form an ion pair.

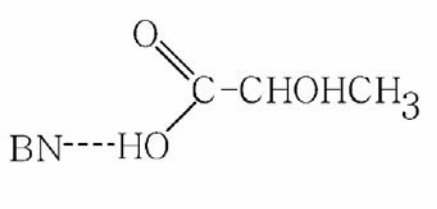


Figure 13 The structure of lactic acid-tertiary amine complex: BN is the tertiary amine (Kailas et al., 2004)

4.4. Lactic acid production by fermentation with addition of ion exchange resin, recovery and partial purification scheme

From previous experiments, the suitable conditions were obtained from each part i.e. fermentation with addition of ion exchange resin, resin elution and regeneration and liquid-liquid extraction. The conditions had been investigated as separated process. In this part, all separated processes were integrated into one single process. The system consisted of (1) lactic acid production by fermentation with addition of ion exchange resin (2) lactic acid recovery from ion exchange resin obtained from the fermentation (3) resin regeneration and (4) extraction of lactic acid from resin eluate and from fermentation broth.

The ion exchange fermentation for lactic acid production by *L lactis* NZ133 was carried out in 500 ml of modified M17 medium containing an initial glucose concentration of 168.2 gl^{-1} and resin of 311.5 g was added in order to achieve higher lactic acid production yield and productivity when compared with batch fermentation at the same initial glucose concentration. The fermentation was completed in 15 h and generated the final cell concentration of 10.8 gl^{-1}. The cell productivity was 0.72 $gl^{-1}h^{-1}$ and the cell yield was 0.07 gg^{-1}. The fermentation was 5 times faster than the batch cultivation alone (section 4.1.1). The viable cell and dried cell weight concentration also had an increasing trend throughout the fermentation time course.

The remaining lactate concentration in the fermentation broth at the end was 64.0 gl^{-1} (Figure 14). This concentration was equivalent to 31.2 g of lactate. In order to calculate for the resin-bound lactate, total lactate production was needed for the calculation. Total lactate production was predicted from resin capacity of 0.15 $gg^{-1}_{resin,\ wet}$. The total lactate of 77.9 g was resulted from the calculation and lactate-bound resin was calculated to be 46.7 g. Therefore, the total lactic acid productivity was 5.16 gh^{-1} and the lactic acid yield was 0.96 gg^{-1}.

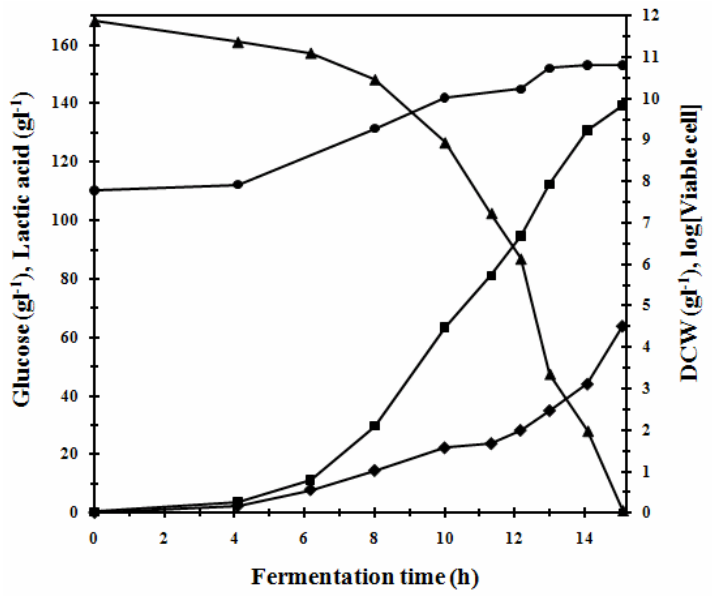


Figure 14 Fermentation profile in batch culture with 168.2 gl^{-1} glucose in M17 modified medium with progressive addition of 311.50 g of IRA-67 resin at 37 °C and pH 6.5: (◆) lactic acid, (▲) glucose, (■) DCW and (●) viable cell

After the fermentation, lactate-bound resin was separated and washed with water. Washing of 721 ml resin was carried out until the wash water was clear. In the washing step, 8.8 g or approximately 19.1% of lactate was lost. The result was similar to Rojan et al. (2008) who reported that the elution recovery of lactic acid from IRA-67 using water was 22.3%. Therefore, the remaining lactate in the resin should be 37.9 g. The resin was then transferred into a column for elution. The large column size was 5.0×43.0 cm. The resin was eluted using 1 M HCl in downward direction at the flow rate of 0.1 $BVmin^{-1}$ (actual value = 70.0 ml min^{-1}) until the yellowness of outlet stream disappeared. The elution result showed that 2,172 ml of HCl was used and the elution took 33.3 min. Total lactate in eluate stream was 37.4 g, which was similar to expected remaining lactic acid of 37.9 g in the resin after being washed. It indicated that lactate could be fully recovered in elution step. According to the use of 1.0 M H_2SO_4 as eluant for lactic acid recovery with IRA-400, almost 100% recovery was achieved by material balance calculation (Cao et al., 2002). Up to the end of resin elution step, the results indicated that 80.9% of the resin-bound lactate could be recovered where majority of lactate loss occurred in washing step. When the resin was regenerated using 1 M NaOH in down flow direction at 0.1 $BVmin^{-1}$ (actual value

= 71.4 ml min^{-1}). The resin was completely regenerated after 32.7 min, using 2,560 ml of 1 N NaOH.

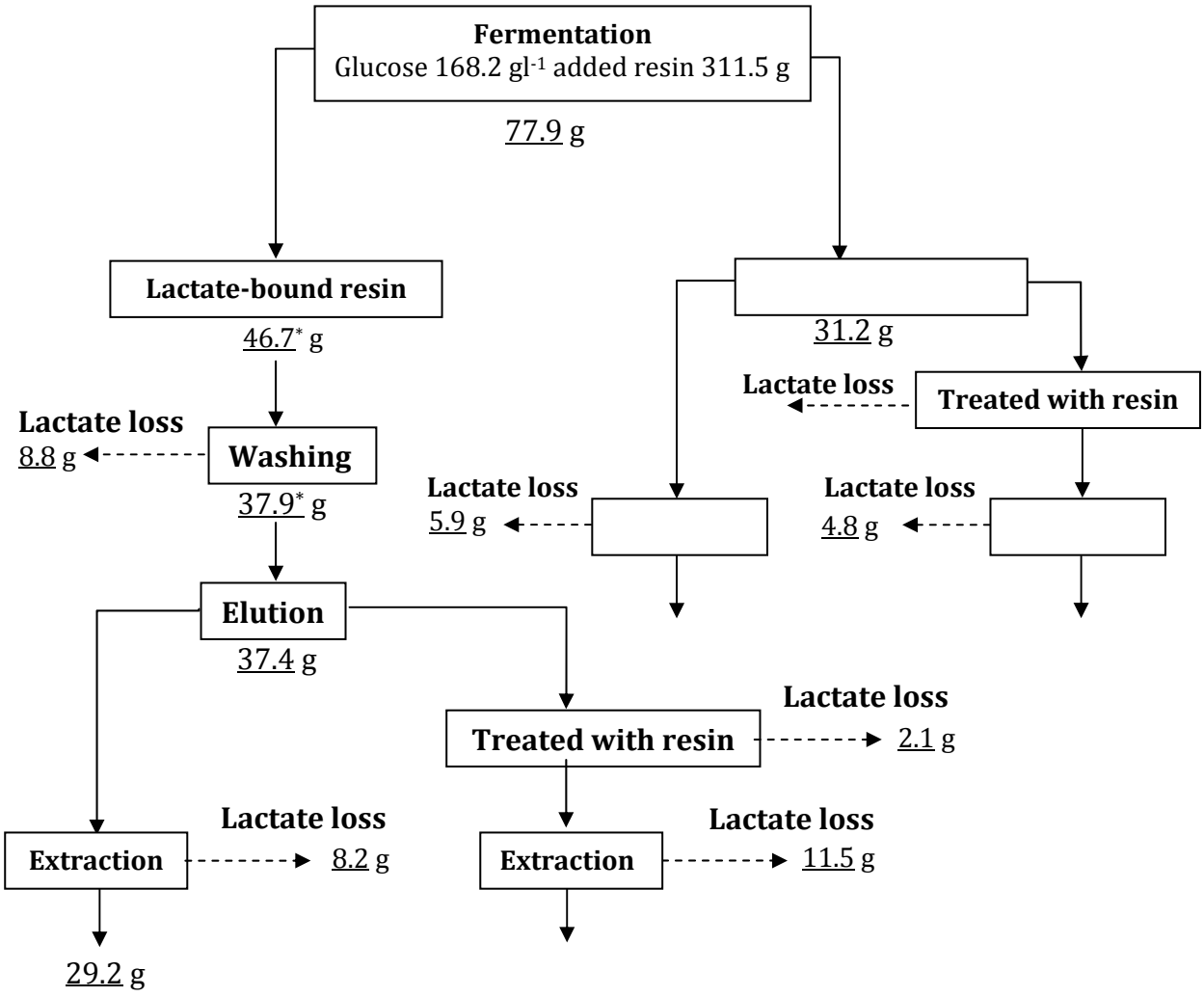
Liquid-liquid extraction was operated in order to partially purify lactate in the eluate and to recover the lactate remained in the fermentation broth. Lactate in the eluate was extracted with or without being treated by cation exchange resin IRA-98 prior to extraction. All solutions were extracted with equal concentration of TOA in octanol at 1:1 organic to aqueous phase volume ratio at 30 °C for 2 h. With untreated eluate, 29.2 g of lactic acid was extracted into the organic phase, indicating 77.0% extraction. The extracion percentage decreased to 67.5% when extracting lactic acid from resin-treated eluate. Treating the eluate by IRA-98 decreased the lactic acid content in eluate to 35.3 g prior to extraction.

The broth from the fermentation with resin addition that initially contained 31.2 g of lactic acid was extracted with or without being treated by IRA-98 resin. When the fermentation broth was extracted with 0.65 M TOA in octanol, 25.3 g of lactic acid was extracted into the organic phase or calculate to be 83.7% extraction. However, the lactate in broth was decreased to 28.8 g after resin treatment. The lactic acid extracted to the organic phase was 24.0 g, which was 83.1% extraction.

When lactic acid extracted from the non-treated eluate and that extracted from the non-treated fermentation broth were combined, the total lactic acid was 54.5 g. The total lactic acid recovery was calculated to be 70.0%. Whereas when lactic acid from the resin-treated eluate was combined with lactic acid from the resin-treated broth, the total lactic acid was 47.8 g, which was accounted for 61.4% recovery. Figure 15 summarised the lactic acid recovery scheme.

Protein concentrations were determined to verify the partial purification by the extraction. The result showed the initial protein concentration of the non-treated eluate to be 1.0 gl^{-1} and the concentration after the extraction was unchanged. The result was the similar in the extraction of the resin-treated eluate that protein concentrations before and after extraction were 1.0 gl^{-1}. The non-treated fermentation broth resulted in the initial protein concentration of 26.5 gl^{-1} which was similar in the final protein concentration after extraction of 26.0 gl^{-1}. Furthermore, the initial protein concentration in the resin-treated fermentation broth was 25.9 gl^{-1} and the final protein concentration after the extraction was 25.5 gl^{-1}. It indicated that the protein was not simultaneously extracted with lactic acid into the organic phase.

Therefore, it could be implied that the liquid extraction of lactic acid by TOA in octanol could provide partial purification to lactic acid solution as protein in the broth was excluded.



* Expected lactate adsorbed in resin

Figure 15 Diagram of proposed lactic acid production process with the recovery and partial purification scheme illustrating mass balance of lactate

5. Summary and Suggestion for Future Researches

In order to achieve high lactic acid production by batch fermentation at high initial glucose concentration, lactate inhibition effect was mitigated by in situ removal of lactate using ion exchange technique. Anion exchange resin, Amberlite® IRA-67, was used for this purpose by being progressively added to the batch fermentation at 111 to 212 gl^{-1} initial glucose. Lactic acid production by fermentation with addition of ion exchange resin can improve lactic acid concentration and lactic acid yield from those obtained from conventional batch, from 102.1 to 151.2 gl^{-1} and from 0.80 to 0.99 gg^{-1}, when using 156 gl^{-1} initial glucose. Furthermore, lactic acid productivities were clearly enhanced in all of initial glucose levels. When using 111, 156 and 212 gl^{-1} glucose, lactic acid productivities were 2.0, 6.2 and 3.3 times higher than the batch at the same glucose, respectively.

After the fermentation, lactate-bound resin was washed in order to clean the resin prior to be eluted. The washing of resin with methanol caused lesser lactic acid loss than with water. However, water was chosen as it could reduce the operational cost of the process.

In the study for elution conditions using HCl as the eluant and resin volume of 100 ml, the flow rate of 0.1 $BVmin^{-1}$ and HCl concentration of 1 M were chosen as it could elute total lactic acid of 4.29 ± 0.59 g at minimal volume of approximately 300 ± 60 ml.

In resin regeneration study using NaOH as the regenerant and resin volume of 100 ml, 1 M NaOH at the flow rate of 0.1 $BVmin^{-1}$ was chosen for regeneration of resin. The resin could be regenerated within 48.8 ± 4.2 min at the minimal regenerant volume of 323 ± 101 ml when using the selected conditions.

In liquid-liquid extraction study using aqueous lactic acid solution (~97 gl^{-1}), trioctylamine (TOA) and octanol were chosen as the extractant and diluent for extracting lactic acid. TOA concentration used in the extraction has to be approximately equal to lactic acid concentration in the solution to be extracted.

Lactic acid extraction was completed in approximately 2 h. Extraction percentage of 81.2% was achieved when using the organic to aqueous phase volume ratio of 1:1. The process could be carried out at room temperature.

These extraction conditions, when applied to solution of lactic acid (~84 gl^{-1}) in M17 broth, caused the volume of organic phase to increase. However, when treating the solution with cation exchange resin, Amberlite® IRA-98, such volume change was not noticed. Treatment of fermentation broth contain lactic acid with resin was required only when lactic acid concentration was high. Extraction percentage of 83.6% was obtained when extracting 96 gl^{-1} lactic acid in M17 broth after treatment with IRA-98 resin.

Furthermore, the extraction conditions were applied to extract the eluate from elution step. Final lactic acid recoveries after the extraction step were 71.9% and 74.2%, with and without resin treatment. Liquid extraction was able to partially purify the lactic acid solutions as the protein in fermentation broth were not transferred to the organic phase with lactic acid.

After separated studies of fermentation, resin elution and extraction, all steps were combined into a single continuous process using selected conditions from each study. When the anion exchange resin (IRA-67) was progressively added to the batch fermentation at 168 gl^{-1} initial glucose, lactic acid yield of 0.96 was obtained. Resin-bound lactate accounted for 59.9% of lactate produced. Washing of lactate-bound resin with water resulted in 19.1% loss of lactic acid. Lactate can be fully recovered from the resin by using 1 M HCl at 0.1 BV min^{-1} (actual value = 70.1 ml min^{-1}). Approximately 70.0% of lactic acid recovery by liquid-liquid extraction was achieved when the eluate and fermentation broth were not treated by IRA-98 resin. Whereas, when the eluate and fermentation broth were treated by IRA-98, 61.4% of lactic acid recovery was resulted.

Further stripping of organic phase to recover the lactic acid and to obtain the reusable extractant would be also required. However, the step is beyond the scope of this study and could be recommended for further study. In addition, online monitoring of elution by optical density to determine end-point of elution could also be investigated in the future.

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7. Appendix

7.1. Calculations of yields and productivities of batch fermentations and batch fermentations with ion exchange resin addition

Table 10 Calculation of resin capacity using the data from elution study.

Average total lactic acid from elution (g) (*A*)	Weight of the resin* (g) (*B*)	Resin capacity ($g_{lactic\ acid}\ g^{-1}_{resin,\ wet}$) (*A/B*)
4.3	25.8	0.17
4.3	30.4	0.14
4.3	29.8	0.14
4.3	31.6	0.14
4.3	26.4	0.16
4.3	31.1	0.14
4.3	27.8	0.15
4.3	29.7	0.14
4.3	26.4	0.16
4.3	28.7	0.15
4.3	29.8	0.14
	Average =	**0.15**

* Based on 100 ml resin

Table 11 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 100 gl^{-1} glucose and 200 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 482.40 ml
Initial glucose level (*B*)	= 56.10 g
Final glucose level (*C*)	= 0.02 g
Glucose loss from sampling (*D*)	= 3.22 g
Actual glucose consumption (*E=B-C-D*)	= 52.85 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 203.02 g
Lactic acid adsorbed in resin (*H=F*G*)	= 30.45 g
Lactic acid in the broth (*I*)	= 17.24 g
Total lactic acid (*J=H+I*)	= 47.69 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 52.85 g
Total lactic acid (*J*)	= 47.69 g
Yield (*J/E*)	= 0.90 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 47.69 g
Fermentation time (*I*)	= 11.08 h
Productivity (*J/I*)	= 4.30 gh^{-1}

Table 12 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 100 gl^{-1} glucose and 250 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 472.60 ml
Initial glucose level (*B*)	= 57.68 g
Final glucose level (*C*)	= 0.02 g
Glucose loss from sampling (*D*)	= 3.13 g
Actual glucose consumption (*E=B-C-D*)	= 54.52 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 255.97 g
Lactic acid adsorbed in resin (*H=F*G*)	= 38.40 g
Lactic acid in the broth (*I*)	= 13.75 g
Total lactic acid (*J=H+I*)	= 52.14 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 54.52 g
Total lactic acid (*J*)	= 52.14 g
Yield (*J/E*)	= 0.96 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 52.14 g
Fermentation time (*I*)	= 11.05 h
Productivity (*J/I*)	= 4.72 gh^{-1}

Table 13 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 100 gl^{-1} glucose and 300 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 472.30 ml
Initial glucose level (*B*)	= 55.78 g
Final glucose level (*C*)	= 0.03 g
Glucose loss from sampling (*D*)	= 2.81 g
Actual glucose consumption (*E=B-C-D*)	= 52.94 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 309.03 g
Lactic acid adsorbed in resin (*H=F*G*)	= 46.35 g
Lactic acid in the broth (*I*)	= 11.56 g
Total lactic acid (*J=H+I*)	= 57.91 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 52.94 g
Total lactic acid (*J*)	= 57.91 g
Yield (*J/E*)	= 1.09 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 57.91 g
Fermentation time (*I*)	= 11.75 h
Productivity (*J/I*)	= 4.93 gh^{-1}

Table 14 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 150 gl^{-1} glucose and 200 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 504.30 ml
Initial glucose level (*B*)	= 75.36 g
Final glucose level (*C*)	= 1.20 g
Glucose loss from sampling (*D*)	= 2.62 g
Actual glucose consumption (*E=B-C-D*)	= 71.54 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 203.78 g
Lactic acid adsorbed in resin (*H=F*G*)	= 30.57 g
Lactic acid in the broth (*I*)	= 40.30 g
Total lactic acid (*J=H+I*)	= 70.87 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 71.54 g
Total lactic acid (*J*)	= 70.87 g
Yield (*J/E*)	= 0.99 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 70.87 g
Fermentation time (*I*)	= 15.23 h
Productivity (*J/I*)	= 4.65 gh^{-1}

Table 15 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 150 gl^{-1} glucose and 250 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 492.60 ml
Initial glucose level (*B*)	= 81.40 g
Final glucose level (*C*)	= 0.04 g
Glucose loss from sampling (*D*)	= 2.86 g
Actual glucose consumption (*E=B-C-D*)	= 78.50 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 264.32 g
Lactic acid adsorbed in resin (*H=F*G*)	= 39.65 g
Lactic acid in the broth (*I*)	= 35.55 g
Total lactic acid (*J=H+I*)	= 75.19 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 78.50 g
Total lactic acid (*J*)	= 75.19 g
Yield (*J/E*)	= 0.96 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 75.19 g
Fermentation time (*I*)	= 16.58 h
Productivity (*J/I*)	= 4.53 gh^{-1}

Table 16 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 150 gl^{-1} glucose and 300 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 489.00 ml
Initial glucose level (*B*)	= 80.45 g
Final glucose level (*C*)	= 0.59 g
Glucose loss from sampling (*D*)	= 2.56 g
Actual glucose consumption (*E=B-C-D*)	= 77.29 g
Resin capacity (*F*)	=0.15 gg^{-1}
Total resin added (G)	= 309.60 g
Lactic acid adsorbed in resin (*H=F*G*)	= 46.44 g
Lactic acid in the broth (*I*)	= 32.01 g
Total lactic acid (*J=H+I*)	= 78.45 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 77.29 g
Total lactic acid (*J*)	= 78.45 g
Yield (*J/E*)	= 1.02 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 78.45 g
Fermentation time (*I*)	= 15.00 h
Productivity (*J/I*)	= 5.23 gh^{-1}

Table 17 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 200 gl^{-1} glucose and 200 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 503.70 ml
Initial glucose level (*B*)	= 110.29 g
Final glucose level (*C*)	= 1.16 g
Glucose loss from sampling (*D*)	= 3.52 g
Actual glucose consumption (*E=B-C-D*)	= 105.61 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 205.72 g
Lactic acid adsorbed in resin (*H=F*G*)	= 30.86 g
Lactic acid in the broth (*I*)	= 58.01 g
Total lactic acid (*J=H+I*)	= 88.87 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 105.61 g
Total lactic acid (*J*)	= 88.87 g
Yield (*J/E*)	= 0.84 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 88.87 g
Fermentation time (*I*)	= 29.08 h
Productivity (*J/I*)	= 3.06 gh^{-1}

Table 18 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 200 gl^{-1} glucose and 250 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 492.10 ml
Initial glucose level (*B*)	= 107.45 g
Final glucose level (*C*)	= 0.31 g
Glucose loss from sampling (*D*)	= 4.91 g
Actual glucose consumption (*E=B-C-D*)	= 102.23 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 257.20 g
Lactic acid adsorbed in resin (*H=F*G*)	= 38.58 g
Lactic acid in the broth (*I*)	= 46.29 g
Total lactic acid (*J=H+I*)	= 84.87 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 102.23 g
Total lactic acid (*J*)	= 84.87 g
Yield (*J/E*)	= 0.83 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 84.87 g
Fermentation time (*I*)	= 30.00 h
Productivity (*J/I*)	= 2.65 gh^{-1}

Table 19 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 200 gl^{-1} glucose and 300 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 492.20 ml
Initial glucose level (*B*)	= 104.92 g
Final glucose level (*C*)	= 0.21 g
Glucose loss from sampling (*D*)	= 4.83 g
Actual glucose consumption (*E=B-C-D*)	= 99.88 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 306.18 g
Lactic acid adsorbed in resin (*H=F*G*)	= 45.93 g
Lactic acid in the broth (*I*)	= 43.76 g
Total lactic acid (*J=H+I*)	= 89.68 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 99.88 g
Total lactic acid (*J*)	= 89.68 g
Yield (*J/E*)	= 0.90 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 89.68 g
Fermentation time (*I*)	= 29.00 h
Productivity (*J/I*)	= 3.09 gh^{-1}

7.2. Washing data and ANOVA of lactic acid recovery and resin regeneration experiments

Table 20 Data of the lactic acid loss when lactate-bound IRA-67 was washed with water in washing step

Fraction number	Volume (ml)			Lactic acid (gl^{-1})		
	Repeat1	Repeat2	Repeat3	Repeat1	Repeat2	Repeat3
1	250	250	250	10.47	10.35	10.24
2	251	250	252	3.80	2.62	8.28
3	250	254	252	1.48	1.49	1.68
4	252	252	251	0.91	0.58	0.79
5	249	250	250	0.73	0.51	0.48
6	249	251	248	0.64	0.50	0.42
7	250	247	251	0.65	0.49	0.36
8	251	249	250	0.61	0.47	0.34
9	252	251	250	0.75	0.76	0.55
10	250	252	249	0.49	0.48	0.36
11	248	251	250	0.73	0.36	0.26
12	250	250	248	0.43	0.34	0.27
13	251	252	250	0.38	0.67	0.28

Table 21 Data of the lactic acid loss when lactate-bound IRA-67 was washed with methanol in washing step

Fraction number	Volume (ml)			Lactic acid (gl^{-1})		
	Repeat1	Repeat2	Repeat3	Repeat1	Repeat2	Repeat3
1	252	251	254	10.26	8.52	9.92
2	252	248	248	3.50	2.54	4.57
3	250	248	251	1.07	1.20	1.86
4	251	251	250	0.54	0.59	0.89
5	250	252	250	0.34	0.30	0.51
6	248	252	248	0.27	0.26	0.36
7	250	250	248	0.25	0.29	0.27
8	252	250	250	0.23	0.23	0.24
9	252	250	251	0.41	0.45	1.60
10	250	251	250	0.26	0.34	1.26
11	248	250	250	0.54	0.90	0.55
12	249	249	251	0.31	0.34	0.33
13	251	249	248	0.25	0.67	0.25

Table 22 ANOVA table for total lactic acid (Response1) in elution step

Source	Sum of Squares	df	Mean Square	F-Value	p-value Prob > F	
Model	2.12	8	0.26	0.37	0.9222	not significant
A-flow rate	0.77	2	0.39	0.54	0.5913	
B-HCl conc.	0.70	2	0.35	0.49	0.6209	
AB	0.65	4	0.16	0.23	0.9193	
Pure Error	12.84	18	0.71			
Cor Total	14.95	26				

The "Model F-value" of 0.37 implies the model is not significant relative to the noise. There is a 92.22 % chance that a "Model F-value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

In this case there is no significant model term.

Values greater than 0.1000 indicate the model terms are not significant.

If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Std. Dev.	0.84	R-Squared	0.1417
Mean	4.26	Adj R-Squared	-0.2398
C.V. %	19.84	Pred R-Squared	-0.9312
PRESS	28.88	Adeq Precision	1.771

A negative "Pred R-Squared" implies that the overall mean is a better predictor of your response than the current model.

"Adeq Precision" measures the signal to noise ratio. A ratio of 1.77 indicates an inadequate signal and we should not use this model to navigate the design space.

Table 23 ANOVA table for volume (Response2) in elution step

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	5.678E+005	8	70972.93	11.60	< 0.0001	significant
A-flow rate	5.619E+005	2	2.809E+005	45.90	< 0.0001	
B-HCl conc.	1098.74	2	549.37	0.090	0.9146	
AB	4813.04	4	1203.26	0.20	0.9370	
Pure Error	1.102E+005	18	6120.85			
Cor Total	6.780E+005	26				

The Model F-value of 11.60 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

In this case *A* are significant model terms.

Values greater than 0.1000 indicate the model terms are not significant.

If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Std. Dev.	78.24	R-Squared	0.8375
Mean	452.48	Adj R-Squared	0.7653
C.V. %	17.29	Pred R-Squared	0.6344
PRESS	2.479E+005	Adeq Precision	8.398

The "Pred R-Squared" of 0.6344 is in reasonable agreement with the "Adj R-Squared" of 0.7653.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 8.398 indicates an adequate signal. This model can be used to navigate the design space.

Table 23(continued) ANOVA table for volume (Response2) in elution step

Term	Coefficient Estimate	df	Standard Error	95% CI Low	95% CI High
Intercept	452.48	1	15.06	420.85	484.11
A	-166.26	1	21.29	-210.99	-121.52
A^2	-19.26	1	21.29	-63.99	25.48
B	-3.59	1	21.29	-48.33	41.14
B^2	-5.37	1	21.29	-50.11	39.36
AB	17.04	1	30.11	-46.23	80.30
A^2B	-20.30	1	30.11	-83.56	42.97
AB^2	4.81	1	30.11	-58.45	68.08
A^2B^2	4.15	1	30.11	-59.12	67.41

Final equation in terms of coded factors:

$$\text{Volume} = 452.48 - 166.26A - 19.26A^2 - 3.59B - 5.37B^2 + 17.04AB - 20.30A^2B + 4.81AB^2 + 4.15A^2B^2$$

Table 24 Optimization of elution step

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Flow rate	Is in range	Level 1 of A	Level 3 of A	1	1	3
HCl conc.	Is in range	Level 1 of B	Level 3 of B	1	1	3
Lactic acid	Maximize	2.91	6.17	1	1	3
Volume	Minimize	220	769	1	1	3

Solutions for 9 combinations of categoric factor levels

Number	Flow rate	HCl conc.	Lactic acid	Volume	Desirability	
1	Level 1 of A	Level 2 of B	4.54	285.667	0.663	Selected
2	Level 1 of A	Level 1 of B	4.29333	299.667	0.602	
3	Level 1 of A	Level 3 of B	4.07333	273.333	0.568	
4	Level 2 of A	Level 3 of B	4.24	458.333	0.480	
5	Level 2 of A	Level 2 of B	4.08333	432	0.470	
6	Level 2 of A	Level 1 of B	3.77	409.333	0.416	
7	Level 3 of A	Level 2 of B	4.63333	623.667	0.374	
8	Level 3 of A	Level 3 of B	4.63	652.667	0.334	
9	Level 3 of A	Level 1 of B	4.05	637.667	0.289	

Table 25 ANOVA table for time (Response1) in regeneration step

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	8294.41	8	1036.80	215.63	< 0.0001	significant
A-flow rate	8211.39	2	4105.69	853.89	< 0.0001	
B-HCl conc.	60.21	2	30.11	6.26	0.0086	
AB	22.80	4	5.70	1.19	0.3506	
Pure Error	86.55	18	4.81			
Cor Total	8380.95	26				

The Model F-value of 215.63 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

In this case *A*, *B* are significant model terms.

Values greater than 0.1000 indicate the model terms are not significant.

If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Std. Dev.	2.19	R-Squared	0.9897
Mean	23.41	Adj R-Squared	0.9851
C.V. %	9.37	Pred R-Squared	0.9768
PRESS	194.73	Adeq Precision	32.125

The "Pred R-Squared" of 0.9768 is in reasonable agreement with the "Adj R-Squared" of 0.9851.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 32.125 indicates an adequate signal. This model can be used to navigate the design space.

Table 25 (continued) ANOVA table for time (Response1) in regeneration step

Term	Coefficient Estimate	df	Standard Error	95% Cl Low	95% Cl High
Intercept	23.41	1	0.42	22.52	24.29
A	24.66	1	0.60	23.41	25.92
A^2	-12.15	1	0.60	-13.40	-10.90
B	1.80	1	0.60	0.55	3.05
B^2	0.057	1	0.60	-1.20	1.31
AB	-1.08	1	0.84	-2.85	0.70
A^2B	1.76	1	0.84	-0.012	3.53
AB^2	0.25	1	0.84	-1.52	2.03
A^2B^2	-0.48	1	0.84	-2.26	1.29

Final equation in terms of coded factors:

$$\text{Time} = 23.41 + 24.66A - 12.15A^2 + 1.80B + 0.057B^2 - 1.08AB + 1.76A^2B + 0.25AB^2 - 0.48A^2B^2$$

Table 26 ANOVA table for volume (Response2) in regeneration step

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	1.173E+006	8	1.466E+005	36.83	< 0.0001	significant
A-flow rate	7.808E+005	2	3.904E+005	98.08	< 0.0001	
B-HCl conc.	2.577E+005	2	1.289E+005	32.38	< 0.0001	
AB	1.344E+005	4	33592.20	8.44	0.0005	
Pure Error	71645.33	18	3980.30			
Cor Total	1.245E+006	26				

The Model F-value of 36.83 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Values of "Prob > F" less than 0.0500 indicate model terms are significant.

In this case *A*, *B*, *AB* are significant model terms.

Values greater than 0.1000 indicate the model terms are not significant.

If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve your model.

Std. Dev.	63.09	R-Squared	0.9424
Mean	521.59	Adj R-Squared	0.9168
C.V. %	12.10	Pred R-Squared	0.8705
PRESS	1.612E+005	Adeq Precision	17.488

The "Pred R-Squared" of 0.8705 is in reasonable agreement with the "Adj R-Squared" of 0.9168.

"Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 17.488 indicates an adequate signal. This model can be used to navigate the design space.

Table 26 (continued) ANOVA table for volume (Response2) in regeneration step

Term	Coefficient Estimate	df	Standard Error	95% Cl Low	95% Cl High
Intercept	521.59	1	12.14	496.08	547.10
A	-186.70	1	17.17	-222.78	-150.63
A^2	-37.93	1	17.17	-74.00	-1.85
B	110.41	1	17.17	74.33	146.48
B^2	16.74	1	17.17	-19.33	52.82
AB	-122.63	1	24.28	-173.65	-71.61
A^2B	25.93	1	24.28	-25.09	76.94
AB^2	14.04	1	24.28	-36.98	65.05
A^2B^2	-0.41	1	24.28	-51.42	50.61

Final equation in terms of coded factors:

$$\text{Volume} = 521.59 - 186.70A - 37.93A^2 + 110.41B + 16.74B^2 - 122.63AB + 25.93A^2B + 14.04AB^2 - 0.41A^2B^2$$

Table 27 Optimization of regeneration step

Name	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
Flow rate	Is in range	Level 1 of A	Level 3 of A	1	1	3
NaOH conc.	Is in range	Level 1 of B	Level 3 of B	1	1	3
Time	Is in range	7.12	51.28	1	1	3
Volume	Minimize	222	990	1	1	3

Solutions for 9 combinations of categoric factor levels

Number	Flow rate	HCl conc.	Time	Volume	Desirability	
1	Level 1 of A	Level 3 of B	47.0367	316.333	0.877	Selected
2	Level 1 of A	Level 1 of B	48.7933	322.667	0.869	
3	Level 2 of A	Level 3 of B	8.12333	331	0.858	
4	Level 1 of A	Level 2 of B	48.38	365.667	0.813	
5	Level 2 of A	Level 2 of B	10.8333	500	0.638	
6	Level 3 of A	Level 3 of B	9.49333	536	0.591	
7	Level 2 of A	Level 1 of B	14.82	620	0.482	
8	Level 3 of A	Level 2 of B	11.1833	749.333	0.313	
9	Level 3 of A	Level 1 of B	12.01	953.333	0.048	

7.3. Calculations of yield and productivity of lactic acid production by fermentation with addition of ion exchange resin, recovery and partial purification scheme

Table 28 Calculations of total lactic acid, yield and productivity from the fermentation of *L. lactis* NZ133 in M17 modified broth with 150 gl^{-1} glucose and 300 g ion exchange resin addition.

1. Calculation of total lactic acid	
Actual volume in the fermenter at the final time point (*A*)	= 486.70 ml
Initial glucose level (*B*)	= 85.35 g
Final glucose level (*C*)	= 0.41 g
Glucose loss from sampling (*D*)	= 4.11 g
Actual glucose consumption (*E=B-C-D*)	= 80.84 g
Resin capacity (*F*)	= 0.15 gg^{-1}
Total resin added (G)	= 311.45 g
Lactic acid adsorbed in resin (*H=F*G*)	= 46.72 g
Lactic acid in the broth (*I*)	= 31.17 g
Total lactic acid (*J=H+I*)	= 77.89 g
2. Calculation of lactic acid yield	
Actual glucose consumption (*E*)	= 80.84 g
Total lactic acid (*J*)	= 77.89 g
Yield (*J/E*)	= 0.96 gg^{-1}
3. Calculation of lactic acid productivity	
Total lactic acid (*J*)	= 77.89 g
Fermentation time (*I*)	= 15.08 h
Productivity (*J/I*)	= 5.16 gh^{-1}