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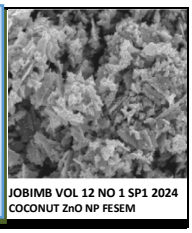

Scaling Up Co-cultivation Fermentation of *Lactococcus lactis* ATCC 11454 and *Lactobacillus rhamnosus* ATCC 7469 with Various Feeding Strategies

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ABSTRACT

This study aimed to analyse the effect of cocultivation between *Lactococcus lactis* ATCC 11454 with *Lactobacillus rhamnosus* ATCC 7469 through their growth profile, riboflavin and nisin production in 2.0 L stirred tank bioreactors. Batch fermentation was employed in the control (C) bioreactor, while fed-batch fermentation employed in bioreactor run (B1) and run 2 (B2), with different medium flow rates of 10 mL/min and 1.6 mL/min, respectively. Results showed that B1 was able to generate an increase of 33.5 % in riboflavin production and a 36% growth rate compared to C and B2. The application in the fermentation process resulted in a prolonged exponential phase, subsequently contributing to a longer metabolite production timeframe. The findings from this study provided an efficient prospect of LAB co-culture, potentially by extending riboflavin production time and increasing vitamin levels in fermented foods or facilitating the production of valuable metabolites.

INTRODUCTION

Lactic acid bacteria (LAB) are a group of bacteria that constitute of genera like *Lactococcus, Lactobacillus, Pediococcus, Streptococcus,* and *Enterococcus*, typically found in dairy fermented products [1]. These genera are safe to be used for human consumption as they have been granted GRAS status by the Food and Drug Administration (FDA). LAB-mediated fermentations have the potential to yield various bioactive by-products with health-promoting effects such as protection against infections, anti-allergic and antioxidant properties, enhanced bioavailability of vitamins/minerals, and anti-anxiety effects [2,3]. Despite their immense potential, the industrial-scale production of these compounds has been hampered by high production costs and low yields from producer cells, thereby limiting their ability to be widely incorporated into food systems [4,5]. In response to these challenges, microbial co-cultivation has emerged as a promising approach for enhancing the production of bioactive compounds from LAB [6,7]. The technique involves the simultaneous cultivation of two or more microbial strains in a fermentation tank at the same time. This novel approach to microbial production platforms allows simultaneous synthesis of multiple valuable compounds, including bacteriocins and vitamins [8].

Scale-up, particularly in microbial processes, is another strategy usually conducted for commercial purposes [9]. The optimization and development in fermentation often play a key role in enhancing bioprocessing, one of which is using bioreactors for scaling up [10]. The use of a traditional shake flask remains important to generate a wide range of data with its simplicity and affordability in the early stage. However, the design, development, and implementation of bioprocess using bioreactors afterward can establish a better approach to enhance

the fermentation yield [11]. Naturally, this tool offers varying degrees of control and monitoring, creating a balance between good data quantity and quality. Expanding production capacities accommodated growing market demands and stimulated progress in biotechnological applications and encouraged sustainable manufacturing practices [12]. This study aims to concurrently enhance the biomass, riboflavin and nisin production by scaling up the cocultivation to 2.0 L bioreactors by utilizing fed-batch fermentation system with varied flow rate.

MATERIALS AND METHODS

A 2.0 L stirred tank bioreactors BIOSTAT® B (B. Braun, Germany) at Fermentation Technology Unit (FTU-FBSB) (Putra Infoport, Universiti Putra Malaysia) was operated to run different parameters of the bioprocess run; Control (C), Bioreactor Run 1 (B1), and Bioreactor Run 2 (B2). The initial working volume of C employing batch fermentation was equal to 1.5 L, whereas B1 and B2 employed fed-batch fermentation where the initial volume was 750 mL. 250 mL of fresh MRS (DeMan, Rogosa and Sharpe) broth was fed three times at 3, 6, and 9 h in B1 (10 mL/min) and at a much slower rate for B2 (1.6 mL/min) as presented in Table 1. The temperature was set at 37°C with stirring speed of 50 RPM. The pH control in the bioreactor was regulated with 2.0 M NaOH and 0.2 M HCl (Merck, Germany), which were set at pH 6.5 at all fermentation hours. The samples were collected at 2, 4, 6, 8, 10 12, and 24 h for all fermentation runs.

Table 1. Batch and fed-batch fermentation with various feeding strategies.

Type of bioprocess	Feeding of medium	Bioreactor	Pump speed (RPM)	Flow rate of MRS (mL/min)	Pump duration (min)
Batch	One-off (initial)	Control	-	-	-
Fed-batch	Sequential	B1	30	10	25
	Continuous	B2	4	1.6	156.25

Inoculum preparation

Lactococcus lactis ATCC 11454 (LL) and *Lactobacillus rhamnosus* ATCC 7469 (LR) were inoculated individually in MRS broth for 24 h to attain 1×10^9 CFU/mL prior to culturing in the bioreactor medium. The bioreactor inoculation time of LR was fixed at 0 h, whereas the LL inoculum was propagated into 10 mL MRS broth at 6 h. A total of 1% inoculum from initial working volume was used to cultivate the fermentation medium.

Cell growth assay

LL+LR from all bioreactors were harvested for 10 mL to measure their growth turbidimetrically (OD600) using a spectrophotometer (GENESYS™ 10S UV-Visible Spectrophotometer, Thermo Scientific, US). The determination of growth was assayed at nine time intervals (0, 2, 4, 6, 8, 10, 12, 24, and 48 h) [13].

Riboflavin quantification

Riboflavin was assayed using Difco™ Riboflavin Assay Medium (RAM) based on the supplier's protocol (Becton, Dickinson and Company, USA). An aliquot of 5 mL bacterial cultures from each respective interval was collected and sedimented by centrifugation (SIGMA 3-18K, Satorius AG, Germany) at room temperature at 9,300 x g (5 min). An aliquot of 20 uL of the cell suspension was inoculated into 5 mL of the RAM and incubated at 37°C for 24 h. 200 µL of the culture was then dispensed into 96-Well (Chimney Well) Black Microplate (Greiner Bio-One GmBH, Austria). Riboflavin level was quantified using a Direct fluorescence spectrophotometer (Gemini XPS Dual Scanning Microplate Spectrophotometer, Molecular Devices, USA) equipped with the data acquisition and analysis software (SoftMax Pro 6, Molecular Devices, Sunnyvale, California). The riboflavin fluorescence was detected upon excitation at a wavelength of 440 nm and emission at a wavelength of 520 nm. All experiments were performed in triplicates.

Nisin activity assay

LL+LR harvested at nine intervals were centrifuged (Sigma 3-18K Superspeed Refrigerated Centrifuge, Sartorius AG, Germany) at 1600×g for 5 min at 4°C. The supernatant was adjusted to the initial pH of the broth (pH 6.5) using 1 M NaOH to eliminate the acidic factor. Nisin activity was determined by the agar well diffusion method [13]. 100 µL of the overnight culture (OD600 0.1) of *Listeria monocytogenes* and *Bacillus subtilis* were spread evenly on Mueller Hinton agar (Oxoid, UK). 5 mm hole was punched aseptically in the agar using sterile metal well borer. Then, 100 µL of the neutralized supernatant was inoculated into designated wells. Inoculated plates were incubated at 37°C for 24 h. 30 µg/mL kanamycin disc (Oxoid, UK) and MRS broth were used as positive and negative controls, respectively. The inhibition zone surrounding the well was measured in diameter (mm) using a calliper. The bacterial growth and nisin activity results were recorded as mean ± SD from three independent trials.

Statistical analysis

The data were expressed as mean values ± standard deviations (SD). One-way analysis of variance (one-way ANOVA) was performed. The significant differences between mean values were considered at a significance level of $p < 0.05$. Minitab software (version 19; USA) was used to perform statistical analysis.

RESULT AND DISCUSSION

Fig. 1a depicts the growth curve of co-cultivation runs in C, B1, and B2. The fermentation period has been adjusted to 24 h instead of 48 h due to no apparent improvement of metabolite production, which was observed 24 h onwards in previous experiments (data not included). The cell growth for all three bioreactors started similarly during the lag and early log phases (2-6 h). Overall, B1 and B2 show almost the same growth pattern and concentration. At 6 h, C showed significantly higher growth by 16.1% and 36.5% compared to B1 and B2 at 10 h.

The pattern was inverse when B1 and B2 displayed significant growth increase, particularly at 24 h by 36% and 34% compared to C, respectively. This shows that employing a fed-batch system enhanced cell growth from OD600 1.9 in shake flask (data not included) to OD600 2.7 and 2.6, in B1 and B2, respectively. Another significant biomass improvement was evident in batch and fed-batch cultures compared to all shake flasks. Notably, there was no cessation of growth even up to 24 h, with biomass continuing to increase towards the end. This prolonged growth could be attributed to the pH being maintained at 6.5, potentially allowing the growth to remain longer in its optimum pH.

Fig. 1b illustrates the riboflavin level C, B1 and B2 produced throughout the fermentation. Riboflavin was synthesized at 6 h with B1 had 33.6% and 21.3% higher than C and B2, respectively.

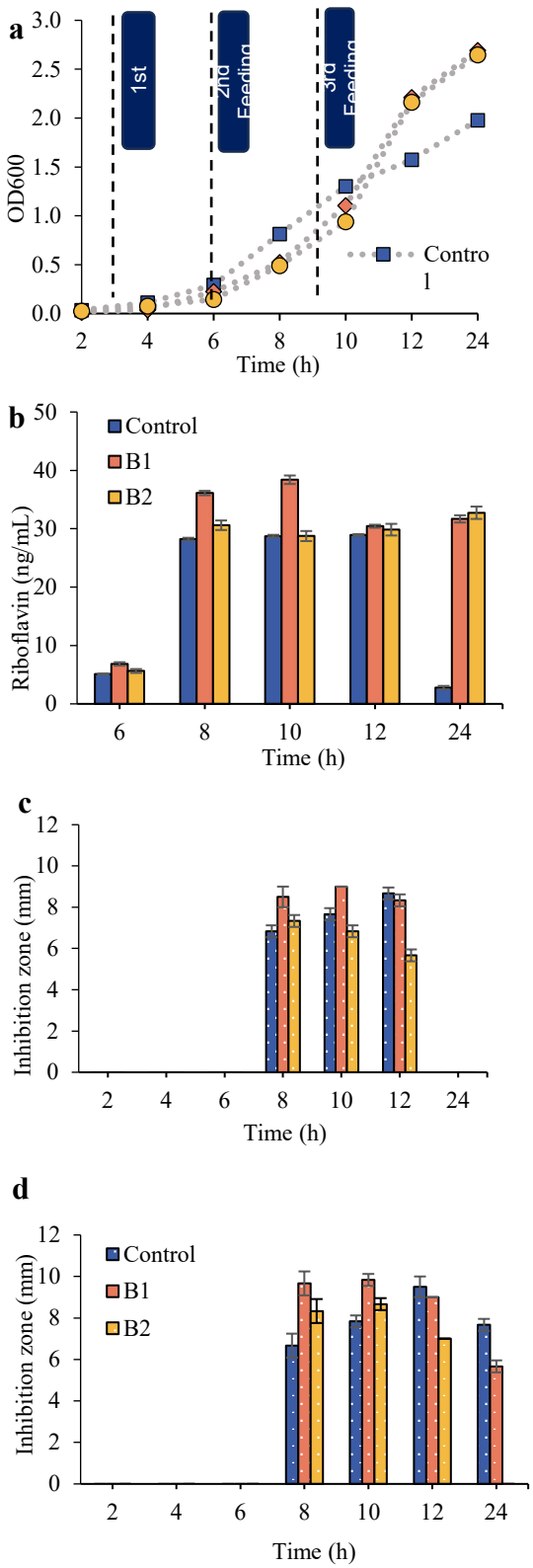

Fig. 1. Fermentation of LL+LR by using 2.0 L stirred tank bioreactor comparing their (a) growth, (b) riboflavin yield, (c) nisin activity against *L. monocytogenes* and (d) *B. subtilis* between C, B1 and B2. Fresh medium was added at 3, 6 and 9 h in B1 and B2 bioreactor. Data are mean ± SD of triplicates (n = 3). Significance was set at $p < 0.05$. Error bars show 95% confidence interval.

The highest riboflavin production was observed at 10 h by B1 (38.39 ng/mL), which was 33.5% higher than C and B2, respectively. The riboflavin production for B1 and B2 were observed to be more consistent, and both could maintain >30 ng/mL, whereas the C was significantly depleted by ~90% at 24 h. The prolonged riboflavin synthesis for up to 24 h by B1 and B2 was more prominent in LL+LR, compared to the limited 12-h yield observed across all shake flasks conditions. Despite being able to sustain a longer production hour, the highest yield achieved by B1 (38.39 ng/mL) and B2 (32.74 ng/mL) did not reach the level attained in shake flasks (46.27 ng/mL) (data not included). Correlating to the higher biomass yield of LL+LR in bioreactors, it could potentially become a limiting factor in riboflavin production as it might have directed more of the carbon flow towards the cell growth upon higher nutrient consumption by the cultures.

The effects on nisin activity are shown in **Fig. 1c** and **1d** against *L. monocytogenes* and *B. subtilis*, respectively. The production of nisin by LL+LR in all three bioreactors became evident only after 8 h. In addition, no nisin activity was observed for all three runs against *L. monocytogenes* at 24 h. The strains' high growth rate possibly affected the limited nisin production shown in all three runs. This observation is similar to the previous study, where the growth rate and nisin production are continuously inverse [13]. C significantly increased from 8 h and peaked at 12 h, with 12.5% and 50% higher nisin yield than B1 and B2, respectively, against *L. monocytogenes*. Despite that, B1 started off with the highest nisin activity from 8-10 h at 9 mm and reduced by 11.1% at 12 h. B2, having the lowest nisin yield, gradually decreased until 12 h, which was 33.3% and 25% lower than C and B1.

A similar pattern was depicted for all three runs against *B. subtilis*, with B1 producing the highest nisin yield from 8-10 h (10 mm). However, B1 declined afterward, with 10% and 25% lower nisin yields than C at 12 h and 24 h, respectively. B2, exhibited higher nisin yield than C against *B. subtilis* from 8-10 h, eventually decreasing and no inhibition zone was observed at 24 h. The slower release of fresh medium in B2 might intensify competition between the strains for access to nutrient sources and space compared to batch (C) and culture with a faster medium release (B1). Nonetheless, C and B1 showed nisin activity against *B. subtilis* throughout 24 h, with C expressing 33.3% higher nisin yield than B1.

Observing the pattern between C, B1, and B2 against both pathogens, we discovered that B1 exhibited better nisin yield after the second and third feeding (8-10 h), followed by B2. This effect could be associated with adding fresh medium at 6 and 9 h, potentially facilitating in regulating nisin synthesis. In addition, C displayed higher nisin activity than B1 and B2 from 12-24 h. This might be possible when there are available residual nutrients in the batch that have not been completely exhausted.

The technological process of riboflavin production here is composed of two main steps: (i) upstream processing which includes developing strains and sterilizing carbon and nitrogen sources, and (ii) fermentation processing which occurs under carefully controlled conditions of optimal temperature, pH, and agitation rates [14], all of which we have optimized in shake flasks. The introduction of fed-batch techniques in the bioreactor has led to higher riboflavin yield as compared to the batch system, which might be attributed to the availability of the immediate riboflavin precursor GTP synthesized from amino acids, tetrahydrofolate derivatives, and CO_2 via threonine, serine,

and glyoxylate cycles [15]. In addition, nisin production followed a pattern similar to a previous study [16], demonstrating that fed-batch production increased the availability of energy sources, amino acids, vitamins, minerals, and peptides. This enrichment stimulates metabolic activity and overcomes the cessation of cell growth for longer.

Previous studies (data not shown in this work) illustrated the process of scaling up from shake flasks into stirred tank bioreactors, which were able to generate maximal metabolite production under batch and fed-batch cultivation, which increased proportionally to cell growth. These controlled conditions in a bioreactor regarding pH, agitation, and feeding strategy can provide the cells with adequate conditions in a better environment for growth and metabolic activities [17].

CONCLUSION

Our investigation revealed that implementing fed-batch fermentation in a bioreactor extends the period of metabolite production to 24 h, compared to the 12 h halt with batch feeding. Additionally, manipulating the feeding flow rate affects the cell growth and enhances their nisin and riboflavin yields. A rapid surge in feeding over a shorter duration facilitated both *L. lactis* and *L. rhamnosus* to consume the substrate with lesser competition in a larger volume, leading to a higher catabolic capability by both strains.

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