fermentation

MDPI

Article

Extractive Fermentation as A Novel Strategy for High Cell Mass Production of Hetero-Fermentative Probiotic Strain *Limosilactobacillus reuteri*

Shanmugaprakasham Selvamani [1,2,3], Solleh Ramli [1], Daniel Joe Dailin [1,2], Khairun Hani Natasya [1], Theodoros Varzakas [1,4], Bassam Abomoelak [5], Dalia Sukmawati [6], Muktiningsih Nurjayadi [6], Siqing Liu [7], Vijai Kumar Gupta [8,9] and Hesham Ali El Enshasy [1,2,10,*]

1 Institute of Bioproduct Development, Universiti Teknologi Malaysia (UTM), Skudai 81310, Malaysia
2 School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia (UTM), Skudai 81310, Malaysia
3 Nutrition Technologies Sdn. Bhd., No 1 & No 3, Jalan Silc 2, Kawasan Perindustrian SiLC, Nusajaya 79150, Malaysia
4 Department Food Science and Technology, University of the Peloponnese, 24100 Kalamata, Greece
5 Arnold Palmer Hospital Paediatric Speciality Diagnostic Laboratory, Orlando, FL 32806, USA
6 Faculty of Mathematics and Natural Science, Universitas Negeri Jakarta, Jakarta Timur 13220, Indonesia
7 Agriculture Research Service, Renewable Product Technology Research Unit, National Center for Agricultural Utilization Research, USDA, 1815 N University St., Peoria, IL 61604, USA
8 Biorefining and Advanced Materials Research Center, SRUC, Kings Buildings, West Mains Road, Edinburgh EH9 3JG, UK
9 Center for Safe and Improved Food, SRUC, Kings Buildings, West Mains Road, Edinburgh EH9 3JG, UK
10 City of Scientific Research and Technology Applications, New Burg Al Arab, Alexandria 21934, Egypt
* Correspondence: henshasy@ibd.utm.my

Abstract: This study reports on a novel technique to enhance the high cell mass and viable cell counts of the heterofermentative probiotic strain, *Limosilactobacillus reuteri*. This is the first report on the cultivation of *L. reuteri*, which was incorporated with weak base anion-exchange resins to remove the accumulating lactic acid in the fermentation broth. Two anion-exchange resins—Amberlite IRA 67 and IRA 96—were found to have a high adsorption capacity with lactic acid. Batch fermentation and fed-batch cultivation were further analyzed using IRA 67 resins, as this application resulted in a higher maximum number of viable cells. The in situ application of anion-exchange resins was found to create shear stress, and thus, it does not promote growth of *L. reuteri*; therefore, an external and integrated resin column system was proposed. The viable cell count from batch fermentation, when incorporated with the integrated resin column, was improved by 71 times ($3.89 \times 10^{11} \pm 0.07$ CFU mL^{-1}) compared with control batch fermentation ($5.35 \times 10^{9} \pm 0.32$ CFU mL^{-1}), without the addition of resins. The growth improvement was achieved due to the high adsorption rate of lactic acid, which was recorded by the integrated IRA 67 resin system, and coupled with the stirred tank bioreactor batch fermentation process.

Keywords: lactic acid bacteria; heterofermentative; high-cell density; extractive fermentation; anion-exchange resins; lactic acid removal

check for updates

Citation: Selvamani, S.; Ramli, S.; Dailin, D.J.; Natasya, K.H.; Varzakas, T.; Abomoelak, B.; Sukmawati, D.; Nurjayadi, M.; Liu, S.; Gupta, V.K.; et al. Extractive Fermentation as A Novel Strategy for High Cell Mass Production of Hetero-Fermentative Probiotic Strain *Limosilactobacillus reuteri*. *Fermentation* **2022**, *8*, 527. https://doi.org/10.3390/fermentation8100527

Academic Editor: Donatella Cimini

Received: 6 September 2022
Accepted: 3 October 2022
Published: 10 October 2022

1. Introduction

The global probiotic market continues to grow rapidly, as does the number of probiotic therapies on offer. In the twenty-first century, the probiotic market, and research relating to the probiotic market, started to look for "smart probiotics"—multi-potential probiotic strains, and "next generation probiotics"—and other potential probiotic strains besides traditional lactic acid bacterial genera [1,2]. Despite new emergent terms, and a growing number of potential strains, the success of the probiotic industries and their products always rely on the number of probiotic cells (in terms of mass) or viable probiotic cells in

their products. The FAO/WHO defines probiotics as "live microorganisms, which, when administrated in an adequate amount, could confer a health benefit on the host" [1,3]. Owing to this fact, the WHO's regulations recommended that functional products or foods that claim to have probiotic benefits should contain probiotic cells in the minimal range of 10^6 to 10^8 CFU [4]. Numerous works and clinical trials have supported these regulations, as the effective functioning of the probiotic mechanism requires the regular consumption of probiotics at an approximate amount of 100 g per day; this enables the successful delivery of about 10^7 viable probiotic cells per day, which will eventually ensure that probiotic benefits are conferred upon the colonic environment [4–6]. This has created great challenges for probiotic industries in that they need to develop various strategies to achieve a high cell density cultivation platform [7–10].

Probiotic cell cultivation often encounters relatively low productivity in the fermentation system, which is mainly due to by-product inhibitions. As most probiotics are lactic acid producers, their cellular growth also produces large amounts of organic acids [10,11]. The high loading of organic acids into the fermentation broth was found to increase the osmotic pressure of the cultivation medium [9,12]. This created an inhibitory effect against cell proliferation. Cytoplasmic acidification also occurs due to organic acids. The undissociated lactic acid diffuses into the cytoplasm of the cells and then eventually dissociates inside [10,13]. This severely affects the trans-membrane pH gradient and reduces the cellular energy needed for cell proliferation [9]; therefore, the fermentation of probiotics is continuously tested with various strategies to improve cellular growth and remove growth-inhibiting factors. One of the most common techniques employed during fermentation is the control of pH by adding NaOH or calcium carbonate [10,14,15]. The pKa value of the acidified fermentation broth results in the addition of large quantities of NaOH [7,15,16]. Eventually, this causes elevated osmotic pressure in the fermentation broth and inhibited cell growth [12]. The addition of calcium carbonate might cause precipitation, thus making it difficult to separate from the fermentation medium [16,17]. Numerous other techniques have also been reported, such as filtration using a specific membrane to separate by-products, including the use of lactate and acetate in a continuous cultivation platform [18,19].

In recent years, fermentations of bacterium, coupled with various adsorbent resins, have been widely used for more efficient product recovery [20–23]. The application of ion-exchange technology with resins is widely used to recover lactic acids from the fermentation medium [23]. There are two types of ion-exchange technology which have been widely used. Cation-exchange resins are explored for the removal of positively charged ions such as calcium, sodium, and copper ions. These cations will be attracted to negatively charged resin surfaces [20–23]. As lactic acid is composed of anionic lactate ions, it can bind to a cationic molecule (exchange resins); this could easily separate the by-products from the fermentation medium. Anion-exchange resins, including Amberlite IRA 67, IRA96, IRA 400, DOWEX-50, and DOWEX-XUS 40, have been previously used for the recovery of lactic acid [23–25]. The removal of lactic acid and other organic acids from the fermentation medium could enhance microbial cell mass production; this is because by-product inhibition will have been reduced [8]. Cui et al. [9] reported the high-cell density cultivation of *Lactobacillus plantarum* using lactic acid removal by anion-exchange resins, and the growth was improved by 13.3 times in the batch that was cultivated with resins [9]. Similar growth improvements of probiotic bacteria *Pediococcus acidilactici* were also reported by Othman et al. [26]. The study performed batch cultivation using the in situ addition of IRA 67 anion resins. This resulted in a 55.5 time improvement in maximum viable cell count compared with methods that employ fermentation without resins. The study was further extended to optimize the effective application of IRA 67 anion-exchange resins with regard to biomass production [8,26].

However, no study on the application of anion-exchange resins on growth performance, using heterofermentative *Lactobacillus reuteri*, has been reported. Chen et al. [27] published the first report on the cultivation of *L. reuteri* incorporated with ion-exchange

resins. The study aimed to remove lactate and acetate, which were found to inhibit the production of the antimicrobial compound, 1,3-propanediol, in *L. reuteri*; however, the study only focused on the metabolic balance of *L. reuteri* and its ability to increase the synthesis of the 1,3-propanediol compound [27]. The heterofermentative *L. reuteri* has widely exploited the synthesis of various short-chain fatty acids (SCFA), as well as higher concentrations of organic acids, vitamins, antimicrobial secondary metabolites, and antimicrobial peptides [5,7,27,28]. Eventually, these heterofermentative potentials turned out to be the growth inhibitory factors that hampered high cell mass production for *L. reuteri* [27]. In this study, the potential use of anion-exchange resin technology to improve the cell mass of *L. reuteri* was investigated. The problems encountered when applying the resins to a bioreactor system will be discussed in this study. The results of this study may provide new innovative strategies for the greater mass production of probiotic cells.

2. Materials and Methods

2.1. Strain and Culture Medium

L. reuteri (DSM 20016^{T}; ATCC 23272) was obtained from the Wellness Industries Culture Collection (WICC), Institute of Bioproduct Development (IBD), UTM, Malaysia. The microbial culture was maintained as a working cell bank by using 15% (*w*/*v*) glycerol, and 5% (*w*/*v*) skimmed milk was used as cryoprotectant at −80 °C until ready for use. For inoculum preparation, 0.5 mL of the frozen working cell bank was distributed in a MRS liquid medium, supplemented with lactose, at 20 g L^{-1}. The inoculated medium was statically incubated at 37 °C for 24 h [7]. The cells that were grown were then transferred to a fresh medium and incubated under similar conditions. For all experiments, including bioreactor cultivations, 10% (*v*/*v*) inoculum was used. The culture medium employed was a modified cultivation medium containing (g L^{-1}) yeast extract, 40.0; dipotassium hydrogen phosphate, 3.0; sodium acetate, 3.0; ammonium citrate, 2.0; magnesium sulphate, 0.5; and manganese sulphate, 0.05. The carbon source for the lactose (40.0 g L^{-1}) was sterilized separately, and then added to the medium before inoculation. The temperature and initial pH were set at 37 °C and 6.5, respectively.

2.2. Batch Fermentation

Cultivation in the bioreactor was carried out in a 6.0 L stirred tank bioreactor, Eppendorf BioFlo®320 bioprocess control station. The bioreactor vessel was made of borosilicate glass and the top plate of the bioreactor vessel was made of stainless steel. The bioreactor was equipped with a temperature monitoring and controlling system. The temperature was maintained, at 37 ± 0.5 °C throughout the fermentation period, by the automatic control system. The bioreactor was also connected to the pH and dissolved oxygen (DO) monitoring and control system. The DO and pH of the fermentation broth were adjusted to the desired initial pH after the transfer of the inoculum. Due to the facultative nature of the bacterium, the compressed sterile air was sparged into the medium at 2.0 vvm until the DO level achieved 100%. Then, air flow was stopped to allow the culture to develop under microaerophilic conditions. The initial pH levels were adjusted to pH 6.5 using 2M NaOH or HCl (after inoculum transfer). Then, the changes in pH were only monitored without a control throughout the fermentation process. Antifoam was added manually to the fermentation broth if a dense foam was observed during cultivation. A base, acid, antifoam, and feed solutions were added into the fermentation medium using calibrated peristaltic pumps. The bioreactor was equipped with two units of six-blade Rushton impellers to homogenize the fermentation broth. The mixing speed was set to 200 rpm. All bioreactor cultivations were inoculated with 10% (*v*/*v*) cells from the exponentially growing pre-cultures and batch fermentation in the bioreactor systems was performed for 24 h.

2.3. Constant Fed-Batch Fermentation

The constant fed-batch fermentations were conducted in two phases. The initial phase comprised a batch fermentation mode with a working volume of 3.5 L until the carbon source was exhausted. The second phase of the fermentation process was initiated in the fed-batch mode. The carbon source was exhausted at approximately 10 h into the initial batch fermentation mode process. During the fed-batch phase, 1 L of concentrated lactose was added continuously into the bioreactor at a constant feed rate of 0.85 mL min^{-1}. Similarly to batch fermentation processes, the fed-batch fermentation in the bioreactor system was performed for 24 h.

2.4. Preparation of Resins

This study used two types of anion-exchange resins: Amberlite IRA 67 and Amberlite IRA 97. These resins were purchased from Sigma, Germany. Both resins are washed until they reach a final pH of 7. The washing procedures followed a sequence that is recommended by most publications [29], and is as follows: first, wash with 1N HCl solution, followed by distilled water; then, wash again with 1N NaOH solution; finally, wash with distilled water again until a final pH of 7 is achieved. The pH-adjusted resins are sieved and then dried in an oven at 100 °C overnight. As both resins are unable to be autoclaved, the resins were sterilized by either immersing them in 3% hydrogen peroxide or exposing them to ultraviolet radiation for 30 to 60 min at room temperature, aseptically in a Class II Biosafety cabinet.

2.5. Anion-Exchange Resin Selectivity

For the sorption equilibrium studies, the MRS medium containing single components of lactose, acetate, and lactate was used to study how selective the resins are towards these components. For each resin, 10 g L^{-1} was added separately into a 50 mL MRS medium containing lactose, acetate, and lactate at an initial concentration of 10 g L^{-1}. The reactions were carried out at an agitation speed of 200 rpm and 37 °C for 5 h. The adsorption capacity of the resins was calculated based on the Langmuir isotherm model, which was determined by following Equation (1):

$$q = (Ci - Ceq)/X \quad (1)$$

From Equation (1), q refers to the specific uptake capacity of lactic acid (gg^{-1}); Ci is the initial concentration of lactic acid (g $L^{-1)}$; Ceq is the equilibrium concentration of lactic acid (g L^{-1}); and X refers to the concentration of resins added (g L^{-1}). To determine the remaining lactic acid in the solution, resins were separated from the fermentation broth by centrifugation at 10,000 rpm for 10 min. The resins were then washed with distilled water, which was followed by regeneration with 4% NaOH to elute the lactic acid from the resin at an ambient temperature.

2.6. Anion-Exchange Resin in Batch and Fed-Batch Fermentation

The high cell-density production of *L. reuteri* during the stirred tank fermentation modes with anion-exchange resins was tested in two conditions: dispersed resin form and separated resin form. In the dispersed mode, 10 g L^{-1} of sterilized anion-exchange resins were aseptically transferred into the culture medium, at the initial point of transfer and at 8h into incubation. For the separated resin form, the sterile resins were aseptically transferred into a sterile stainless-steel column which was then connected to the bioreactor. The fermentation broth was pumped from the bioreactor into a resin flask and circulated back via a peristaltic pump.

2.7. Recovery of the Anion-Exchange Resins

The resins were collected from the fermentation broth via sieving, after incubation was complete. Resin can be reused again, after completing several washing steps, and if it is followed by regeneration, as previously mentioned [30]. Briefly, the collected anion resins were washed with filtered distilled water until the solution appeared clear. Then, a

1M hydrochloric acid solution was used as an eluent to extract lactic acid from resins. A series of washing and elution was repeated until the lactic acid was completely extracted. Finally, the resins were regenerated using a 4% NaOH solution in ambient conditions. The regeneration was confirmed by using a silver nitrate solution with the collected outlet solution. The white precipitate of AgCl would be absent upon the complete regeneration of anion-exchange resins. The regenerated anion-exchange resins were washed with filtered distilled water several times to remove the excess NaOH, and then they were completely dried under a benchtop oven at 60 °C.

2.8. Analytical Procedure

Samples were collected from the bioreactor at appropriate time intervals, centrifuged at 10,000 rpm for 10 min, and stored at −20 °C until they were required for further analysis. Prior to centrifugation, the biomass produced was quantified by the optical density of collected samples at 600 nm. The spectrophotometric absorbance was correlated using cell dry weight (CDW), in accordance with a previously obtained calibration line. To obtain a viable cell count, samples were serially diluted with a sterile phosphate buffer and plated in an MRS agar plate. All plates were incubated at 37 °C for 48 h and the total number of colonies formed was quantified. The amount of lactose, lactic acid, and acetic acid was determined using a high-performance liquid chromatography technique and an Agilent 1100 HPLC system equipped with a 300 mm × 7.8 mm Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA). A 5 mM sulfuric acid was used for the mobile phase at a flow rate of 0.6 mL min^{-1} and at a temperature of 65 °C. Components were identified and quantified using a refractive index (RI) measurement with suitable standards.

3. Results and Discussion

3.1. Selection of Anion-Exchange Resins, Conditions to Use, and Their Adsorption Capacity

The selection of suitable resins plays a significant role in the successful removal of lactic acid that is produced in the fermentation broth. In this study, two types of weak base anion-exchange resins were utilized for the adsorption of lactic acids from the *L. reuteri* fermentations (Table 1). Based on the previous literature, the adsorption of lactic acid is more efficient when weak base anion resins are used compared with the use of strong base resins [22,30,31]. This is due to a higher resistance to oxidation and the organic fouling properties of the weak base anion-exchange resins [24,25]. The type of supporter matrix and organic acid exchange capacity are important criteria for the selection of resins, as per the results of previous studies [9,26]. The Amberlite IRA 67 anion resins are made up of an acrylic gel type matrix, and the Amberlite IRA 96 resins are composed of a styrene-divinylbenzene macroporous matrix. Table 1 summarizes the physiochemical properties of both resins. The idiosyncratic capabilities of both anion-exchange resins had been demonstrated widely in the previous literature [23].

Table 1. Physiochemical properties of the anion-exchange resins used in this study.

Type of Resin	Amberlite™ IRA 67	Amberlite™ IRA 96
Description	Weakly basic anion resins	Weakly basic anion resins
Co-polymer	Cross-linked acrylic	Styrene-divinylbenzene
Matrix	Gel	Macroporous
Functional group	Tertiary amine	Tertiary amine
Physical form	White, translucent, spherical beads	White to tan, opaque, spherical beads
Total exchange capacity	≥1.6 eq/L	≥1.3 eq/L
Ionic form in the packaging	Moist free basic	Moist free basic
pH stability range	0–6	0–7
Water retention capacity	56–62%	57–63%
Temperature range	5–60 °C	5–60 °C
Particle diameter	500–700 µm	550–750 µm
Uniformity coefficient	≤1.8	≤1.6

One of the arduous tasks that one faces when using resins involves finding a suitable method to prepare the resins. Many types of anion- or cation-exchange resins were non-autoclavable [31,32]. The ion-exchange resins are susceptible to microbial contaminations, and thus, incorrect sterilization techniques could destroy the resins. In this study, the sterilization of anion-exchange resins was achieved using two methods: (a) immersed in 3% hydrogen peroxide, and (b) exposed to UV radiation. The hydrogen peroxide method recommended by Cui et al. [9] would have been a very effective sterilization method during the resin regeneration stage; however, it was not suitable for the in situ loading of resins into bioreactors, as the wet resins after washing were highly susceptible to contaminations. Reducing the number of washing steps also impacted cell growth as a result of trace amounts of hydrogen peroxide. For the regeneration step, soaking in hydrogen peroxide is highly recommended after the elution of acids, which should then be followed by washing. It was recommended that the wet resins should be completely dry before UV exposure steps. For the in situ loading of anion resins into a sterile medium for microbial fermentation, this dry resin–UV exposure technique was more relevant in terms of achieving an aseptic environment.

In this study, both resins were exposed to UV radiation for 1 to 2 h, in a biological safety cabinet, as suggested by previous publications [26,29]; however, this changed the appearance of the Amberlite IRA 67 anion resins, as the exposure caused the white gel-type structure to produce reddish burnt-like conditions. This physiological change did not significantly impact the adsorption capacity in this study (data are not shown here); however, we may speculate that a longer exposure time could affect the regeneration of resins. Secondly, after sterilizing wet resins, they required strenuous efforts to maintain that sterility. Wet resins tend to stick to the container, and thus, they required scraping; therefore, dry resins were more convenient and effective to handle during and after sterilization. During all resin preparations, the resins were completely air dried before UV sterilization. Both resins were prepared in very thin layers, in sterile containers, and exposed to UV for a maximum of 30 min. The sterility of the resins was confirmed by sterility testing using a microbiological nutrient medium.

Table 2 summarizes the sorption study of the two weak base anion-exchange resins evaluated in this study. Both resins achieved higher lactic acid sorption capacities, although the resins are not significantly different from one another. The IRA 67 and IRA 96 anion resins had shown a maximum lactic acid extraction up to 77%. In contrast, the adsorption of acetic acid was slightly different for both resins. The IRA 96 resins provided a maximum adsorption of acetic acid at 0.342 ± 0.002 gg^{-1} whereas a maximum value of 0.241 ± 0.015 gg^{-1} was obtained for the IRA 67 resins. These findings were supported by the previous literature. A recent study compared lactic and acetic acid adsorption using various anion-exchange resins [26]. The study had proven that weak base anion resins, especially IRA 67 resins, had a higher lactic acid sorption capacity. The IRA 67 resins are made up of a matrix of cross-linked acrylic gel, which caused the resins to be more hydrophilic, and thus, they had a higher affinity toward organic acids; however, Bishai et al. [29] demonstrated that IRA 96 resins had more macro-reticular and porous structures than gel matrix resins, which accounted for a higher lactic acid adsorption capacity. In this study, both resins show a (very) minimal adsorption capacity for lactose, thus indicating that IRA 67 and IRA 96 resins preferentially adsorb organic acid anions [29]. This is supported by Cui et al. [9], who highly recommended that the selection of extractive resins should have a high specificity to avoid adsorbing carbon and amino acids in the fermentation broth.

Table 2. Adsorption capacity of anion-exchange resins.

Type of Resins	Component	Adsorption Capacity (g L^{-1})	Percentage of Removal (%)
Amberlite™ IRA 67	Lactic acid	0.784 ± 0.002	77.93 ± 0.05
	Acetic acid	0.241 ± 0.015	23.80 ± 2.69
	Lactose	0.060 ± 0.014	6.00 ± 1.41
Amberlite™ IRA 96	Lactic acid	0.736 ± 0.011	77.37 ± 0.39
	Acetic acid	0.342 ± 0.002	36.80 ± 1.56
	Lactose	0.117 ± 0.005	9.67 ± 0.94

The initial concentration of the components was adjusted to 10 g L^{-1} and the resins were added at a loading amount of 10 g L^{-1}.

3.2. Flask Cultivation of L. reuteri with Anio-Exchange Resins

The growth of *L. reuteri*, and the lactic acid accumulation in the presence of anion-exchange resins, were evaluated during flask cultivation. Cells were grown in a 50 mL medium using a 150 mL Erlenmeyer flask at 37 °C for 24 h. The flasks were incubated statically and agitated at 200 rpm in an incubator shaker. The anion-exchange resins were added in the amount of 10 g L^{-1} into the flask at 0 h. Based on Table 3, there were no significant differences in terms of viable probiotic cell number (CFU mL^{-1}) between *L. reuteri* cells grown in the presence of IRA 67 and IRA 96 anion resins; however, the presence of anion-exchange resins in the cultivation medium gave significant viable probiotic cell numbers compared with control flasks without resins. This explained the by-product inhibitory effect found in the fermentation of lactic acid bacteria. The presence of resins had successfully eliminated lactic acid which is the by-product of lactose fermentation using *L. reuteri*. This enables more cell division to occur and increased the number of viable cells.

Table 3. Viable probiotic cell count (CFU mL^{-1}) obtained via the in situ addition of anion-exchange resins in flask cultivation of *L. reuteri*.

Type of Resins	Viable Cells (CFU mL^{-1})		Lactic Acid Accumulated (g L^{-1})	
	Static	Agitated	Static	Agitated
Without resins				
Control	$2.55 \times 10^7 \pm 0.18$	$1.46 \times 10^7 \pm 0.26$	11.26 ± 0.05	22.32 ± 0.43
With resins				
Amberlite™ IRA 67	$4.31 \times 10^8 \pm 0.63$	$4.21 \times 10^8 \pm 0.13$	8.75 ± 0.01	4.24 ± 0.02
Amberlite™ IRA 96	$4.10 \times 10^8 \pm 1.58$	$4.07 \times 10^8 \pm 1.61$	8.82 ± 0.02	4.41 ± 0.01

Agitating the culture also caused a greater quantity of lactic acid to be produced compared with static cultivation conditions. In control studies, lactic acid production was doubled compared with the static cultivation of *L. reuteri*. Static cultivation was proposed for this bacterium to create microaerophilic conditions [7]; however, agitation accounted for the rapid intake of lactose, thus impacting the metabolism, and resulting in the accumulation of lactic acid during fermentation. Moreover, this also lowered the viability of probiotic cells in agitated cultures. In contrast, agitated cultures favored the higher adsorption of lactic acids into anion-exchange resins. As summarized in Table 3, nearly 80% of lactic acid produced was removed from the fermentation medium, and this enhanced the viability of probiotic cell mass.

Although there was no significant difference between the performances of both anion resins, the IRA 67 resins resulted in more noteworthy viable probiotic cell numbers and the better adsorption of lactic acid (Table 3). The utilization of IRA 67 resins is highly cited in the fermentation of *Lactobacillus* genera [30,33–35]; however, these studies are mainly devoted to the production and extraction of lactic acids from the fermentation process. The strategies for improving the production of probiotic cell mass using the IRA

67 anion-exchange resins were discussed in lesser detail [35]. Othman et al. [26] compared the potential of the IRA 67 resins with other anion resins in the high-cell density cultivation of the probiotic strain *P. acidilactici*. The study claimed that IRA 67 resins had improved the viable cells by 67 times compared with the control without resins. Among the five different types of anion-exchange resins tested, the IRA 67 resins achieved the highest removal of lactic acid from the culture at a loading concentration of 10 g L^{-1} [26]. This is the first study to demonstrate a potential strategy whereby anion-exchange resins are incorporated into the high cell-density fermentation process of the probiotic strain of *L. reuteri*.

3.3. Batch Fermentation with and without Anion-Exchange Resins

Figure 1 shows the growth profile of the batch cultivation of *L. reuteri*, with and without resins. The growth of the bacterium was evaluated using the cell dry weight (CDW g L^{-1}) at appropriate time intervals. The growth pattern is also supported by changes in the pH of the fermentation broth. Concentrations of the lactose and lactic acid available in the fermentation broth were also determined and plotted. In the first stage of batch cultivation, 10 g L^{-1} of IRA 67 resins were directly added into the cultivation medium after 0 h of fermentation; however, no significant growth was recorded when compared with the control batch, which ran in parallel to the cultivation medium (Figure 1b). After 10h of cultivation, no further cell mass production was observed. A similar output was obtained when the experiment was repeated with 10 g L^{-1} of IRA 96 resins (data not shown here). It was concluded that loading anion resins in the early stage of cultivation negatively impacted the growth of *L. reuter*, where no cell growth was observed (Figure 1b); therefore, batch cultivation was repeated, and anion resins were introduced only after 8 h of cultivation (Figure 1c). The decision to load the resins at the eighth hour was made based on the exponential cell mass production that was observed in the control batch fermentation process, which was performed without resins. Figure 1a illustrates that growth inhibition was observed after 14 h of batch fermentation without resins. The highest cell density was obtained at 12 h, (4.02 ± 0.03 g L^{-1}) and was reduced by about 24.3% within the next 10 h of fermentation. As well as exhausting lactose concentration, the accumulation of lactic acid also greatly affected the *L. reuteri* cell mass. This was observed at lactic acid concentrations above 40 g L^{-1}, which is comparable to the reported growth inhibition concentration for this bacterium. In contrast, the addition of anion-exchange resins (at 8 h—Figure 1c) rapidly removed the accumulated lactic acids, and this favored a stable stationary phase during cell mass production after 14 h of fermentation.

However, the growth of L. reuteri was not severely affected by the in situ addition of anion resins at 0h or during the 8h after inoculation. This could be related to the shear stress created by resin collisions. This hypothesis is based on the agitation speed of 200 rpm employed in this study; however, these results were contrasted with the previous findings [27]. Othman et al. [26] evaluated that different agitation speeds resulted in an even distribution of resins and the higher adsorption of lactic acids into the resins in a 2 L stirred tank bioreactor. The study also reported that agitation speeds below 200 rpm caused the sedimentation of resins and speeds above 200 rpm promoted cell growth. In addition, the study reported that only agitation speeds above 400 rpm had caused shear stress that affected resin morphology [26]. In the current study, the 6L stirred tank bioreactor that was used was equipped with two Rushton impeller blades with baffles. This created an even distribution of resins at agitation speeds above 200 rpm (Figure 2) compared with 400 rpm. After an examination of the previous literature, with regard to lower agitation speeds, it was expected that the strong shear stress had been created based on the growth performance of *L. reuteri*; therefore, batch fermentation with the in situ addition of resins was tested again at slower agitation speeds. Cultivation was performed at an agitation speed of 100 rpm and the in situ addition of resins was completed after 8 h of cultivation. At this agitation speed, the resins were distributed as sediments, and had not significantly improved production cell mass.

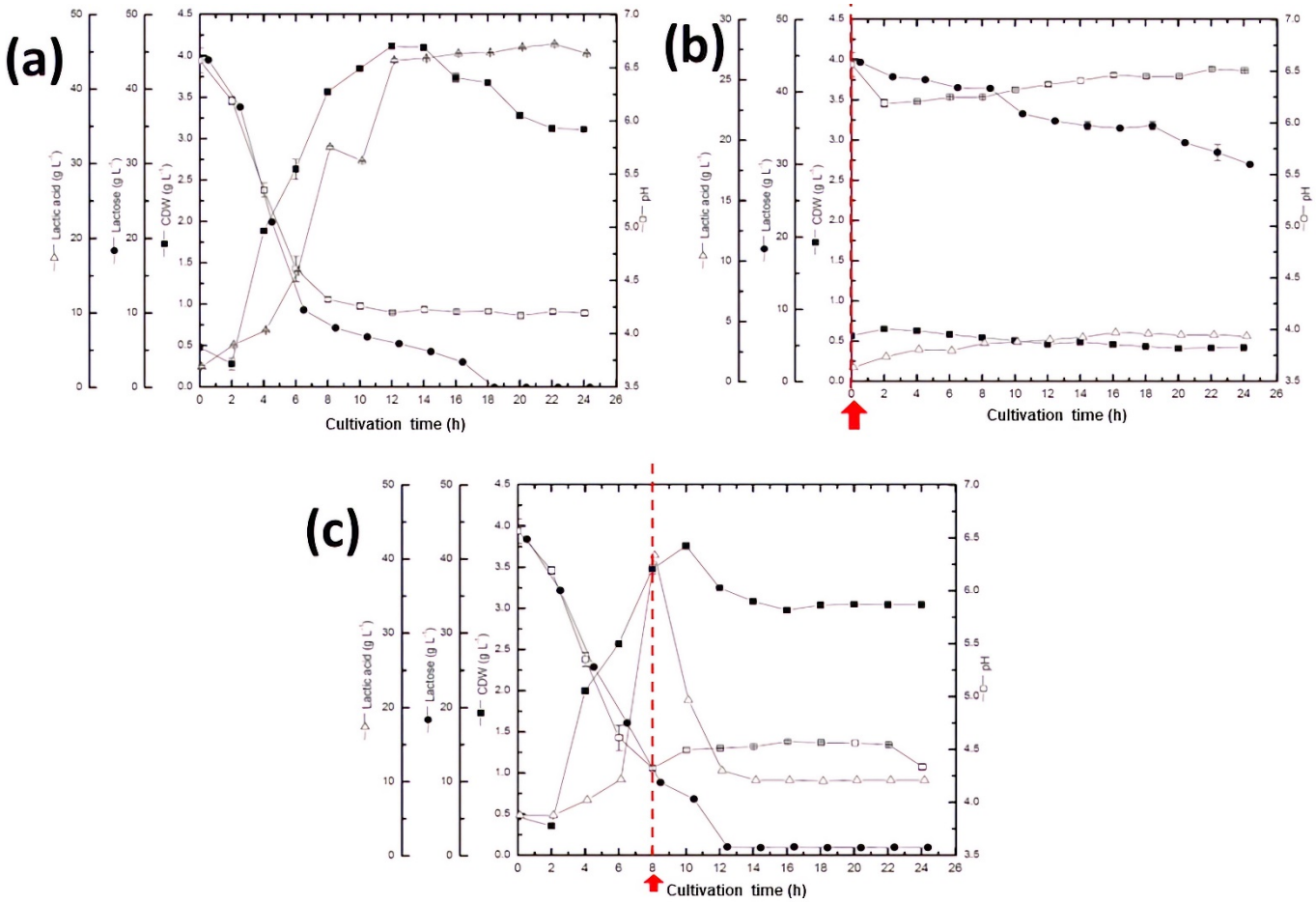

Figure 1. Cell mass production during batch fermentation of *L. reuteri*: (**a**) without (control) and with (**b**) in situ addition of IRA 67 resins at 0 h and (**c**) in situ addition of IRA 67 resins after 8 h of fermentation (arrow and dashed lines show a time of addition of resins). All batch fermentations were performed without pH control and at an agitation speed of 200 rpm. For (**b**,**c**), anion-exchange resins (10 g L^{-1}) were aseptically introduced in situ to the fermentation broth at 0 h and 8 h of incubation.

To overcome the problem of shear stress created by the in situ addition of resins, an integrated resin column system was investigated. In this system, the anion-exchange resins were added into a separate column, then the fermented liquid broth was drawn from the bioreactor and allowed to pass through the resin column before being redirected back into the bioreactor. The 6L stirred tank bioreactor that was utilized in this study was not able to be modified to have an internal resin column; therefore, the integrated resin system was demonstrated as an external component of the bioreactor system. The fermented liquid broth was drawn to the maximum flow rate of a peristaltic pump and reverted into the bioreactor via gravitation flow (the resin column system was fixed at a higher position than the bioreactor top cover). The IRA 67 anion resins were only employed to evaluate the effectiveness of this integrated resin column for the batch fermentation of *L. reuteri*. Figure 3 illustrates batch cultivation with an integrated resin column.

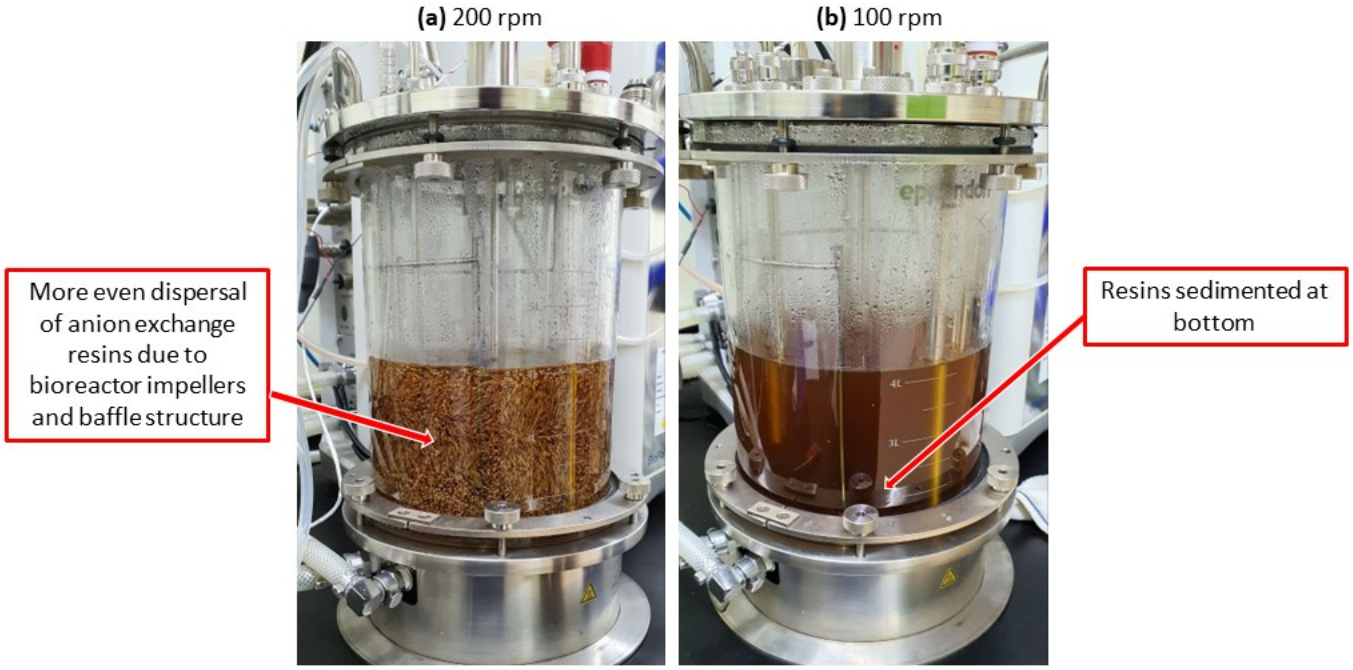

Figure 2. The dispersal of anion-exchange resins at 200 rpm (**a**) and 100 rpm (**b**).

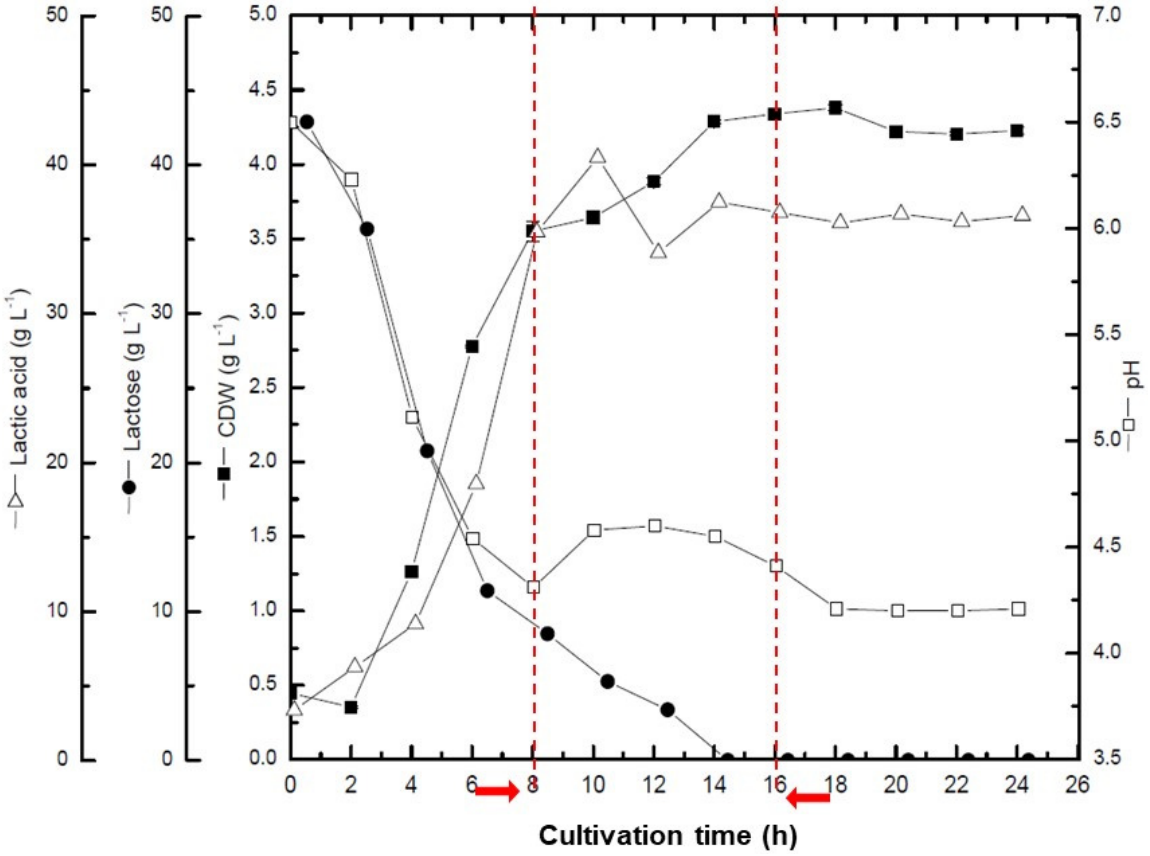

Figure 3. Cell mass production during the batch fermentation of *L. reuteri* with an integrated anion-exchange resin column (10 g L^{-1} Amberlite IRA 67). The batch fermentations were performed without a pH control and at an agitation speed of 200 rpm. The fermentation broth was circulated continuously from 8 h of cultivation to 16 h, which are shown with an arrow and dashed line in the graph.

The integrated resin column had recorded a better improvement in cell mass production compared with cell mass production after the in situ addition of resins. The highest cell mass production (4.38 ± 0.017 g L^{-1}) level was recorded after 18 h of fermentation. This indicated that the resin column had overcome the shear stress and removed growth inhibiting lactic acid accumulation. The integrated resin column improved the cell mass of *L. reuteri* by 9% compared with the control batch fermentation process performed without resins (Figure 1a); however, lactic acid was not rapidly adsorbed into anion resins, as was the case with the previous batch cultivation process (Figure 1c). This could be explained by the time frame required for the complete adsorption of lactic acid in the resin column during the circulation of the fermentation medium. With no shear stress and efficient lactic acid removal, the cell growth rapidly increased with a greater metabolic rate. The lactic acid was continuously produced in the fermentation medium, albeit with the complete depletion of lactose in the fermentation medium. As the fermentation medium was circulated over the resin column from 8 h to 16 h, the pH of the fermentation broth was observed to be slightly elevated compared with its previous lower acidic levels. The addition of anion-exchange resins had been previously reported to reduce the acidity of fermentation [32]. The free-basic form of the resins was proposed as an alternative to the addition of NaOH during the pH-controlled batch fermentation of lactic acid bacteria [35].

Several studies recommended the utilization of column resins for bioreactor cultivations [23,24]. Jianlong et al. [20] demonstrated the use of the ion-exchange resin column for the integrated product recovery of fine mycelial pellets of *Aspergillus niger*, cultivated during batch fermentation, in order to produce citric acid. In another study, the bench-top bioreactor was designed to be equipped with an internal resin column for prodigiosin pigment recovery of *Serratia* sp. using batch fermentation [21]. Cui et al. [9] also demonstrated the batch fermentation of *L. plantarum* with a 40 cm length × 10 cm diameter external resin column attached to a 3.5 L bench-top bioreactor. The circulation of the fermentation medium into the sterile resin column was made to be automatic, and it was incorporated with a changing pH value. An on–off valve was set to open, in order to allow the medium to flow into the external resin column when the pH dropped below 6.5. The valve shut off when the pH increased to 6.6, which occurred due to the efficient removal of lactic acid [9]. Moreover, the study by Othman et al. [26] utilized an internal resin column attached to a 2 L stir tank bioreactor. This system claimed to reduce the problem of shear force created by resins in dispersed conditions. The application of the internal resin column recorded the highest growth improvement (by 94 times), compared with control fermentation without resins, and it performed 1.4 times better than dispersed resin cultivation.

An external resin system is easier to use than a bench-top stir tank bioreactor system. Some of the ion-exchange resins reported in the literature were sensitive to sterilization by autoclaving; therefore, the external columns to store resins proved to be more efficient, and their operations in aseptic conditions were more convincing. In this study, batch fermentation in a 6L stirred tank bioreactor was performed using various methods to incorporate anion-exchange resins in a more effective way. These steps were prerequisites to the establishment of a successful high-cell density, fed-batch cultivation of *L. reuteri*. The precis of this experiment is as follows: (i) in situ addition of anion-exchange resins from 0 h of cultivation could impact growth and cell mass production; (ii) the late exponential stage could be the best time to introduce anion resins; and (iii) an integrated resin column is more effective for the reduction of shear-stress caused by the agitation of resins.

3.4. Fed-Batch Fermentation with and without Anion-Exchange Resins

Cell mass production as a result of the batch fermentation of *L. reuteri* is always limited by the carbon source required for cell proliferation; therefore, fed-batch cultivations are designed to eliminate the lactose limitations during cell mass production.; however, the large amount of lactic acid production caused by cell growth also affected the probiotic cell mass of *L. reuteri* [7] Based on data obtained from previous batch experiments, fed-batch cultivations were conducted with anion-exchange resins in the integrated resin column.

Figure 4 shows the results of fed-batch fermentation performed without pH control, an agitation speed at 200 rpm, and a feeding rate of 0.05 L h^{-1}.

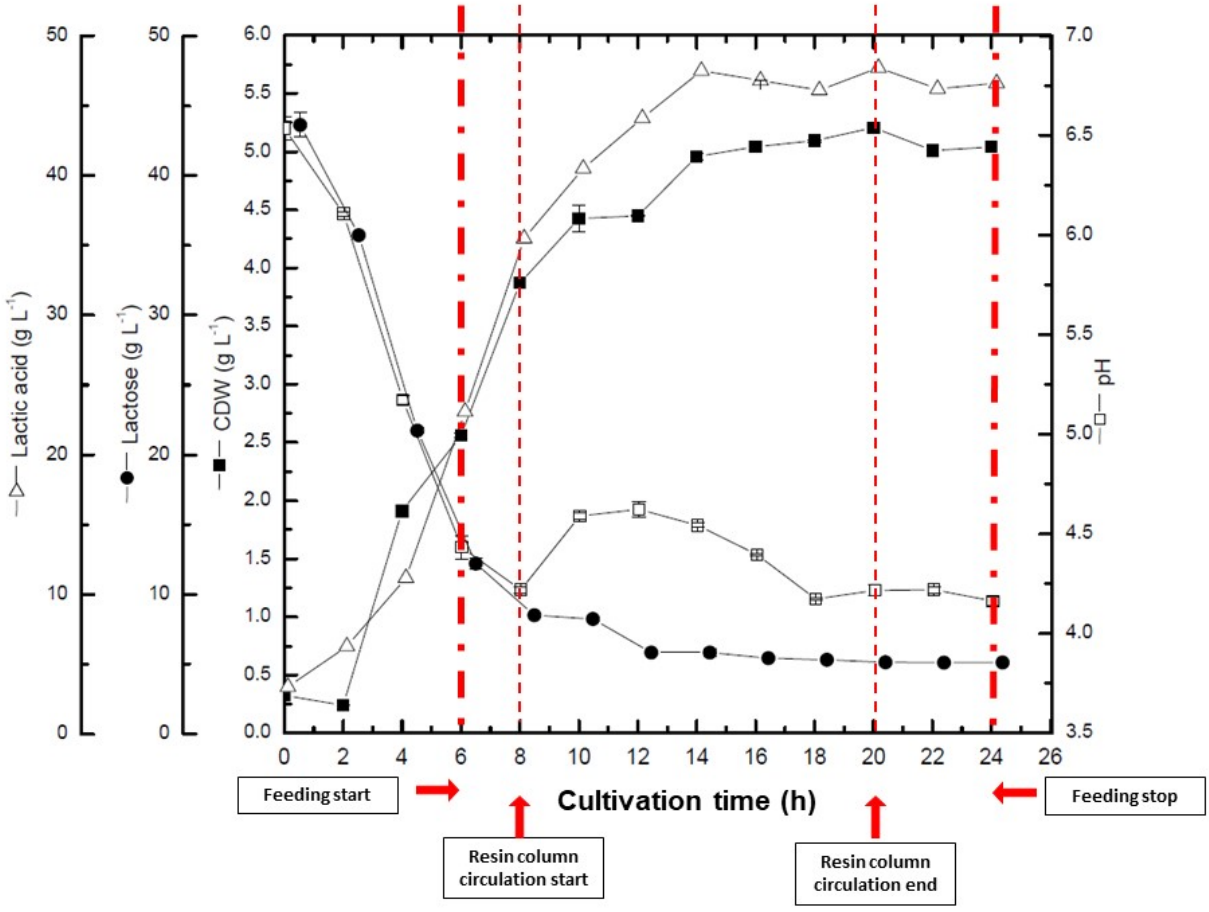

Figure 4. Fed-batch cultivation of *L. reuteri* with an integrated anion-exchange resin column (10 g L^{-1} Amberlite IRA 67). Fermentation started in batch mode, and then the feeding of lactose started in the sixth hour (as shown by the arrow and dash-dotted lines on the graph) of fermentation as the lactose concentration in the medium had depleted. Both the batch and fed-batch phases of fermentation were performed without pH control and at an agitation speed of 200 rpm. The fermentation broth was circulated continuously from 8 h of cultivation to 20 h, which are shown by the arrow and dashed lines on the graph.

Based on the obtained results, the constant fed-batch fermentation process had enhanced higher cell mass production, coupled with reduced by-product inhibition. The growth improvement of *L. reuteri* was achieved through optimal cultivation conditions, where lactose was continuously supplied for cell metabolism, and the accumulation of lactic acid in the medium was maintained below inhibitory levels. Lactic acid was not fully absorbed into the anion resin; however, the increasing cell mass supported the hypothesis of extractive fermentation. As the resins removed fermentative by-products from the cultivation medium, this enhanced its continued production with an accelerated cell growth and metabolic rate. The cell mass production was delayed in the fed-batch fermentation of *L. reuter*, as the highest cell mass (5.23 $\pm$ 0.03 g L^{-1}) was obtained after 20 h of fermentation. This could be explained by the time required for the complete circulation of the fermentation medium through the external resin column. The production of the probiotic biomass of *L. reuteri* was improved by about 19.5% compared with the batch cultivation processes that have an integrated resin column system. This fed-batch culture yielded a biomass of *L. reuteri* that was about 30.1% greater than conventional batch fermentation without resins.

Table 4 summarizes the maximum number of viable cells obtained under different bioreactor systems which were established for the high-cell density cultivation of *L. reuteri*. No significant differences were observed between the highest cell masses collected during the three different modes of fermentation using a 6L stir tank bioreactor; however, the viable cell counts had ratified the significant impact of anion resins on the growth performance of *L. reuteri*. As shown in Table 4, the viable cell count obtained from the batch fermentation process which incorporated the integrated resin column was improved by 71 times ($3.89 \times 10^{11} \pm 0.07$ CFU mL^{-1}) compared with the control batch fermentation ($5.35 \times 10^{9} \pm 0.32$ CFU mL^{-1}) process that did not add resins. Similarly, the highest adsorption of lactic acid was recorded by the integrated IRA 67 resin system, coupled with batch fermentation, compared with fed-batch cultivations alone; however, the fermentation time was delayed by 6 h to achieve the highest cell mass and viable count, respectively.

Table 4. Comparison of the cultivation of *L. reuteri* under different bioreactor systems for higher probiotic cell mass production, coupled with lactic acid removal.

Parameters	Batch Fermentation without Resin	Batch Fermentation with Resin Column	Fed-Batch Fermentation with Resin Column
The highest cell mass (CDW) collected [g L^{-1}]	4.02 ± 0.03	4.38 ± 0.017	5.23 ± 0.03
Cultivation time reaching highest CDW [h]	12	18	20
Viable cell count [CFU mL^{-1}]	$5.35 \times 10^{9} \pm 0.32$	$3.89 \times 10^{11} \pm 0.07$	$1.30 \times 10^{13} \pm 0.05$
Lactic acid accumulated [g L^{-1}]	42.70 ± 0.03	36.61 ± 0.01	57.70 ± 0.05
Estimated total lactic acid production [g L^{-1}]	42.70 ± 0.03	47.31 ± 0.02	67.72 ± 0.02

The growth of *L. reuteri* was improved by 33 times when a fed-batch cultivation process coupled with a resin system was used, compared with when batch fermentation with a resin system was used. This could be related to the low lactic acid adsorption observed in the fed batch-resin system. Continuous feeding of lactose might be adsorbed into the resins during the circulation of the fermentation broth. The study by Garret et al. [32] also evaluated that other molecules produced during fermentation could interfere and compete with lactic acid for adsorption. Moreover, Chen et al. [27] reported that the removal of major by-products (lactate and acetate) had enhanced the production of other metabolites, such as ethanol, in *L. reuteri*. This hypothesis is based on the heterofermentative nature of the bacterium and it is due to an unbalanced ratio between the total lactic acid produced and the supplemented lactose. Ethanol production could be possible, though it impacts cell mass production along with cell viability. In addition, foam production was also observed during fermentation, which could be related to the production of carbon dioxide as a co-product of ethanol.

4. Conclusions

The generation of lactic and acetic acids during lactose fermentation severely impacts the high-cell density cultivation of the probiotic strain, *L. reuteri*. These results clarified that anion-exchange, which is usually utilized for the recovery of metabolites could reduce the by-product inhibition of lactic acid and acetic acid. Extractive fermentation was performed using 10 g L^{-1} of Amberlite™ IRA 67, and it had shown a 1.1 time improvement with regard to cell mass (g L^{-1}), and a 71 time improvement with regard to viable cell counts (CFU mL^{-1}), compared with batch fermentations conducted with and without resins. The in situ addition of resins had created shear stress that severely impacted growth, and this finding is contradictory to previous similar works. This could be explained by the different impeller and baffle systems of bioreactors, which are made for the homogenization of the fermentation medium; however, the application of anion-exchange resins in an integrated column, attached externally to the stirred tank bioreactor, managed to overcome the shear

force problem. As the anion-exchange resins are reusable after the elucidation of acids, the application of the resins could be a low-cost strategy to improve the growth performance of *L. reuteri*.

Author Contributions: Conceptualization, supervision, project administration, H.A.E.E., D.J.D., V.K.G. and S.S.; methodology, analysis, writing—original draft, S.S., S.R., K.H.N. and T.V.; funding acquisition, H.A.E.E. and B.A.; Formal analysis, S.S., H.A.E.E., B.A. and D.S.; writing—reviewing and editing, S.S., T.V., B.A., D.S., M.N., S.L., V.K.G. and H.A.E.E. All authors have read and agreed to the published version of the manuscript.

Funding: Authors would like to thank the Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM) for the financial support with the industrial fund R.J130000.7609.4C336 and R.J130000.7609.4C465.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: S.S. would like to extend their thanks to the School of Postgraduate Studies (SPS), Universiti Teknologi Malaysia (UTM), for the financial support of the Zamalah Ph.D. Scholarship session 2017/2018. S.S. also would like to express his gratitude to Hesham Ali El-Enshasy and Bassam Abomoelak for the great opportunity. Thanks to Thevarajoo, S. for technical advice on the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. El Hage, R.; Hernandez-Sanabria, E.; Van de Wiele, T. Emerging trends in "smart probiotics": Functional consideration for the development of novel health and industrial applications. *Front. Microbiol.* **2017**, *8*, 1889. [CrossRef] [PubMed]
2. O'Toole, P.W.; Marchesi, J.R.; Hill, C. Next-generation probiotics: The spectrum from probiotics to live biotherapeutics. *Nat. Microbiol.* **2017**, *2*, 17057. [CrossRef] [PubMed]
3. Hill, C.; Guarner, F.; Reid, G.; Gibson, G.R.; Merenstein, D.J.; Pot, B.; Morelli, L.; Canani, R.B.; Flint, H.J.; Salminen, S. The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* **2014**, *11*, 506–514. [CrossRef] [PubMed]
4. Galdeano, C.M.; Cazorla, S.I.; Dumit, J.M.L.; Vélez, E.; Perdigón, G. Beneficial effects of probiotic consumption on the immune system. *Ann. Nutr. Metab.* **2019**, *74*, 115–124.
5. Buckley, M.; Lacey, S.; Doolan, A.; Goodbody, E.; Seamans, K. The effect of Lactobacillus reuteri supplementation in Helicobacter pylori infection: A placebo-controlled, single-blind study. *BMC Nutr.* **2018**, *4*, 48. [CrossRef]
6. Selvamani, S.; Mehta, V.; El Enshasy, H.A.; Thevarajoo, S.; El Adawi, H.; Zeini, I.; Pham, K.; Varzakas, T.; Abomoelak, B. Efficacy of Probiotics-Based Interventions as Therapy for Inflammatory Bowel Disease: A Recent Update. *Saudi J. Biol. Sci.* **2022**, *29*, 3546–3567. [CrossRef]
7. Selvamani, S.; Dailin, D.; Rostom, M.; Malek, R.; Gupta, V.; El-Enshasy, H. Optimizing medium components to enhance high cell mass production of biotherapeutic strain Lactobacillus reuteri DSM 20016T by statistical method. *J. Sci. Ind. Res.* **2020**, *79*, 798–803.
8. Othman, M.; Ariff, A.B.; Wasoh, H.; Kapri, M.R.; Halim, M. Strategies for improving production performance of probiotic Pediococcus acidilactici viable cell by overcoming lactic acid inhibition. *AMB Express* **2017**, *7*, 215. [CrossRef]
9. Cui, S.; Zhao, J.; Zhang, H.; Chen, W. High-density culture of Lactobacillus plantarum coupled with a lactic acid removal system with anion-exchange resins. *Biochem. Eng. J.* **2016**, *115*, 80–84. [CrossRef]
10. Wang, T.; Lu, Y.; Yan, H.; Li, X.; Wang, X.; Shan, Y.; Yi, Y.; Liu, B.; Zhou, Y.; Lü, X. Fermentation optimization and kinetic model for high cell density culture of a probiotic microorganism: Lactobacillus rhamnosus LS-8. *Bioprocess Biosyst. Eng.* **2020**, *43*, 515–528. [CrossRef]
11. Schiraldi, C.; Adduci, V.; Valli, V.; Maresca, C.; Giuliano, M.; Lamberti, M.; Cartenì, M.; De Rosa, M. High cell density cultivation of probiotics and lactic acid production. *Biotechnol. Bioeng.* **2003**, *82*, 213–222. [CrossRef] [PubMed]
12. Dailin, D.J.; Elsayed, E.A.; Malek, R.A.; Hanapi, S.Z.; Selvamani, S.; Ramli, S.; Sukmawati, D.; Sayyed, R.; El Enshasy, H.A. Efficient kefiran production by Lactobacillus kefiranofaciens ATCC 43761 in submerged cultivation: Influence of osmotic stress and nonionic surfactants, and potential bioactivities. *Arab. J. Chem.* **2020**, *13*, 8513–8523. [CrossRef]
13. Gao, X.; Kong, J.; Zhu, H.; Mao, B.; Cui, S.; Zhao, J. Lactobacillus, Bifidobacterium and Lactococcus response to environmental stress: Mechanisms and application of cross-protection to improve resistance against freeze-drying. *J. Appl. Microbiol.* **2022**, *132*, 802–821. [CrossRef] [PubMed]

14. Lobeda, K.; Jin, Q.; Wu, J.; Zhang, W.; Huang, H. Lactic acid production from food waste hydrolysate by Lactobacillus pentosus: Focus on nitrogen supplementation, initial sugar concentration, pH, and fed-batch fermentation. *J. Food Sci.* **2022**, *87*, 3071–3083. [CrossRef]
15. Machado, J.; Rossi, D.M.; Ayub, M.A.Z. Batch and fed-batch strategies of lactic acid production by Lactobacillus plantarum BL011 using soybean hull hydrolysates as substrate. *Biomass Convers. Biorefin.* **2022**. [CrossRef]
16. Gao, M.-T.; Shimamura, T.; Ishida, N.; Takahashi, H. pH-uncontrolled lactic acid fermentation with activated carbon as an adsorbent. *Enzym. Microb. Technol.* **2011**, *48*, 526–530. [CrossRef]
17. Yang, P.-B.; Tian, Y.; Wang, Q.; Cong, W. Effect of different types of calcium carbonate on the lactic acid fermentation performance of Lactobacillus lactis. *Biochem. Eng. J.* **2015**, *98*, 38–46. [CrossRef]
18. Komesu, A.; Wolf Maciel, M.R.; Rocha de Oliveira, J.A.; da Silva Martins, L.H.; Maciel Filho, R. Purification of lactic acid produced by fermentation: Focus on non-traditional distillation processes. *Sep. Purif. Rev.* **2017**, *46*, 241–254. [CrossRef]
19. Chen, G.Q.; Eschbach, F.I.I.; Weeks, M.; Gras, S.L.; Kentish, S.E. Removal of lactic acid from acid whey using electrodialysis. *Sep. Purif. Technol.* **2016**, *158*, 230–237. [CrossRef]
20. Jianlong, W.; Xianghua, W.; Ding, Z. Production of citric acid from molasses integrated with in-situ product separation by ion-exchange resin adsorption. *Bioresour. Technol.* **2000**, *75*, 231–234. [CrossRef]
21. Bae, J.; Moon, H.; Oh, K.-K.; Kim, C.-H.; Sil Lee, D.; Kim, S.-W.; Hong, S.-I. A novel bioreactor with an internal adsorbent for integrated fermentation and recovery of prodigiosin-like pigment produced from Serratia sp. KH-95. *Biotechnol. Lett.* **2001**, *23*, 1315–1319. [CrossRef]
22. Zhang, Y.; Qian, Z.; Liu, P.; Liu, L.; Zheng, Z.; Ouyang, J. Efficient in situ separation and production of l-lactic acid by Bacillus coagulans using weak basic anion-exchange resin. *Bioprocess Biosyst. Eng.* **2018**, *41*, 205–212. [CrossRef] [PubMed]
23. Jatoi, A.S.; Baloch, H.A.; Mazari, S.A.; Mubarak, N.; Sabzoi, N.; Aziz, S.; Soomro, S.A.; Abro, R.; Shah, S.F. A review on extractive fermentation via ion exchange adsorption resins opportunities, challenges, and future prospects. *Biomass Convers. Biorefin.* **2021**. [CrossRef]
24. Din, N.A.S.; Lim, S.J.; Maskat, M.Y.; Mutalib, S.A.; Zaini, N.A.M. Lactic acid separation and recovery from fermentation broth by ion-exchange resin: A review. *Bioresour. Bioprocess.* **2021**, *8*, 31. [CrossRef]
25. Othman, M.; Ariff, A.B.; Rios-Solis, L.; Halim, M. Extractive fermentation of lactic acid in lactic acid bacteria cultivation: A review. *Front. Microbiol.* **2017**, *8*, 2285. [CrossRef]
26. Othman, M.; Ariff, A.B.; Kapri, M.R.; Rios-Solis, L.; Halim, M. Growth enhancement of probiotic Pediococcus acidilactici by extractive fermentation of lactic acid exploiting anion-exchange resin. *Front. Microbiol.* **2018**, *9*, 2554. [CrossRef]
27. Chen, G.; Lin, M.; Fang, B. Inhibition and in situ removal of organic acids during glucose/glycerol co-fermentation by Lactobacillus reuteri. *Biochem. Eng. J.* **2015**, *99*, 93–98. [CrossRef]
28. Atilola, O.A.; Gyawali, R.; Aljaloud, S.O.; Ibrahim, S.A. Use of phytone peptone to optimize growth and cell density of Lactobacillus reuteri. *Foods* **2015**, *4*, 318–327. [CrossRef]
29. Bishai, M.; De, S.; Adhikari, B.; Banerjee, R. A platform technology of recovery of lactic acid from a fermentation broth of novel substrate Zizyphus oenophlia. *3 Biotech* **2015**, *5*, 455–463. [CrossRef]
30. Boonmee, M.; Cotano, O.; Amnuaypanich, S.; Grisadanurak, N. Improved lactic acid production by in situ removal of lactic acid during fermentation and a proposed scheme for its recovery. *Arab. J. Sci. Eng.* **2016**, *41*, 2067–2075. [CrossRef]
31. Demir, Ö.; Gök, A.; Kırbaşlar, Ş.İ. Optimization of protocatechuic acid adsorption onto weak basic anion exchange resins: Kinetic, mass transfer, isotherm, and thermodynamic study. *Biomass Convers. Biorefin.* **2022**. [CrossRef]
32. Garrett, B.G.; Srinivas, K.; Ahring, B.K. Performance and stability of Amberlite™ IRA-67 ion exchange resin for product extraction and pH control during homolactic fermentation of corn stover sugars. *Biochem. Eng. J.* **2015**, *94*, 1–8. [CrossRef]
33. Bernardo, M.P.; Coelho, L.F.; Sass, D.C.; Contiero, J. L-(+)-Lactic acid production by Lactobacillus rhamnosus B103 from dairy industry waste. *Braz. J. Microbiol.* **2016**, *47*, 640–646. [CrossRef] [PubMed]
34. Moldes, A.; Alonso, J.; Parajo, J. Recovery of lactic acid from simultaneous saccharification and fermentation media using anion exchange resins. *Bioprocess Biosyst. Eng.* **2003**, *25*, 357–363. [CrossRef] [PubMed]
35. Zaini, N.A.B.M.; Chatzifragkou, A.; Charalampopoulos, D. Microbial production of d-lactic acid from dried distiller's grains with solubles. *Eng. Life Sci.* **2019**, *19*, 21–30. [CrossRef]