ENHANCED PRODUCTION OF RIBOFLAVIN AND NISIN THROUGH COCULTIVATION OF *Lactococcus lactis* AND *Lactobacillus rhamnosus*

By

NADRAH BINTI ABDUL HALID

Thesis Submitted to School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

May 2024

FSTM 2024 8

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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May 2024

Chairman : Hanan bin Hasan, PhD
Faculty : Food Science and Technology

Microbial co-cultivation from different species of lactic acid bacteria (LAB) has the potential mean to trigger its quorum sensing to induce the production of targeted metabolites and possibly increasing its yield. The interspecies relationship between *Lactococcus lactis* ATCC 11454 and *Lactobacillus rhamnosus* ATCC 7469 were evaluated by comparing the growth profile (OD 600), specific growth rate (μ_{max}), riboflavin yield (vitamin B2), and nisin activity (against *Listeria monocytogenes* and *Bacillus subtilis*). Fermentation was performed between the monocultures of *L. lactis* (LL) and *L. rhamnosus* (LR), and their co-culture (LL+LR). The fermentation conditions (temperature, pH, agitation, carbon and nitrogen sources, inoculation ratio and time) were varied to attain optimum bacterial growth, riboflavin and nisin yield. Subsequently, fermentation in 2.0 L stirred tank bioreactors were investigated using the combination of optimized factors in shake-flask scale. Batch fermentation was implemented in the control (C) bioreactor, while fed-batch fermentation was employed in bioreactor run 1 (B1) and run 2 (B2), with different medium flow rates of 10 mL/min and 1.6 mL/min, respectively. The most profound factors to enhance the riboflavin yield were temperature 37°C at pH 6.5, without additional glucose and yeast extract. The cell growth, nisin activity and most significantly riboflavin yield in LL+LR increased by 58% and 57% as compared to LL and LR, respectively. Furthermore, B1 was able to generate an increase of 33.5% riboflavin and 36% growth rate than C and B2. The application of bioreactor in the fermentation process resulted in a prolonged exponential phase which subsequently contributed to a longer timeframe for metabolite production. The findings from this study provided an efficient future prospect of LAB co-culture, potentially by extending riboflavin production time and increasing vitamin level in fermented foods or facilitating the production of valuable metabolites.

Keywords: fermentation, lactic acid bacteria, co-cultivation, riboflavin, nisin

SDG: GOAL 3: Good Health and Well-Being

Abstrak tesis yang dikemukakan kepada Senat of Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENINGKATAN PENGELUARAN RIBOFLAVIN DAN NISIN MELALUI PENGKULTURAN-BERSAMA *Lactococcus lactis* DAN *Lactobacillus rhamnosus*

Oleh

NADRAH BINTI ABDUL HALID

Mei 2024

Pengerusi : Hanan bin Hasan, PhD
Fakulti : Sains dan Teknologi Makanan

Pengkulturan-bersama mikrob daripada spesies bakteria asid laktik (LAB) yang berbeza mempunyai potensi untuk merangsangkan penderiaan "quorum" untuk mendorong penghasilan and meningkatkan metabolit yang diinginkan. Hubungan antara spesies *Lactococcus lactis* ATCC 11454 dan *Lactobacillus rhamnosus* ATCC 7469 telah dinilai dengan membandingkan profil pertumbuhan (OD 600), kadar pertumbuhan spesifik (μ_{max}), pengeluaran riboflavin (vitamin B_2) dan nisin, iaitu bacteriocin yang diuji terhadap *Listeria monocytogenes* dan *Bacillus subtilis.* Penapaian dijalankan secara pengkulturan-individu untuk *L. lactis* (LL), *L. rhamnosus* (LR), dan pengkulturan-bersama kedua-dua LAB (LL+LR). Faktor penapaian (suhu, pH, pergaulan, sumber karbon dan nitrogen, nisbah inokulasi dan masa) dipelbagaikan untuk mencapai pertumbuhan bakteria yang optimum dan pengeluaran riboflavin dan nisin yang lebih tinggi. Ini diikuti dengan perpindahan penapaian ke dalam bioreaktor 2.0 L dengan menggunakan gabungan faktor yang dioptimumkan di dalam skala kelalang. Penapaian kelompok dilaksanakan di dalam bioreaktor kawalan (C), manakala penapaian kelompok suapan digunakan di dalam Bioreaktor 1 (B1) dan 2 (B2) dengan menggunakan kadar aliran dan susbtrat yang berbeza dengan masing-masing sebanyak 10 mL/min dan 1.6 mL/min. Faktor yang paling ketara memberi kesan terhadap produksi riboflavin dapat dilihat pada suhu 37°C di pH 6.5 tanpa penambahan glukosa dan ekstrak yis. Pertumbuhan sel, aktiviti nisin dan paling signifikan, produksi riboflavin di LL+LR menaik sebanyak 58% dan 57% berbanding LL dan LR. Tambahan pula, bioreaktor berskala yang dijalankan di dalam B1 mampu menjana peningkatan 33.5% riboflavin dan 36% kadar pertumbuhan daripada sistem C dan B2. Penggunaan bioreaktor dalam proses penapaian mampu menghasilkan fasa pertumbuhan yang berpanjangan dan seterusnya dapat menyumbang kepada jangka masa yang lebih lama untuk pengeluaran metabolit. Penemuan daripada kajian ini boleh memberikan prospek masa depan yang baik bagi pengkulturan bersama bakteria asid laktik, berpotensi memanjangkan masa pengeluaran riboflavin dan meningkatkan tahap vitamin dalam makanan yang ditapai, atau memudahkan pengeluaran metabolit yang bermutu.

Kata kunci: penapaian, bakteria asid laktik, kultivasi-bersama, riboflavin, nisin

SDG: MATLAMAT 3: Kesihatan Baik dan Kesejahteraan

ACKNOWLEDGEMENTS

In the name of Allah, The Most Gracious, The Most Merciful.

All praise and thanks are due to Allah, for giving me the blessings, strength, knowledge and perseverance to pursue and complete my master's degree.

I would like to extend my deepest gratitude to my supervisor, Dr. Hanan Hasan, for the unwavering support and guidance throughout this academic journey. His dedication to both his students' research and welfare is nothing short of great, and I am forever grateful. Thank you for creating a supportive environment where I can get feedback, insights and invaluable advice that comes with compassion in challenging times.

I would like to thank my co-supervisors, Dr. Hafiz Rahim and Prof. Madya Ts. Dr. Mohamad Faizal Ibrahim for constantly encouraging me in my research process, offering advice and support whenever their guidance is needed.

I am forever indebted to my lab mates, two families that I have worked with for these few years. Kak Rani, Epeng, Chong, Kierthy, Wendy, Sheera, Jiangshan, and Nurul, Syafiqah, Kak Hajar, Khudyr, Arif, Alia, Anis. Thank you for making my lab life so much lighter and manageable. From the quick run to get some coffee and pastries, to those late nights working together, and everything in between; I will cherish them all. Special thanks to Ummu and Zafirah, I am following in your footsteps, both of you. Thank you for the endless support. You guys are a blessing to me.

I would also like to thank the lab officers who have contributed in one way or another, especially the help and insights I could get during the HPLC and bioreactor times.

Finally, to my Mama and Abah. You two are my greatest source of strength and comfort. Mama, thank you for standing strong for your kids and believing in me. Having you around is enough to keep me going. Abah, you were my inspiration since the very beginning, and you still are. I hope this could at least make you proud. Your girl did it. This is for you. To my family, my siblings, my cheekiest nephews and niece, thank you for being the pillars in my journey. I couldn't have made it without you guys.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

Hanan bin Hasan, PhD
Senior Lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Chairman)

Muhamad Hafiz bin Abd Rahim, PhD
Senior Lecturer
Faculty of Food Science and Technology
Universiti Putra Malaysia
(Member)

Mohamad Faizal bin Ibrahim, PhD
Assoc. Prof. Ts. Dr
Faculty of Biotechnology and Biomolecular Sciences
Universiti Putra Malaysia
(Member)

ZALILAH MOHD SHARIFF, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 10 October 2024

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LIST OF ABREVIATIONS

ATCC	American Type Culture Collection
B. subtilis	*Bacillus subtilis*
BHI	Brain Heart Infusion
BLIS	Bacteriocin-like inhibitory substance
GRAS	Generally Recognized as Safe
L. monocytogenes	*Listeria monocytogenes*
LAB	Lactic Acid Bacteria
LB	Luria-Bertani
LL	*Lactococcus lactis*
LL+LR	Cocultivation of *Lactococcus lactis* and *Lactobacillus rhamnosus*
LR	*Lactobacillus rhamnosus*
MRS	de Man Rogosa Sharpe
NaOH	Sodium hydroxide
OD_{600}	Optical density at wavelength 600 nanometre
p	p-value
RAM	Riboflavin Assay Medium
SD	Standard deviation
USP	United States Pharmacopeia

CHAPTER 1

INTRODUCTION

1.1 Background

Lactic acid bacteria (LAB) are a group of microorganisms that are commonly utilized in various industries including food, clinical and agricultural sectors (Abedin et al., 2023; Bintsis, 2018). They are the most common starter culture main for food preservation and have been recognized as Generally Recognized as Safe (GRAS) status (Ayivi et al., 2020, Plavec & Berlec, 2020). In recent years, LAB has garnered interest from researchers for their significant ability to synthesize various beneficial metabolites. These metabolites showcase unique bioactivity and contribute to a diverse range of health-promoting effects, including vitamins, bacteriocins, organic acids, exopolysaccharides, gamma-aminobutyric acid and flavor compounds (Hakim et al., 2023; Mathur et al., 2020).

Their highly diverse properties have laid the foundations for the food industry to enhance the sensory quality by regulating the taste and texture in their food products (Hoxha et al., 2023; Mokoena, 2017). To date, the ability of LAB to naturally produce various compounds has gained attraction in the manufacturing industries to improve the shelf-life, quality, safety, and nutritional value of fermented products (Albano et al., 2020; Ponzio et al., 2024).

Certain LAB strains exhibit the capacity for biosynthesis of essential B group vitamins, essential micronutrients that play a critical role in several metabolic and regulatory processes necessary for human health, including folates, cobalamin, and riboflavin. Among these vitamins, riboflavin is particularly important for various physiological functions (Levit et al., 2021; Thakur et al., 2015). Ariboflavinosis can occur when there is a deficiency in riboflavin consumption which is linked to ocular complications, cardiovascular risks, preeclampsia, anaemia, hepatic and dermal harm, as well as alterations in cerebral glucose metabolism (Suwannasom et al., 2020; Thakur et al., 2017).

Traditionally, riboflavin has been synthesized for fortification purposes through chemical means (Liu et al., 2020; You et al., 2021). However, in recent years, there has been a significant change towards using microbial techniques to produce riboflavin. Riboflavin-supplying LAB, such as *Lactobacillus rhamnosus* ATCC 7469, offers a clear advantage over chemical synthesis by increasing the nutritional value of food using cleaner and sustainable methods (Hasan et al., 2023; Zhang et al., 2022).

Furthermore, LAB also synthesize natural antimicrobials compounds, which is known as bacteriocins or bacteriocin inhibitory like substances (BLIS) that can be used for food preservation (Barcenilla et al., 2022; Manna & Jana, 2024). In the last decade, bacteriocins have been clinically tested as controlling agents for bacterial, biofilm, and viral infections (Fernandes & Jobby, 2022). As an ideal biopreservative agent, the strategy is to only exhibit antimicrobial activity against targeted pathogens but also did not present any detrimental effects to the intestinal microbiome of consumers (Pisoschi et al., 2018). This is due to the properties of bacteriocin which can exhibit non-toxicity towards eukaryotic cells and are inactivated by digestive proteases. Besides possessing a broad antimicrobial spectrum against food-borne pathogens and spoilage bacteria, it exhibits bactericidal effects on the bacterial cytoplasmic membrane, and show no cross-resistance with antibiotics (Bhattacharya et al., 2022; Lahiri et al., 2022).

Bacteriocins are significantly better compared to conventional antibiotics due to its stability in a broad range of physicochemical conditions. It has demonstrated inhibitory effects against intestinal pathogens such as *Clostridium defficile, Staphylococcus aureus, Listeria monocytogenes, Salmonella enteritidis* and vancomycin-resistant enterococci (VRE) (Darbandi et al., 2022). *Lactococcus lactis* subsp. *lactis* is a common and high producer of bacteriocin called nisin (Rasid et al., 2023; Zhao et al., 2021), capable of inhibiting closely related Gram-positive bacteria such as *Listeria monocytogenes* and *Bacillus subtilis* (Kondrotiene et al., 2023; Suzuki and Suzuki, 2021). It has demonstrated a profound effect on hampering pathogenic growth, thereby extending the shelf life of perishable foods (Nie et al., 2023).

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 in food systems (Garsa et al., 2014; Juturu & Wu, 2018; Zhao et al., 2021). In response to these challenges, microbial co-cultivation has emerged as a promising approach for enhancing the production of bioactive compounds from LAB (Canon et al., 2020; Cui et al., 2020; Selegato & Castro-gamboa, 2023).

Co-cultivation involves the simultaneous cultivation of two or more microbial strains in a fermentation tank at the same time. This allows for the induction and stimulation possibly through quorum sensing of secondary metabolic pathways and the production of diverse valuable metabolites. This technique offers a novel approach to microbial production platforms, allowing for the simultaneous synthesis of multiple valuable compounds, including both bacteriocins and vitamins (Aguilar et al., 2021; Guo et al., 2024; Kapoore et al., 2021; Peng et al., 2021).

There are limited number of studies focusing on the interaction and behaviour of LAB strains in a co-cultivation system to simultaneously enhance the synthesis of nisin and riboflavin. This knowledge gap highlights the importance of conducting thorough studies to determine the practicality and effectiveness of growing industrially important LAB species together, such as the nisin producer *Lactococcus lactis* ATCC 11454 and the riboflavin producer *Lactobacillus rhamnosus* ATCC 7469 to increase it biosynthesis.

1.2 Problem statement

The issues and challenges to be addressed are as follows:

1. The monoculture system is prone to contamination by other microorganisms. This can cause drawbacks like lower productivity, lower quality of biomass and culture collapse. Thus, the idea of implementing co-cultivation system can create a stronger and more robust microbial consortium.

2. The challenge of scaling up lies in the optimization process and strategies, which are different from that of shake flasks. The control process, key parameters and feed fermentations should be taken into consideration. These are crucial as metabolites production depends heavily on the scaling up strategy.

1.3 Hypothesis

The hypotheses of this study are:

1. The co-cultivation between *L. lactis* ATCC 11454 and *L. rhamnosus* ATCC 7469 will enhance cell growth and the production of riboflavin and nisin concurrently compared to the monoculture of both LABs.
2. Upscaling the volume of co-cultivates with various feeding strategies will increase the cell growth and metabolites production as compared to the lab scale shake flasks.

1.4 Objectives

The objectives of this study are:

1. To elevate the cell growth, riboflavin and nisin production through co-cultivation of both *L. lactis* ATCC 11454 and *L. rhamnosus* ATCC 7469 using various fermentation factors.

2. To enhance the cell growth, riboflavin and nisin production through bioreactor scale-up using different feeding strategies in fed-batch cultivation.

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