Lactobacillus delbrueckii subsp. *bulgaricus* as a Microbial Chassis for D-Lactic Acid Biosynthesis: Strain Improvement, Metabolic Engineering, and Development of Molecular Tools

A thesis

Submitted for the partial fulfilment of the requirements for the degree of

Doctor of Philosophy

By

Payal Mukherjee

Department of Biosciences and Bioengineering

Indian Institute of Technology Guwahati

Guwahati-781039, Assam, India

April 2025

Dedicated to My Beloved Parents

Indian Institute of Technology Guwahati
Guwahati 781039, Assam, India

DECLARATION

I hereby declare that the research findings presented in this thesis, entitled **"*Lactobacillus delbrueckii* subsp. *bulgaricus* as a Microbial Chassis for D-Lactic Acid Biosynthesis: Strain Improvement, Metabolic Engineering, and Development of Molecular Tools"** is the result of research work carried out by me under the supervision of **Prof. Senthilkumar Sivaprakasam** in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, for the award of the degree of Doctor of Philosophy.

I also declare that the contents of this thesis have not been submitted, in whole or in part, for any other degree or membership of any Institute or University to the best of my knowledge and belief. In keeping with academic norms for reporting research, due acknowledgments have been made wherever the findings of other researchers have been cited in this thesis.

Date: 08.04.2025

Place: Guwahati

Payal Mukherjee

Payal Mukherjee

Roll: 196106017

Indian Institute of Technology Guwahati
Guwahati 781039, Assam, India

CERTIFICATE

This is certified that the work described in this thesis entitled "***Lactobacillus delbrueckii* subsp. *bulgaricus* as a Microbial Chassis for D-Lactic Acid Biosynthesis: Strain Improvement, Metabolic Engineering, and Development of Molecular Tools**" by **Ms. Payal Mukherjee** for the award of the degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India. The contents of this thesis have not been submitted, either in part or in full, to any other University or Institute for the award of any degree.

Date: 08.04.2025

Prof. Senthilkumar Sivaprakasam

(Thesis Supervisor)

Acknowledgement

I begin by expressing my deepest gratitude to the divine presence for bestowing upon me the strength, resilience, and guidance needed to navigate this remarkable journey. Each day unfolded as an opportunity to learn, grow, and broaden my horizons, shaping both my understanding and my character. The challenges I faced became stepping stones, and the moments of triumph served as reminders of the grace that accompanies perseverance.

I owe my deepest gratitude to my supervisor, Prof. Senthilkumar Sivaprakasam, for providing me with this invaluable opportunity. I am grateful for the freedom he provided to pursue my work independently, along with his immense kindness and unwavering support throughout my academic journey. His guidance has been crucial to the successful completion of this research and to my growth as an independent researcher. With insightful feedback and access to all necessary resources, he has played a vital role in enabling me to accomplish this work.

I extend my heartfelt gratitude to my doctoral committee chairperson, Dr. Selvaraju Narayanasamy, and committee members, Prof. Ajaikumar B. Kunnumakara and Dr. Soumen Kumar Maiti, for their invaluable time and kind words of encouragement. Their insightful guidance, constructive suggestions have been instrumental in shaping and successfully executing this work.

A special note of thanks to Prof. Ayigari Ramesh for his inspiration, motivation, and guidance during critical moments of my research journey. I would also like to sincerely thank Prof. Ajaikumar B. Kunnumakara for generously granting me access to his laboratory facilities, particularly the PCR thermal cycler, which was essential for the molecular biology work in this thesis.

I express my heartfelt gratitude to Dr.Emmanuelle Maguine, INRAE, France for providing essential strain at the right moment, which proved crucial for the success of my experiments and this complete thesis work.

I am deeply grateful to all the faculty members and staff members of the Department of Biosciences and Bioengineering (BSBE), IIT Guwahati, and the Central Instruments Facility, whose support and infrastructure enabled this research.

I am thankful to the Ministry of Human Resource Development for providing the fellowship and the DBT Project (BT/PR24592/NER/95/764/2017), which supported this research.

I would also like to extend my thanks to the BioPAT Lab members, both past (Dr. Srikant Katla, Dr. Naresh Mohan, Dr. Subbi Rami Reddy Tadi, Dr. Ganesh Nehru, Dr. Kiran Kumar Gali, Dr. Nivedhitha Swaminathan and Dr. Allampali Satya Sai Pavan) and present (Siddharth, Naveen, Ankita, Poulami, Ajay, Sushanth, Thilak and Vasantha) for their camaraderie and contributions.

A special mention to Pallabi Seal and Sanjana Pal for their assistance in my work, and to Nirmal and Meghana, whose collaborations added value to my research.

I would also like to express my sincere gratitude to Ansuman Sahoo for his invaluable assistance with the bioreactor experiments. I extend my heartfelt thanks to all my friends and batchmates—Parama, Ayushi, Kalpana, Vishnu, Robin, Malaya, and many others—who offered their unwavering support and made my time at IIT Guwahati truly memorable.

I am profoundly grateful to my dearest friends, Shilpi and Surabhi, for their unwavering care and for sharing in the little moments that brought joy and warmth, making this journey truly special from day one.

A special note of thanks to Satyam for his unwavering support, constant presence, keeping me up-to-date with his technical expertise and for helping me wholeheartedly in countless ways throughout this journey. To my parents, especially my father, who has been the best dad in the world, I cannot thank you enough for being my pillar of strength, unwavering support, and for showering me with unconditional love, encouragement, and immense belief in me. To my mother, for her boundless love, care, and constant support, I am so blessed.

Finally, I extend my heartfelt gratitude to my beloved family, friends, and everyone who has been a part of this journey, directly or indirectly. Each one of you has played a unique and significant role in helping me reach this milestone and shaping me into the person what I am today.

" I may not know where this path leads, but with passion for my work, I trust it will take me where I'm meant to be, and success will follow."

Payal Mukherjee

Synopsis

D-lactic acid (DLA) is an industrially significant organic acid with diverse applications in the production of biodegradable polymers, such as polylactic acid (PLA), a sustainable alternative to traditional plastics. The optical purity of DLA is a critical factor influencing the thermal stability and crystallinity of PLA, which are essential for applications like food packaging. Microbial fermentation using lactic acid bacteria (LAB) offers an eco-friendly and economically viable alternative to conventional chemical synthesis for DLA production. LAB, particularly homofermentative strains, possess inherent metabolic pathways to synthesize DLA with high optical purity from renewable feedstocks, including lignocellulosic biomass, food waste, and agro-industrial residues.

Advancements in metabolic engineering have enabled the enhancement of DLA biosynthesis through strategies such as gene overexpression, pathway optimization, and targeted knockouts to eliminate by-product formation. Emerging synthetic biology tools and genome-scale metabolic models have further improved the efficiency of strain development. The integration of dynamic metabolic engineering, employing feedback-regulated circuits and inducible systems, can facilitate precise control over DLA production, ensuring high yields and reduced metabolic burdens. LAB present challenges as hosts for genetic engineering due to limited molecular cloning tools available for many strains. *Lactococcus lactis* and *Lactobacillus plantarum* are the primary host organisms extensively studied and engineered LAB due to their relatively well-developed genetic systems. However, *Lactobacillus delbrueckii*, an important member of the LAB family, holds significant potential for producing high titre of optically pure DLA due to its metabolic capacity. Despite this, the genetic manipulation of *L. delbrueckii* remains challenging due to the lack of efficient molecular cloning tools and reproducible transformation methods. Although substantial progress has been made in developing advanced genetic tools and synthetic biology approaches for LAB, including CRISPR-based genome editing, the availability of these tools for *L. delbrueckii* is still limited. Addressing these challenges by optimizing transformation protocols and developing strain-specific molecular tools is crucial for unlocking the full potential of *L. delbrueckii* as a robust microbial cell factory for industrial applications including DLA biosynthesis.

Chapter 1 of this thesis deals with introduction and the state-of-the-art literature for DLA biosynthesis through microbial fermentation route using LAB as a potential host. Chapter 2 of this thesis focuses on the initial screening of multiple *Lactobacillus delbrueckii* strains to identify suitable candidates for enhanced DLA production. Strains *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 and VI104 demonstrated superior lactose utilization and DLA production under static and shaking culture conditions. Among these, strain VI104 exhibited the highest DLA titre of 1.98 g L^{-1} and an optical purity of 99.09% under shaking conditions in presence of 2%(w/v) lactose-Man Rogosa Sharpe (MRS) media, making it a prime candidate for metabolic engineering. Conversely, ATCC 11842, while capable of moderate DLA production, exhibited low electroporation efficiency and poor transformation reproducibility, limiting its immediate applicability for direct genetic manipulation. Optimization of electroporation protocols for the shuttle vector pLEM415 revealed critical parameters for enhanced transformation efficiency, including harvesting cells during the early exponential phase and applying pulse voltages within the range of 1.5–1.8 kV. The P_{ldhL} promoter from *Lactobacillus sakei* was validated for robust recombinant protein expression in VI104, establishing it as the optimal host for metabolic engineering. ATCC 11842, despite its limitations, was selected for random mutagenesis due to its potential for improvement through strain adaptation strategies.

Building on the findings of Chapter 2, Chapter 3 delves into the metabolic reprogramming of *L. bulgaricus* VI104 to enhance overall DLA production. It explores targeted metabolic engineering of *L. bulgaricus* VI104 to overcome lactose assimilation bottlenecks and improve DLA production. The heterologous expression of galactokinase (*galK)* and galactose-1-phosphate uridyltransferase (*galT)* constructed the complete Leloir pathway, significantly reducing galactose accumulation, a common byproduct of lactose hydrolysis in *L.bulgaricus*. To mitigate ATP imbalances caused by redirected metabolic flux, overexpression of the pyruvate kinase (*pyk)* gene was employed, enhancing acid tolerance under high DLA concentrations. Overexpression of key glycolytic enzymes (d-lactate dehydrogenase (*dldh)*, phosphofructokinase (*pfk)*, and phosphoglycerate kinase (*pgk*)) and the major *dldh* homologs (*Ldb0101* and *Ldb1010*) sequentially further improved metabolic flux, leading to a 240% increase in DLA titres compared to wild VI104. The engineered strain, LdbVI104_07, achieved a maximum DLA production of 9.39 g L^{-1} with a yield of 0.188 g g^{-1} and a 273% enhancement in acid tolerance. Transcriptomics and enzymatic assays substantiated these improvements, revealing elevated expression levels of the key glycolytic genes including *dldh*. This metabolic

reprogramming identifies VI104 as a highly promising strain for further enhancement and optimization in industrial applications.

Despite the significant advancements in strain performance achieved in Chapter 3, the constitutive overexpression of heterologous enzymes introduced a metabolic burden, limiting further improvements in productivity. Chapter 4 addresses this challenge by employing dynamic metabolic engineering to regulate metabolic activity in response to DLA levels. The development of an engineered DLA-inducible promoter-repressor system enabled real-time modulation of enzyme expression, reducing the metabolic load during the growth phase and maximizing DLA production during the production phase. This promoter-repressor system, derived from *Pseudomonas fluorescens* A506, was re-engineered for functional efficacy in VI104. Codon optimization enhanced regulatory element performance, with peak activity observed at DLA inducer concentrations between 60–100 mM. The engineered promoter-repressor system enabled precise modulation of *dldh* expression as an application, autonomously regulating the transition between growth and production phases. This dynamic control reduced overall metabolic load during the growth phase while maximizing DLA biosynthesis during the production phase. Fluorometry and microscopy validated the engineered promoter repressor system functionality, while molecular docking elucidated critical noncovalent interactions between the D-*LldR* repressor and the operator sequence in the absence of DLA. Upscaling to lab-scale bioreactor resulted in a 1.63-fold increase in DLA production, achieving a maximum titre of 9.02 g L^{-1}. The autonomous dynamic regulatory system (ADR) also extended the fermentation time and improved overall productivity compared to constitutive expression systems. These results highlight the potential of dynamic regulatory systems to optimize biosynthesis and enhance industrial applicability. Cross-species validation and molecular docking analyses expanded the utility of this synthetic circuit, enhancing metabolic flexibility and productivity in LAB.

While the engineered VI104 strain achieved impressive results, the mutagenesis approach outlined in Chapter 5 aimed to enhance the performance of ATCC 11842, a strain initially identified in Chapter 2 with moderate DLA production capacity. Due to the extremely low transformation efficiency of ATCC 11842, an alternative strain improvement strategy was employed to enhance its DLA biosynthetic potential. Synergistic use of ultraviolet (UV) irradiation and chemical mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) generated a mutant strain, Mut_N23, with significantly enhanced metabolic capabilities. Mut_N23 exhibited a 97% increase in DLA production and a 37% improvement in glucose

uptake rates. Optimization of fermentation parameters using One-Factor-At-a-Time (OFAT) and Response Surface Methodology (RSM) led to a maximum DLA titre of 7.88 g L^{-1}, a 300% improvement compared to the wild-type ATCC 11842 strain, with a specific productivity of 0.110 g g^{-1} h^{-1}. Mut_N23 also demonstrated versatility by utilizing whey permeate (WP), a cost-effective carbon source, to produce 4.89 g L^{-1} of DLA with 99.09% optical purity as an application. Characterization of purified DLA using Fourier Transform Infrared (FTIR) and proton Nuclear Magnetic Resonance (NMR) spectroscopy confirmed its equivalence to commercially available standards. The transcriptomic analysis of Mut_N23 revealed upregulated expression of key genes associated with lactose metabolism and DLA biosynthesis, providing insights into the molecular basis of its improved performance.

Overall, this thesis focused on exploring *Lactobacillus delbrueckii* subsp. *bulgaricus* as a potential host for industrial applications beyond its traditional role in dairy industries. Through systematic strain screening, metabolic engineering, construction of dynamic regulatory systems, and mutagenesis approaches, this work highlights the versatility and adaptability of *L. bulgaricus* for producing high-purity DLA. Importantly, a foundational framework has been established by developing molecular cloning tools, such as optimized electroporation protocols and synthetic regulatory systems, to facilitate further genetic modifications in this organism. The findings of this study pave the way for leveraging *L. bulgaricus* in applications far beyond DLA production. The groundwork laid here not only advances its use in industrial bioproduction but also opens avenues for exploring this organism for potential therapeutic and probiotic applications in the future. This thesis thus serves as a significant step toward realizing the untapped potential of *L. bulgaricus* as a versatile microbial cell factory.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

Abbreviation	
ADR	Autonomous Dynamic Regulation
ALE	Adaptive Laboratory Evolution
Ald	Aldolase
Amp^r	Ampicillin Resistance
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
ATR	Attenuated Total Reflectance
ATCC	American Type Culture Collection
BOD	Biological Oxygen Demand
BSA	Bovine Serum Albumin
CAGR	Compound Annual Growth Rate
CcpA	Catabolite Control Protein A
CCD	Central Composite Design
CFU	Colony Forming Units
COBRA	Constraint-Based Reconstruction and Analysis
COD	Chemical Oxygen Demand
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
csp	Cold Shock Protein
DBR	DNA Binding Domain
dldh	D-Lactate Dehydrogenase
DLA	D(+) -Lactic Acid
EB	Electroporation Buffer
Emr	Erythromycin Resistance
Eno	Enolase
EMS	Ethyl Methanesulfonate
EPS	Exopolysaccharides
fba	Fructose 1,6-Bisphosphate Aldolase
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectroscopy

Abbreviation

galK	Galactokinase
galM	Galactose Mutarotase
galT	Galactose-1-Phosphate Uridyltransferase
gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase
GEMs	Genome-Scale Metabolic Models
glk	Glucokinase
GMOs	Genetically Modified Organisms
Gpma	2,3-Bisphosphoglycerate-Dependent Phosphoglycerate Mutase
GRAS	Generally Recognized as Safe
HFLAB	Homofermentative Lactic Acid Bacteria
HlbA	Histone-like protein A
HPLC	High-Performance Liquid Chromatography
IgG	Immunoglobulins
LAB	Lactic Acid Bacteria
ldh	Lactate Dehydrogenase
LB	Luria-Bertani
LF	Lactoferrin
LLA	L(-) -Lactic Acid
LPO	Lactoperoxidase
MPC	Milk Protein Concentrate
Mrfp	Monomeric Red Fluorescent Protein
mRCM	Modified Reinforced Clostridial Medium
MS	Mass Spectrometry
MWCO	Molecular Weight Cut-Off
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide (NAD) + Hydrogen (H)
NBRC	Biological Resource Center, NITE
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NICE	Nisin-Controlled Gene Expression
NIRS	Near-Infrared Spectroscopy
NMR	Nuclear Magnetic Resonance
NTG	N-Methyl-N′-Nitro-N-Nitrosoguanidine
OFAT	One-Factor-At-A-Time
PBS	Phosphate Buffered Saline

Abbreviation

PDLA	Poly-D-Lactic Acid
PEP	Phosphoenolpyruvate
pfk	Phosphofructokinase
pgk	Phosphoglycerate Kinase
PLA	Poly Lactic Acid
PLIP	Protein-Ligand Interaction Profiler
PLLA	Poly-L-Lactic Acid
PVDF	Poly(Vinylidene Fluoride)
pyk	Pyruvate Kinase
RBS	Ribosome Binding Site
RID	Refractive Index Detector
RSM	Response Surface Methodology
SCP	Single Cell Protein
SDS PAGE	Sodium Dodecyl-Sulfate-Polyacrylamide Gel Electrophoresis
SMP	Skim Milk Powder
SSF	Simultaneous Saccharification and Fermentation
tpi	Triosephosphate Isomerase
UF	Ultrafiltration
USD	United States Dollar
UV	Ultraviolet
WP	Whey Permeate

CHAPTER 1:

Introduction and Review of Literature

1.1 Lactic acid and its isomers

Lactic acid is an organic compound commonly found in various biological processes and industries. It is a hydroxycarboxylic acid with the chemical formula ($C_3H_6O_3$). Lactic acid exists in two stereoisomers: L(-)-lactic acid (LLA) and D(+)-lactic acid (DLA), which are mirror images of each other (*Fig 1.1*). These isomers differ in their spatial arrangement of atoms, making them chiral. LLA is predominantly found in biological systems, such as muscle metabolism and fermentation, while DLA has applications in certain industrial processes. Together, these isomers play a significant role in fields ranging from biochemistry to material science. Lactic acid finds applications across diverse industries due to its versatile properties. In the food and beverage sector, it is used as a preservative, flavor enhancer, and pH regulator. In pharmaceuticals and cosmetics, lactic acid serves as a moisturizing agent and exfoliant in skincare products. Its biodegradable nature makes it valuable in the production of polylactic acid (PLA), a sustainable bioplastic used in packaging and medical devices. Additionally, lactic acid is employed in agriculture as a feed additive and in the textile industry for dyeing and finishing processes. Its wide range of applications highlights its importance in both traditional and modern industries.

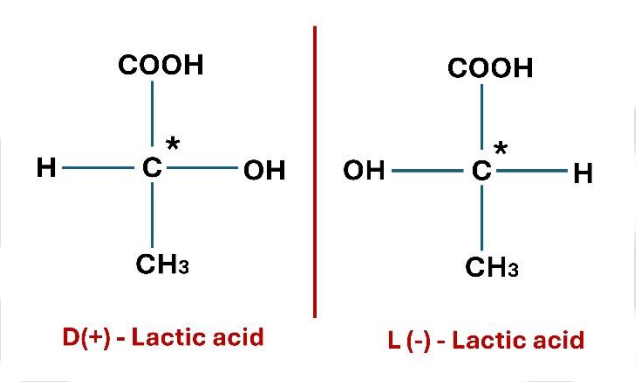


Figure 1.1 Structure of lactic acid isomers

1.1.1 D-lactic acid

DLA is an organic acid that holds substantial industrial significance and is widely utilized in various sectors such as the chemical, pharmaceutical, agriculture, textile, leather, and polymer industries[1]. In recent times, there is a surge in interest surrounding DLA primarily because of its application in the development of thermostable biodegradable polymer known as PLA [2]. PLA has gained prominence as a highly promising and eco-friendly substitute to polythene, demonstrating its potential to serve as a viable material for various food packaging applications. PLA is synthesized by the process of polymerization using lactic acid as its monomer [3]. However, properties of PLA

are hindered primarily due to its optical purity of lactic acid. PLA, a compound with chirality, consists of two enantiomers: DLA and LLA. The specific characteristics of PLA are influenced by the relative proportions of DLA and LLA [4]. Consequently, it becomes crucial to generate lactic acid with the highest possible optical purity to facilitate optimal PLA production [1]. Thermal stability is a crucial requirement for the successful utilization of PLA in the food industry. Traditional production methods of poly-L-lactic acid (PLLA) have involved employing LLA as the monomer, ensuring the desired thermal stability for PLA applications. However, its application has been restricted due to its thermal instability above 173-178 °C. In addition, using DLA as a monomer, the melting temperature of the Poly-D-lactic acid (PDLA) can be increased by 40-50° C. Proportionately, the enhanced thermal steadiness of PLA has found its application as a food packaging material in food industry [5].

Through precise control of fermentation conditions and the strategic selection of host organisms, it becomes feasible to exclusively obtain specific lactic acid isomers using fermentation technology. Lactic acid bacteria and other microbes are naturally capable of producing both isomers of lactic acid from various carbohydrate sources. Leveraging genetically modified microbes further enhances lactic acid production capabilities. The formation of amorphous polymers occurs when a racemic mixture of LLA or DLA is polymerized. On the other hand, to produce crystalline and stable polymers like PLLA or PDLA, it is necessary to initiate the process implementing optically pure LLA or DLA, respectively. Different concentrations of the enantiomers applied manipulate specific attributes of the polymers, such as crystallinity. When PLLA is blended with PDLA, for example, it elevates the polymer's melting point by 50^{o}C, resulting in a highly regular complex with high degrees of crystallinity and thermal stability [6]. Consequently, it is increasingly necessary to manufacture optically pure PDLA that can be used to enhance the melting temperature employing DLA. The growing demand for PLA is projected to drive its market size from USD 1,096.31 million in 2019 to USD 2,424.62 million by 2025, reflecting a compound annual growth rate (CAGR) of 14.14%. Additionally, the DLA market is expected to reach USD 293.1 million by the end of 2026, with a CAGR of 7.2% during the period of 2021-2026.

1.1.2 Production methods and commercialization

Industrially, DLA can be produced through either a conventional chemical route or the microbial fermentative route. The former case uses non-renewable petrochemical feedstock as raw material. It uses hazardous chemicals during the chemical synthesis yielding a racemic mixture of lactic acid whereas, the microbial fermentation route produces enantiomerically pure lactic acid isomers using

abundantly available feedstocks at low cost [7]. The commercial production of chemical synthesis depends on the formation of lactonitrile. In this procedure, initially, hydrogen cyanide is introduced to acetaldehyde in the presence of a catalyst to synthesize lactonitrile. Then the lactonitrile is hydrolysed by the addition of concentrated H_2SO_4 to produce lactic acid and ammonium salt. Additionally, the process involves the esterification of methanol to yield methyl lactate, which is subsequently hydrolysed in the presence of an acid catalyst to produce methanol and lactic acid [8]. Microbial production of DLA using homo-fermentative lactic acid bacteria (HFLAB) is a much more techno-economically viable strategy for DLA production due to their ability to convert sugar solely into DLA effectively by reducing other by-products. Different renewable resources such as lignocellulosic biomass, starchy biomass, etc., were studied for optically pure DLA production in the last two decades [9] [10]. However, its pre-treatment involves the use of costly enzymes. On the other hand, the dairy industry's by-product, whey, is considered a lucrative substrate for DLA production apart from its wide availability.

The Food and Drug Administration (FDA) recognizes lactic acid and its calcium, sodium, and potassium salts as food additives that are Generally Recognized as Safe (GRAS)[11]. DLA bioproduction is being optimized at different levels for lessening overall manufacturing cost and production of highly optically pure isomers. The downstream processes for separation and purification of DLA at high scale for commercialization is the major cause for incrementing the overall cost factor. DLA production has been previously achieved through metabolic engineering strategies, adaptive evolution in host organisms, and the implementation of various bioreactor systems. Furthermore, strategies that involve the co-cultivation of symbiotic microbes have been investigated for their potential use in this context [12]. The utilization of innovative immobilization techniques and compatible support materials within bioreactor systems has the potential to yield substantial economic benefits for production of lactic acid isomers.

Prominent lactic acid and PLA manufacturers include Archer Daniels Midland Company (USA), NatureWorks LLC (USA), Purac (The Netherlands), Galactic S.A. (Belgium), as well as various Chinese companies such as CCA (Changzhou) Biochemical Co. Ltd., Henan Jindan Lactic Acid Co. Ltd., and Musashino Chemical Co. Ltd (China). The cost estimates for food-grade lactic acid fall within the range of 1.38 US$ kg-1 (at 50% purity) to 1.54 US$ kg-1 (at 88% purity). Presently, prominent producers of D-lactic acid (DLA) include Corbion (Netherlands), Musashino Chemical, Galactic, Yancheng Huade Biology (China), and Shandong Shouguang Juneng Golden Corn (China).

1.1.3 Advances in DLA production from renewable sources

Renewable feedstocks used for DLA production till date include lignocellulosic biomass, food waste, sugar and starch materials, macroalgae, microalgae, and glycerol, offering sustainable and cost-effective alternatives through microbial fermentation[13][14][15]. Lignocellulosic biomass, such as bagasse, grass, straw, corn cob, wood waste, molasses, sugar beet pulp, and coconut pulp, requires pretreatment to break down its complex structure into fermentable sugars. Food waste, rich in carbohydrates, can be sourced from households, cafeterias, restaurants, bakeries, and food industries, while sugar and starch materials, including molasses, whey, and agricultural fruit waste, also serve as substrates, needing pretreatment to remove inhibitors. Macroalgae and microalgae, which do not require fertile land or freshwater, offer high carbohydrate content and require thermal acid hydrolysis for fermentation [16][17]. Glycerol, a by-product of biodiesel and bioethanol production, can be converted into DLA using LAB or through chemical reactions with catalysts and enzymes [18]. Recent advancements have focused on utilizing lignocellulosic biomass and agro-industrial waste streams, which not only provide sustainable feedstocks but also reduce production costs. For instance, lignocellulosic biomass, rich in glucose and xylose, has been identified as a valuable resource due to its abundance and low cost. Engineered strains of *Escherichia coli* have been shown to effectively ferment glucose-xylose mixtures, achieving DLA production levels of up to 87.5 g L^{-1} [19]. Studies on agro-industrial wastes, such as coffee mucilage and rice husk hydrolysates, have demonstrated their potential as alternative carbon sources, significantly improving lactic acid yields [20]. A recent study using a three-stage carbon source utilization strategy with *Zizania latifolia* waste and cane molasses achieved lactic acid production levels of 129.47 g L^{-1} after 86 hours of fermentation [21]. Similar strategies can be implied for DLA biosynthesis as well. Overall, the shift towards renewable resources for DLA production reflects a growing commitment to sustainable practices in the chemical industry, addressing environmental concerns while meeting the increasing demand for biodegradable materials.

1.1.4 Advances in DLA production through metabolic engineering strategies

The prime objective of metabolic engineering is to overproduce a certain metabolite having desired applications. Each metabolite has a predetermined pathway for biosynthesis. Enzymes have the most basic function in metabolite synthesis post transcriptionally. Overexpression of genes expressing these enzymes significantly increases the yield of the target metabolite is the most feasible strategy being done frequently in this field using suitable molecular biology tools till date. Since overexpression of target genes may result in additional side effects such as metabolic burden, an imbalance in total energy, and cellular stress, fine tuning of metabolic flux is necessary. Gene knockout and gene silencing done using advanced genome editing tools can selectively cut-off by-

product formation shifting carbon flux towards product of interest (*Fig.1.2*). Through using strong constitutive promoters and inducible promoters the metabolic shunting of pathways can also be controlled through dynamic metabolic engineering strategies via toggle switches.

Engineering strains capable of producing the required product effectively enough to meet the criteria for large-scale bioprocessing is a critical task of metabolic engineers in the establishment of commercial microbial cell factories. The choice of the base strain for alteration is the first key step in the metabolic engineering process. Isolation and evaluation of DLA producing microbes from environment and dairy wastewater samples could be desirable for selecting an efficient wild strain with enhanced DLA production. Since it is unlikely that a single wild-type organism will have a phenotype that satisfies all of the requirements for the production of a variety of products, starting strains should be chosen based on metabolic capacity for the desired product, bioprocess compatibility, metabolic and genetic engineering ease, and the ability to use low-cost feedstocks, among other factors. Computational genome-scale metabolic modelling and simulation can be a valuable resource in selecting an ideal production organism by allowing comparison of metabolic capacities of different species of organisms.

Certain major glycolytic genes along with the key enzymes *d-ldh* and beta-galactosidase (*β-gal*) overexpression can highly increment DLA production in presence of lactose-based substrate in host organism lactic acid bacteria (Table 1.1). Efficient lactose-based substrate utilization through creating galactose utilization pathways is a viable option for enhancing DLA production which has been addressed in this thesis work. Adaptive evolution studies reflect the flexibility of microbes to adapt to certain stress conditions can be applied for inheriting acid resistant and wide range temperature resistance impacting target metabolite biosynthesis. A common challenge encountered during the overproduction of certain natural metabolites is the occurrence of feedback inhibition and transcriptional attenuation control in the production pathway, which is triggered by the accumulation of the desired product. To address this issue, strain development involves the implementation of dynamic metabolic engineering strategies to optimize the production process. Several elegant and effective genetic tools for LAB have been discovered during the last few decades [22]. Previous published literature reports have described several synthetic and natural promoters for efficient gene expression in *Lactococcus lactis* and several other LAB. *Lactococcus lactis and Lactobacillus plantarum* have been the primary choice for genetic and metabolic studies so far due to their significance as starter cultures and probiotics, coupled with the availability of ample genetic tools and high transformation efficiencies. However apart from inducible gene expression systems, specific gene regulatory elements in LAB have been developed and

characterised, including synthetic promoter libraries, inducible promoters (e.g. *PlacA, PlacSynth, and PxylA*) and constitutive promoters for controlled gene expression in several LAB [23].

Over the last few decades, substantial work has been devoted to the development of marker less LAB genome manipulation. More advanced application of LAB genome engineering has been reported, employing single-stranded oligonucleotide-mediated recombineering rather than double-stranded DNA [24]. The demand for a large amount of oligonucleotide, as well as the low recombineering efficiency, are some of the drawbacks of this method. The introduction of Clustered regularly interspaced short palindromic repeats (CRISPR) technology has also expanded the scope for manipulating genomic DNA of several LAB which offers several prospects for enhancing starter culture functional properties, probiotic qualities, and the generation of native or non-native metabolites. Except for overexpression and downregulation of genes, there are very limited published reports on advanced metabolic engineering of LAB for enhanced DLA synthesis due to lack of well-established molecular biology tools.

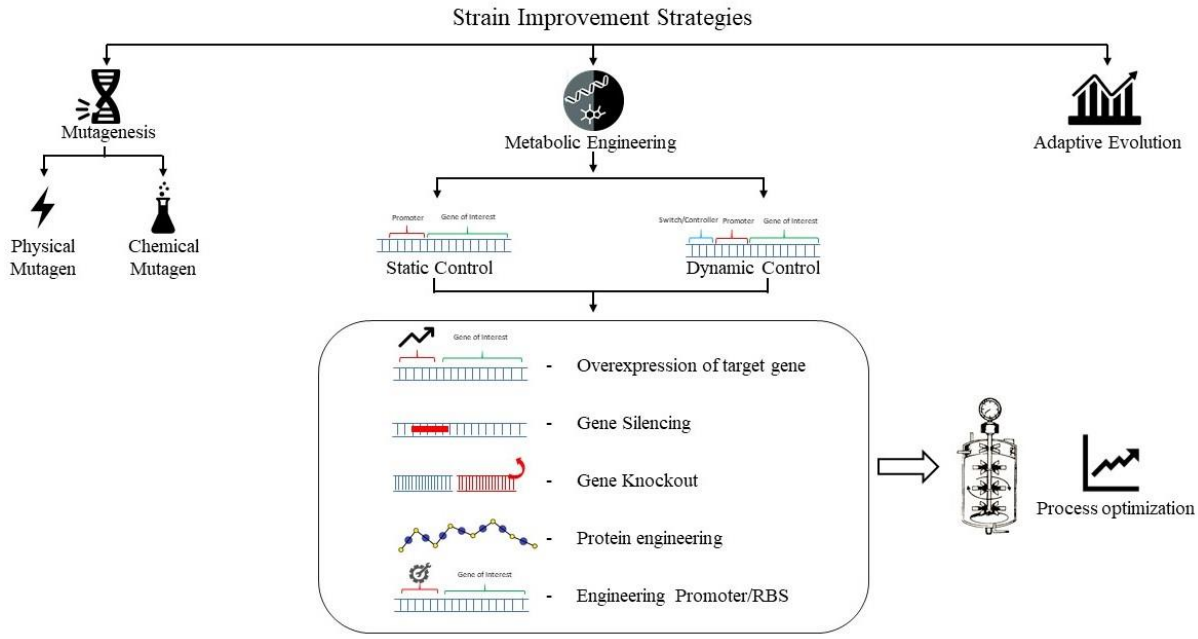


Figure 1.2 Metabolic engineering strategies for strain improvement

Table 1.1: An overview of the literature on metabolically modified strains for increased DLA production.

Host organism	Regulated genes	Media	Yield of DLA (gg^{-1})	DLA titre (gL^{-1})	Reference
***Bacillus subtilis* strain 168**	Overexpressed genes: • D- Lactate dehydrogenase (*ldhA*) • glycerol dehydrogenase (*gldA101*)	LB	0.96	87	[25]
Saccharomyces cerevisiae	Overexpressed genes: • D- Lactate dehydrogenase (*ldhA*) Downregulated gene: • Pyruvate Decarboxylase 1 (*PDC1*)	YPD	0.61	62	[26]
Escherichia coli	Downregulated gene • synthase gene (*msgA*)	Minimal	0.98	118	[27]
Bacillus coagulans QZ19	Downregulated gene • L-lactate dehydrogenase (*ldh)* • Acetolactate synthase (*alsS*)	LB	0.96	99	[28]
Saccharomyces cerevisiae	Overexpressed genes • D- lactate dehydrogenase (*ldhA, LEUM 1756*) Downregulated genes • Pyruvate decarboxylase (*PDC1*) • Monocarboxylate transporter (*JEN1*) • Alcohol dehydrogenase (*ADH1*)	YP	0.8	112	[29]
***Klebsiella oxytoca* KMS004**	Downregulated genes • Alcohol dehydrogenase (*ADH1*) • phospho-transacetylase/acetate kinase A genes (*pta-ackA)*	Mineral salt	0.92	34	[30]
	Downregulated genes • Fumarate reductase (*FrdABCD*) • Pyruvate formate lyase (*PflB*)	Low salt	0.98	133	[31]
Lactobacillus plantarum	Overexpressed genes				

	• Xylose isomerase and Xylokinase (*XylAB*) Downregulated genes • L- lactate dehydrogenase (*L-ldh*)	Corn stover waste	0.53	47.2	[32]
Corynebacterium glutamicum	Overexpressed genes • Glycolytic pathway enzymes • Entner–Doudoroff (ED) pathway enzymes Downregulated genes • Lactate dehydrogenase (*ldh*) • Phosphophenolpyruvate carboxylase (*ppc*)	Rich and minimal	0.95	264	[33]
Saccharomyces cerevisiae	Overexpressed genes • Glycolytic pathway enzymes Downregulated genes • Pyruvate Decarboxylase 1 (*PDC1*) • Alcohol dehydrogenase (*ADH1*)	YPD	0.646	60.3	[34]
Saccharomyces cerevisiae	Overexpressed genes • *Issatchenkia orientalis* glycosylphosphatidylinositol-anchored protein (*IoGas1*) • D- Lactate dehydrogenase (*d-ldh*) Downregulated genes • Pyruvate decarboxylase (*PDC1, PDC6*) • *JEN1* (a monocarboxylate transporter) • L-lactate cytochrome-c oxidoreductase (*CYB2*) • Alcohol dehydrogenase 1(*ADH1*)	YPD	0.7	92.0	[35]
Synechocystis sp. PCC 6803	Overexpressed gene • Novel glycerol dehydrogenase (*gldA101*)	BG-11 rich medium	NA	2.17 (with acetate)	[36]
Saccharomyces cerevisiae	Overexpressed gene • D-lactate dehydrogenase (*ldh*) Downregulated gene • Glycerol pathway genes (*GPD 1, GPD 2*) • Alcohol dehydrogenase 1(*ADH1*)	Yeast minimal media	0.81	40.03	[37]

1.1.5 Bioprocessing strategies and purification

To improve yield and cost-effectiveness in biosynthesis of DLA, integrated bioprocesses have been proposed. The chemical and biological production processes are being encouraged to implement one step integrated operations to replace two stage reactions attributed to the rising

economic and ecological demands. The downstream and purification techniques, and less on the process intensification segment, are main crucial contributors of the overall cost factor [38]. To enhance output and productivity, reduce process time, and minimise operating expenses and capital investment, there is a significant need for intensification and integration of processing steps. Successful process integration requires increased process sturdiness, thorough process knowledge, simple process development tactics, effective troubleshooting, and a better comprehension of the system. DLA has been reported to produce in lab scale via both wild and metabolically engineered strains [39] [40] [40] [41]. Continuous platforms provide several conveniences for small operations in a straightforward and streamlined manner. In terms of cost, flexibility, and productivity, continuous bioprocessing is a more preferable solution for DLA over batch production, especially with process intensification techniques that need the treatment of large volumes of feedstocks throughout the year. Although continuous lactic acid production coupled with process intensification by membrane-based separation [42] [43] has been reported so far, there are relatively few reports available in the area of effective controlling and online monitoring that will effectively address the performance glitches. In order to achieve optimal control and monitoring of continuous DLA production, advanced sensors and actuators are required to measure and regulate key process variables such as pH, temperature, substrate concentration, product concentration, biomass concentration and dissolved oxygen. These key variables affect the growth and metabolism of the microorganisms that could impact the quality and yield of DLA. Deployment of online analytical tools such as near-infrared spectroscopy (NIRS), mass spectrometry (MS) and high-performance liquid chromatography (HPLC) could facilitate real-time monitoring of residual concentration of limiting nutrients and the quality (optical/chemical purity) of DLA. Moreover, these analytical tools also enable the implementation of model-based control strategies that can optimize the performance of the bioprocess by adjusting the operating conditions according to the desired objectives. On operational perspective, online monitoring and control can facilitate rapid detection and correction of faults or disturbances that may occur during the continuous operation, such as contamination, equipment failure, or fluctuations in feed quality or flow rate.

The flexible operation, high productivity, and decreased cost with quality, as well as process integration facilities, are the crucial elements underlying the biotech sectors that are leveraging continuous platforms over batch. The challenges associated with maintaining homogeneity, regulating nutrient intake over extended periods with stringent sterility requirements, and

addressing genetic instability in cells underscore the need for rigorous control procedures, accompanied by robust online monitoring and control strategies.

Lactic acid purification involves a chemical reaction with concentrated sulfuric acid (H_2SO_4), which yields calcium sulfate ($CaSO_4$) or gypsum as a byproduct. This purification step is one of the basic and age-old procedures for separating and purifying lactic acid (*Fig 1.2*) in various industries, where lactic acid is used as a key ingredient in products ranging from food and pharmaceuticals to cosmetics and biotechnology applications. Recent separation and purification technologies like reactive extraction, in-situ adsorption, ion-exchange chromatography, electrodialysis or crystallization when integrated along with the fermentation process can certainly enhance efficiency [44] [45].

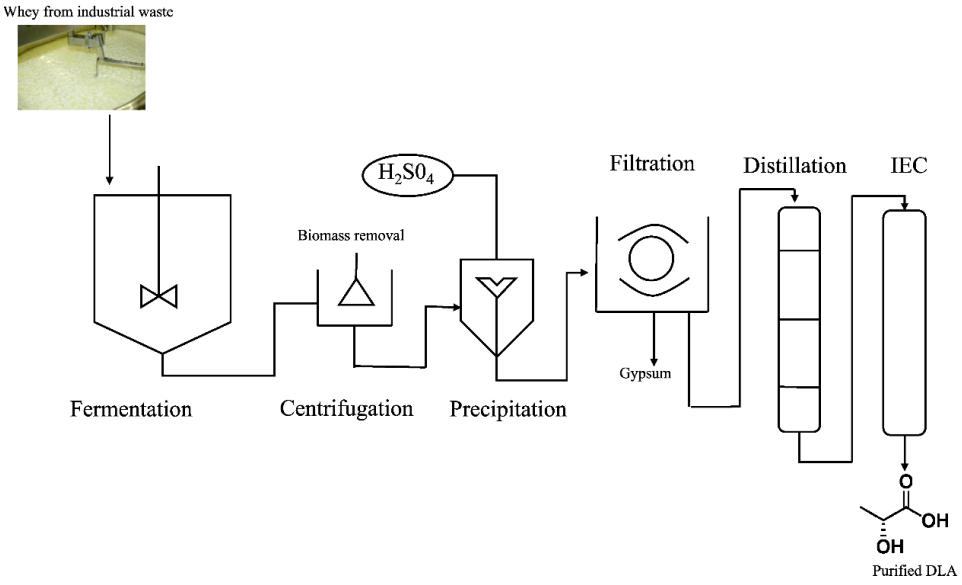


Figure 1.3 An overview of integrated process technology for converting WP (renewable source) into DLA.

Efficient purification methods play a crucial role in lactic acid production, also require ensuring its economic feasibility. The significant disparity in boiling points and lactic acid's strong affinity for water present considerable challenges in achieving proper separation and the production of high-purity lactate. Lactic acid and its isomers in technical grade (22-44 % w/w) are mostly separated through precipitation process with generation of huge amounts of solid waste. For generation of highly optically pure lactic acid, process optimized liquid-liquid extraction, adsorption processes and membrane-based separation are mostly preferred.

Esterification reactions accompanied with reactive distillation processes are prominent currently [46] [47].

Two important parameters for bioproduction of DLA includes optical purity and titre. Optical purity of DLA from several lactic acid bacteria totally depends on the NAD dependent lactate dehydrogenase gene and can be improved through genetic engineering strategies only [48]. While high concentrations of acid can be improved by continuous product removal techniques since acid accumulation hampers biomass. Process optimization through membrane-based separation of DLA can prove to be more effective in reducing overall cost factor. The cross-flow velocity and transmembrane pressure are equally key elements in the separation of acid by membrane integration. These attributes are important in separating efficiency, which has an indirect influence on fermentation dilution rate. All these challenges can be efficiently addressed by implementing good controlling approaches with effective online sensing instruments that forecast feed rates and product concentrations. Process intensification strategies including continuous fermentation coupled with suitable less cost separation and purification technology is what is required for bringing out more potential of DLA.

1.2 Lactic acid bacteria as potential host

LAB comprise a group of gram-positive bacteria that are non-motile and microaerophilic. This category encompasses various genera *(Enterococcus, Lactobacillus, Pediococcus, Leuconostoc, Oenococcus, Lactococcus, Streptococcus, Weissella,* etc., within the order *Lactobacillales*) associated to the phylum Firmicutes, and anaerobic Bifidobacterium genus under the phylum Actinobacteria and have low G-C content. LAB have been involved with food fermentation and preservation from time immemorial, and they are now the most significant category of emerging industrial microorganisms being used as starter cultures in food sector. The confirmed GRAS status of most LAB, their ability to withstand various stress conditions, their uncomplicated metabolism, and their capability to metabolize a wide range of carbon sources are characteristics that significantly contribute to their widespread application in various industrial settings [49]. LAB genomes exhibit a notable phenomenon known as reductive evolution, characterized by the loss of numerous metabolic genes, biosynthetic constraints, and the presence of several pseudogenes in comparison to other organisms [50]. In the food sector, there has been an ongoing effort to enhance LAB probiotics through strain improvement, which aims to enhance product qualities, including texture, taste, reduced additives, calorie content, acid compositions modulation, and the elimination of undesirable traits like antibiotic resistance [51]. In a similar way, LAB including major probiotics could be

exploited as an efficient cell factory to make bulk and fine bio-chemicals including pyruvate-dissipating end products, exopolysaccharides, bacteriocins, vitamins, low-calorie carbohydrates, complex flavour compounds, and polylactic acid or polylactide. Presently, *Lactobacillus* strains have found widespread industrial applications with successful market integration across various sectors. In the food and beverage industry, products like Yakult, containing *Lactobacillus casei*, have achieved remarkable commercial success, with daily sales of over 40,000 bottles in Japan, while companies like Amul in India have introduced probiotic ice creams to meet growing consumer demand for functional foods [52]. In the pharmaceutical sector, the Indian probiotic drug market has expanded rapidly, with products like Bifilac, Darolac, and Sporlac achieving significant sales, reflecting the increasing demand for probiotics to treat antibiotic-associated diarrhoea and gut health issues. Additionally, *Lactobacillus* strains are extensively used as starter cultures in fermented meat products, such as dry sausages, enhancing food safety and quality by inhibiting spoilage microorganisms. Moreover, *Lactobacillus* plays a crucial role in industrial lactic acid production, a key raw material for biodegradable plastics and other products, with the global lactic acid market projected to reach 2.8 million tons by 2030 [53]. These examples demonstrate how *Lactobacillus* has achieved remarkable industrial success, highlighting its potential for future biotechnological application

LAB can be broadly categorized into two types: Homofermenters and heterofermenters, which is determined by the type of end product they produce. The homofermenters produce lactic acid as the major product generated from pyruvate while heterofermenters produce other by-products (acetate/ ethanol/ formate etc.,) along with lactic acid. Homofermenter helps in directing the carbon flux from the carbon substrate only towards lactic acid production and can be efficiently used for DLA fermentation. These homofermentative organism includes *Lactobacillus acidophilus*, *Lactobacillus amylophilus, Lactobacillus bulgaricus*, *Lactobacillus helveticus* and *Lactobacillus salivarius* while heterofermentative organisms include *Lactobacillus fermentum, Lactobacillus xylosus, Lactobacillus plantarum, Lactobacillus casei and Lactococcus lactis*. DLA has been reported to be produced by several species of LAB, in particular *Lactobacillus delbreuckii* [54] [55] [56], *Lactobacillus coryniformis* subsp. *torquens* [57] [54] *Leuconostocmesenteroides* subsp. *mesenteroides, Leuconostocmesenteroides* subsp. *dextranicum, Leuconostoccarnosum, Leuconostocfallax , Lactobacillus bulgaricus* [58], as well as genetically modified *Lactobacillus plantarum* [59] [60]. Furthermore, it's worth noting

that certain LAB strains have the remarkable ability to exclusively produce optically pure DLA (*Fig 1.3*).

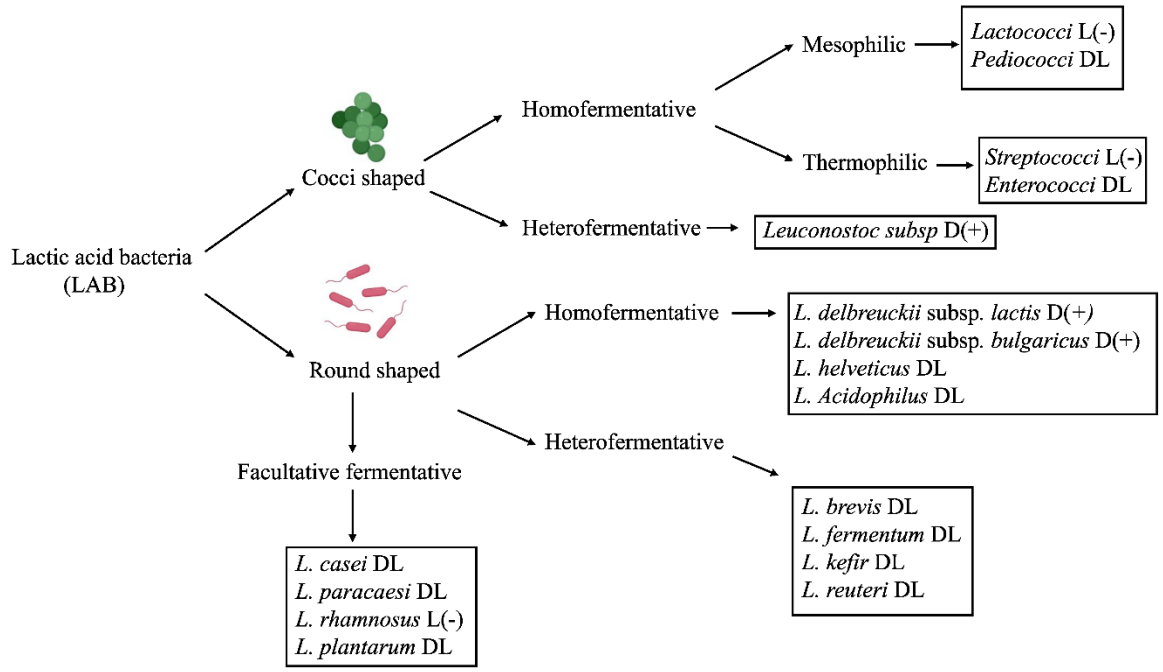


Figure 1.4 Classification of LAB based on their end-product

The increasing global demand for PLA has resulted in a rapid surge in the demand for lactic acid. LAB produces DLA naturally through their cellular metabolism from carbon sources, which accounts for certain enzymes comprising glycolytic pathway and the key enzyme D-lactate dehydrogenase (*d-ldh*) accomplishing the purpose. The number of competent pathways for by-product formation is reduced for homo-lactic acid bacteria, for which they are mostly preferred when compared to other species of bacteria like *Escherichia coli*, *Corynebacterium glutamicum*, *Saccharomyces cerevisiae*, *Bacillus* species, etc. Genetically modified organisms for future manufacturing and commercialization of DLA needs to fulfil specific requirements, including its safety, stability, and efficacy. Although certain wild HFLAB can produce only DLA isomer but for its application in industries, productivity and yield required be improved further through genome modification and process optimization strategies.

Since LAB are considered GRAS organisms, their use for producing industrially important compounds and therapeutics can be justified well. Although the FDA does not permit the easy marketing of genetically modified organisms, LAB can be considered because of its safe category and basic metabolic alteration. Aside from that, LAB has quite a limited biosynthetic

capacity, metabolic versatility, and simple physiology which are essential characteristics in host organisms makes it suitable for metabolic engineering [61]. Furthermore, within LAB, energy metabolism and the process of biosynthesis are relatively distinct processes, allowing sugar catabolism to be manipulated without affecting biosynthesis and vice versa. The efficiency of metabolic engineering in these bacteria has been aided by a thorough understanding of their physiology and genetics and there is no concern on multiplicity of genes.

Emerging molecular cloning tools for engineering LAB and presence of strong constitutive promoters makes its more suitable host for efficient strain development through metabolic engineering strategies [62] [63] [22] [64]. The total genomic sequences of 25 commonly used LAB are already annotated and published in database of National Centre for Biotechnology Information (NCBI) which makes them more suitable for manipulations. Genomics and functional genomics study provide us an opportunity to even get a broad understanding of LAB's physiological and metabolic capacities.

1.2.1 Metabolic pathways for DLA production in LAB

In LAB, the metabolism of hexoses primarily occurs through two major pathways: the glycolytic pathway (Embden-Meyerhof pathway) and the phosphoketolase pathway [65][66]. Lactic acid is the major metabolite in homofermentative metabolism through glycolytic pathway while acetic acid, propionic acid, CO_2, ethanol, and other by-products are formed through heterofermentative metabolism in phosphoketolase pathway. Two moles of lactic acid per mole of glucose is formed from homolactic fermentation theoretically but experiment yields are lower due to biomass formation. LAB produces lactic acid in two forms: L- and D-isomer. While DLA is harmful to human health, LLA is preferred for use in food and pharmaceuticals, as well as a starting material in the production of biopolymers. DLA is synthesized mainly through glycolysis, which commences with glucose and finishes with pyruvate. D-*ldh*, a key enzyme found in LAB which owns stereospecific NAD^+-dependent activity, converts this pyruvate to optically pure DLA in a reversible reaction. Pyruvate can also be converted to a variety of different products via other processes, depending on the growth circumstances and characteristics of the organism. Numerous research teams have directed their efforts toward enhancing the production of optically pure lactic acid using different LAB strains. This is achieved through the disruption or deletion of the lactate dehydrogenase gene (*l-ldh/d-ldh*), which encodes the enzyme responsible for the formation of the undesired isomer. [67][68][69][70].

Apart from *d-ldh*, phosphofructokinase enzyme (*pfk*) was discovered to play a key role in the EM route flow, with decreased or increased activity resulting in proportionately lower or higher flux and lactate production [71]. Under situations of carbon limitation, poor growth rates, and changes in oxygen content, homofermentative bacteria have been shown to transition to mixed acid fermentation *(Fig.1.4)*. Also, in presence of galactose utilization pathways, homolactic acid bacteria shifts its metabolic flux towards production of acetic acid. Increased NADH oxidase (*Nox*) activity in presence of oxygen causes competition for available NADH and, as a result, a switch in metabolism to generate a mixture of products [72].

Pyruvate kinase (*pyk*) is a pivotal enzyme within the glycolytic pathway, playing a crucial role in cellular metabolism by generating ATP and pyruvate from phosphoenolpyruvate. This enzyme has acid tolerance property in few of the LAB which provides low pH resistance along with shifting carbon metabolic flux towards DLA production [73]. Another enzyme that has been found to produce high yield homofermentative lactic acid is fructose-1,6-biphosphate aldolase (*fba*). Dual expression of phosphofructokinase (*pfk*) and aldolase (*ald*) in presence of strong constitutive promoters and carbon substrate might increase D-lactic acid yield as per reported study [71].

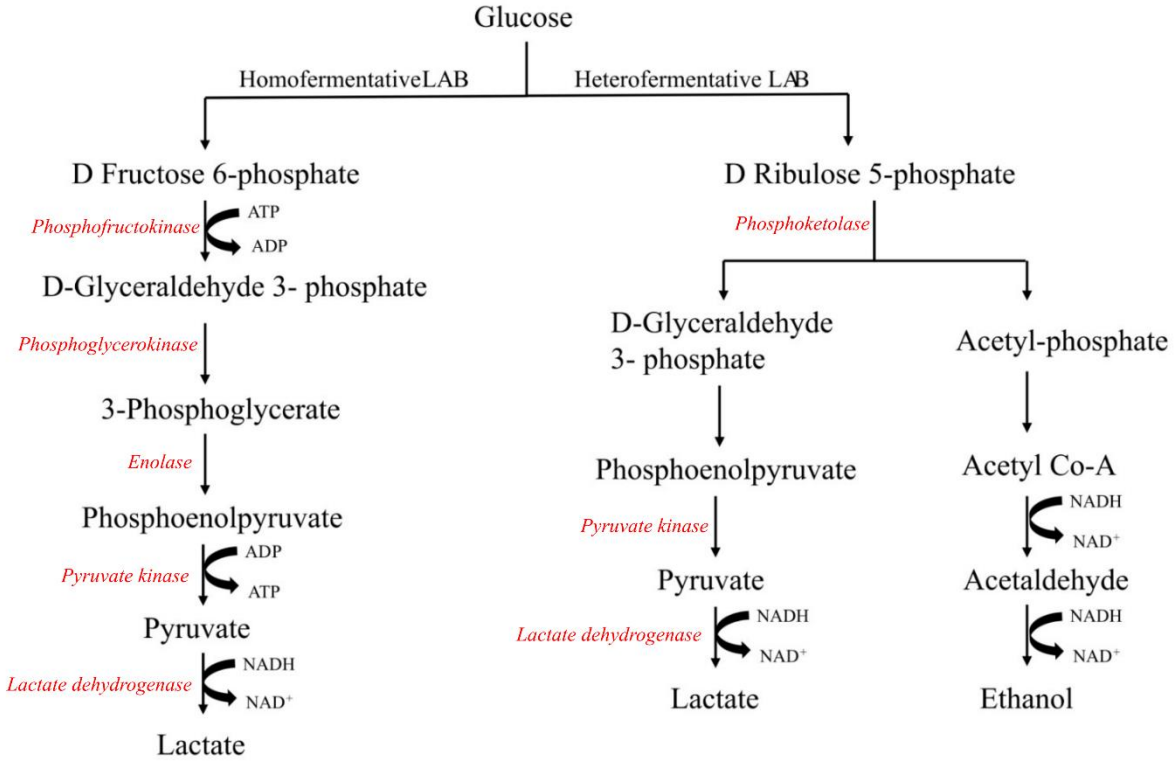


Figure 1.5 Brief overview of metabolic pathways for lactate production in homo and hetero-fermentative lactic acid bacteria

1.2.2 Metabolic engineering strategies for increased DLA titre and productivity in LAB

Traditional and conventional methods for enhancing strains to produce D-lactic acid (DLA) have primarily focused on fermentation-based optimization, selective adaptation, mutagenesis, and process engineering. Fermentation remains the core strategy, with *Lactobacillus* species such as *Lactobacillus delbrueckii* and *Lactobacillus bulgaricus* widely utilized in batch, fed-batch, and continuous fermentation systems to improve DLA yield and productivity [74]. Selective adaptation has been employed to enhance strain tolerance to high DLA concentrations and improve substrate utilization [75]. Random mutagenesis using UV irradiation or chemical agents like ethyl methanesulfonate has led to high-yielding mutant strains with improved acid tolerance and reduced by-product formation [76]. Process optimization, including the use of low-cost feedstocks such as cassava starch hydrolysate and lignocellulosic hydrolysates, has further improved economic feasibility [5]. Additionally, immobilized cell fermentation using alginate beads or polymer matrices has enhanced lactic acid production by maintaining high cell densities and enabling continuous fermentation [77]. These traditional approaches have significantly contributed to industrial-scale DLA production, paving the way for further advancements through metabolic engineering and synthetic biology.

Enhancing the production of DLA in LAB requires targeted metabolic engineering strategies that focus on key metabolic pathways and enzymatic activities. Since, DLA production pathways are almost similar in different LAB strains, a generalized central approach involves increasing the activity of *dldh*, the key enzyme catalyzing the conversion of pyruvate to DLA similar to previous reports [78]. Strategies such as gene overexpression of the *dldh* gene, utilizing strong promoters and optimized ribosome binding sites compatible with the specific LAB strain, can significantly boost DLA production (Table 1.2) [79]. Additionally, enzyme engineering techniques, including directed evolution and rational design, can generate *dldh* variants with improved catalytic efficiency [80]. Furthermore, enhancing NADH regeneration through the overexpression of associated enzymes can support higher DLA yields by ensuring adequate cofactor availability during the metabolic process [78], since *dldh* the key enzyme for DLA biosynthesis is dependent on this cofactor.

To maximize DLA production, it is also crucial to optimize the supply of key precursors, particularly pyruvate. Metabolic pathway engineering can facilitate this by modifying glycolytic flux or reducing competing pathways through targeted overexpression or gene

knockouts, thereby directing more carbon toward DLA synthesis similar to previous reports [81]. The choice and concentration of carbon sources also play a significant role as well; optimizing these factors can lead to enhanced pyruvate availability and increased DLA titres [2]. A thorough investigation of different carbon sources and their impact on LAB metabolism can yield insights into optimal growth conditions for DLA production. Reducing byproduct formation is another essential strategy in metabolic engineering for DLA production. Gene knockouts targeting enzymes involved in competing pathways can redirect metabolic flux toward DLA synthesis, minimizing unwanted byproducts. Employing metabolic flux analysis can provide a detailed understanding of the cellular metabolism and identify critical bottlenecks, allowing for a rational approach to optimizing the metabolic network. These insights can lead to the development of engineered strains that maintain high DLA production rates while minimizing the accumulation of undesired metabolites.

Finally, enhancing cell growth and viability is critical for achieving high DLA titres. Optimizing the culture medium composition by adding essential nutrients and growth factors can significantly improve both cell density and DLA yield. Additionally, fine-tuning fermentation parameters such as temperature, pH, and dissolved oxygen levels can create optimal growth conditions for LAB. The integration of systems biology approaches, utilizing omics data to understand the broader metabolic network, can further refine these strategies, leading to more effective metabolic engineering frameworks. By leveraging these multifaceted strategies, researchers can significantly enhance the productivity and titre of DLA in LAB, paving the way for more efficient bioproduction processes. Limited literature exists on the metabolic engineering of LAB for DLA production till date. Among these, one notable study reported the engineering of *Lactococcus lactis* for enhanced galactose and glucose utilization, combined with the overexpression of key *dldh* genes, resulting in improved DLA production [79]. However, there are very few studies where other Lactobacillus species have been engineered for lactic acid biosynthesis. One significant study focused on the engineering of *Lactobacillus helveticus* CNRZ32 for the production of pure LLA. Researchers constructed two *ldhD*-negative strains through gene replacement, resulting in strains GRL86 and GRL89. Among these, GRL89 exhibited a 93% increase in *l-ldh* activity compared to the wild-type strain, demonstrating enhanced LLA production [82].

Table 1.2: A comparative analysis of different strains of LAB and various metabolic engineering approaches for enhanced production of target metabolite

Strain	Product of interest	Technique	Cloning tools	Titre	References
P. acidilactici DQ2	L-/DLA	*ldh* gene disruption	pSET4E plasmid, a temperature-sensitive knockout vector modified to include an erythromycin resistance gene (*Em*^{r}).	76 g /L	[83]
***Lactobacillus plantarum* NCIMB 8826 (ΔldhL1)**	DLA	*ldhL1* gene disruption and expression of secreted endoglucanase (CelA)	pCU plasmid, an expression vector with erythromycin resistance gene (*Em*^{r})	1.47 g/L	[84]
***Lactobacillus fermentum* GRL1032**	Mannitol and pyruvate	Additional disruption of *ldhL* gene in the *ldhD*-deficient mutant using targeted gene replacement	Integration vector pKTH5097 with erythromycin resistance for targeted gene disruption	Reduced mannitol productivity;	[85]
***Lactobacillus plantarum* NCIMB 8826**	DLA	Disruption of *ldhL1* gene replaced with an α-amylase-secreting expression cassette to enable direct starch utilization	Modified *ldhL1* disruption cassette integrated into the genomepG+host9 plasmid (temperature-sensitive vector for homologous recombination)	73.2 g/L	[86]
***Lactobacillus paracasei* NCBIO01-M2**	LLA	Knockout of *ldhD* gene and overexpression of *ldhL1* gene using CRISPR-Cas9 gene editing	pNcas-ΔldhD-ldhL1 plasmid (containing sgRNA for *ldhD*, homology arms, and *Amp*^{r} resistance gene)	221 g/L	[87]
Lactococcus lactis NZ9000	DLA	Replacement of the endogenous *l*-lactate dehydrogenase (*l*-Ldh) gene with a heterologous *d*-	pAH4 plasmid for homologous recombination (replacement of *l*-Ldh with *d*-Ldh);	15.0 g/L	[88]

		lactate dehydrogenase (*d*-Ldh) gene from *Lactobacillus delbrueckii* subsp. *lactis* JCM 1107 for AH1; additional introduction of an α-amylase gene from *Streptococcus bovis* NRIC 1535 for AH2 to enable starch utilization.	pNZ8048 plasmid for α-amylase gene expression		
Lactobacillus saerimneri TBRC 5746	DLA	CRISPR/dCas9-assisted transcriptional repression of two *Lldh* genes to enhance optical purity	Modified CRISPR/dCas9 plasmid derived from pHSP02, containing crRNA for *Lldh* genes and chloramphenicol resistance marker	9.4 g/L	[89]
Lactobacillus plantarum NCIMB 8826 ΔldhL1	DLA	Disruption of *ldhL1* gene; introduction of xylose-assimilating genes (*xylAB* encoding xylose isomerase and xylulokinase) to enable utilization of pentose sugars for fermentation	pLEM415 vector with the constitutive clpC promoter for gene expression; erythromycin resistance marker (Em^r)	61.4 g/L	[90]

Similarly, there are few more literature reports where lactobacillus species has been engineered for enhanced LLA biosynthesis. The strategies used for engineering *Lactobacillus plantarum* include deleting the gene encoding *d-ldh* (*ldhD*) and disrupting the operon encoding lactate racemase (*larA*-E)[91]. Similarly, the strategies used for engineering *Lactobacillus paracasei* include establishing a CRISPR-Cas9 gene editing platform and applying adaptive evolution to obtain a high-performance strain capable of efficiently producing LLA at high temperatures [92]. Despite these advancements in LLA production, the number of engineered Lactobacillus strains for DLA production remains extremely limited. Among the available studies, *Lactococcus lactis* and *Lactobacillus plantarum* are the most extensively engineered strains for metabolic pathways aimed at DLA production as mentioned before [93]. Apart from this, one recent study also focussed on increasing DLA in *Lactobacillus saerimneri* TBRC 5746 through

CRISPR/dCas9-assisted transcriptional repression of the two *l-ldh* genes responsible for LLA production which resulted in a 38% increase in DLA production and improved the optical purity[94]. Overall, this highlights the significant gap in research and the potential for further exploration of other *Lactobacillus* species for this purpose.

1.2.3 Synthetic biology tools available for engineering LAB

The development of synthetic biology tools for LAB has significantly advanced the field of microbial engineering. These tools include a variety of genetic and genomic techniques that enable precise manipulation of LAB for various applications.

1.2.3.1 Expression Systems

A cornerstone of synthetic biology is the forward engineering of cellular behavior using well-defined parts libraries. *Lactococcus lactis* (*L. lactis*) has emerged as a model and platform LAB for synthetic biology due to its importance in the fermented foods industries. Early gene cloning vectors constructed using plasmids isolated from *L. lactis* strains, such as pWV01 and pSH71, are still the backbone for more advanced genetic tools for gene expression, inducible systems, chromosomal integration, and recombineering [95]. Constitutive promoters and terminators of varying strength have been isolated from *L. lactis*, providing sufficient gene expression for initial characterization studies. Inducible promoters, such as the nisin-controlled expression (NICE) system, offer conditional expression and are widely used in synthetic biological applications. Other inducible systems include the PZn-zitR expression system and the agmatine-controlled expression (ACE) system, which provide tight co-expression and dose-responsive alternatives to the NICE system [96]. However, in contrast, only a limited number of well-established molecular cloning tools are currently available for *Lactobacillus* species.

One recent publication discusses the development of synthetic LactoSpanks promoters for *Lactobacillus gasseri*, a model commensal bacterium from the human gut [97]. These inducible promoters, controlled by the *LacI* repressor from *E. coli* and induced by IPTG, offer a range of expression levels with reduced leakage. This advancement supports various applications in *L. gasseri* and potentially other LAB and Gram-positive bacteria, enhancing the precision and efficiency of gene expression control in synthetic biology. Promoters of elongation factor Tu, such as P_{tuf33} from *Lactobacillus plantarum*, P_{tuf34} from *Lactobacillus buchneri*, and P_{tufR} from *Lactobacillus reuteri*, are commonly derived from housekeeping genes essential for fundamental cellular functions are some of the strong constitutive promoters used in lactobacillus [98]. However, natural promoters often lack the precise control needed for optimal

gene expression. To address this limitation, engineered promoters have been developed to fine-tune gene regulation. For example, a synthetic promoter library was created for *Lactobacillus* strains by randomizing the non-consensus spacer sequences of rRNA constitutive promoters, enabling enhanced versatility in controlling gene expression[99].

1.2.3.2 Genome Editing Tools

Chromosomal integration has long been achieved in *L. lactis* for both knock-out and knock-in functionalities. Recent advancements include the use of single-stranded DNA (ssDNA) recombineering combined with CRISPR-Cas9 technology, which has vastly improved mutational efficiency [100]. The pathway engineering vehicle for lactic acid bacteria (PEVLAB) system leverages species-specific plasmid copy control to accommodate large DNA manipulation and chromosomal integration through homologous recombination.

1.2.3.3 Synthetic Biological Circuits

Synthetic gene circuits offer tunable autonomous decision-making for successful delivery and production of biotherapeutics. These circuits can provide added protection through containment mechanisms, such as quorum-sensing molecule-induced autolysis and spatial patterning using dual inducing and antimicrobial activities of nisin. Various 'kill-switches' developed for *E. coli* may also be adaptable to LAB [101]. Overall, the development of these synthetic biology tools has provided a strong foundation for engineering LAB for therapeutic, preventative, and diagnostic applications, paving the way for the next generation of living therapeutics.

1.2.3.4 Application of Genome-Scale Metabolic Models (GEMs) and Constraint-Based Reconstruction and Analysis (COBRA)

Genome-scale metabolic models (GEMs) and Constraint-Based Reconstruction and Analysis (COBRA) have become invaluable tools in understanding and optimizing the metabolic capabilities of LAB. These models integrate genomic, biochemical, and physiological data to simulate and predict the metabolic behavior of LAB under various conditions. GEMs provide a comprehensive representation of the metabolic network of LAB, including all known metabolic reactions and associated genes. By using COBRA, researchers can apply constraints based on experimental data, such as nutrient availability and environmental conditions, to predict the fluxes of metabolites through the network. This approach allows for the

identification of key metabolic pathways and bottlenecks, enabling targeted genetic modifications to enhance desired traits.

One significant application of GEMs and COBRA is in predicting the nutrient requirements of LAB. By simulating growth under different nutrient conditions, researchers can determine the minimal set of nutrients required for optimal growth and productivity[102]. This information is crucial for designing cost-effective fermentation media and improving the efficiency of industrial fermentation processes. Additionally, GEMs and COBRA can be used to predict the metabolic patterns of LAB under stress conditions, such as high temperature, low pH, or nutrient limitation [103]. These predictions help in understanding how LAB adapt to adverse environments and can guide the development of more robust strains with enhanced stress tolerance. Overall, the application of GEMs and COBRA in LAB research has provided deep insights into the metabolic capabilities and requirements of these bacteria, paving the way for the development of improved strains for various industrial applications.

1.2.4 Challenges in engineering LAB

While the engineering of LAB through synthetic biology tools has shown great promise, several challenges remain that hinder the full realization of their potential. Understanding and addressing these challenges is crucial for advancing the application of engineered LAB in various fields, including food production, probiotics, and therapeutic uses.

1.2.4.1 Genetic stability and expression control

One major challenge in the engineering of LAB is maintaining genetic stability over time. LAB often exist in complex environments, which can impose selective pressures that may lead to the loss of engineered traits. Additionally, the expression of heterologous genes may be inconsistent due to variations in promoter efficiency and the competition for cellular resources. This instability can undermine the reliability of LAB as production organisms for industrial applications.

1.2.4.2 Limited genetic tools and resources

Despite recent advancements, the availability of genetic tools specific to LAB is still limited compared to other microorganisms, such as *Escherichia coli* or *Saccharomyces cerevisiae*. The lack of efficient methods for gene editing and transformation in LAB strains poses significant hurdles for researchers. Many LAB species exhibit natural competence, but the efficiency of

transformation can be low, necessitating the development of more robust genetic systems to facilitate the introduction and expression of desired traits.

1.2.4.3 Metabolic burden and pathway engineering

Another challenge faced in LAB engineering is the metabolic burden associated with introducing new pathways. The incorporation of heterologous genes can divert resources away from essential metabolic processes, leading to decreased growth rates and product yields. Optimizing metabolic pathways to balance the production of desired compounds while maintaining cellular fitness is a complex task that often requires iterative rounds of engineering and selection. Moreover, understanding the intricate metabolic networks within LAB is still an evolving area of research.

1.2.4.4 Regulatory and safety concerns

The use of genetically modified organisms (GMOs), including engineered LAB, raises regulatory and safety concerns, particularly in food applications. Regulatory frameworks for the use of GMOs vary by region, and navigating these regulations can be a significant barrier to the development and commercialization of engineered LAB. Furthermore, the safety of these organisms must be thoroughly evaluated to ensure they do not pose risks to human health or the environment. This necessitates comprehensive risk assessments and long-term studies to establish the safety profiles of engineered LAB strains.

1.2.4.5 Consumer acceptance and market readiness

Consumer acceptance of engineered LAB is another critical challenge. While there is a growing interest in probiotics and functional foods, the idea of consuming genetically modified organisms can be met with skepticism. Educating consumers about the benefits and safety of engineered LAB is essential for fostering acceptance. Additionally, the market readiness for such products requires collaboration between scientists, industry stakeholders, and regulatory bodies to develop clear guidelines and standards for the use of engineered LAB in food and health applications.

1.3. *Lactobacillus delbrueckii* subsp. *bulgaricus*: A potential host

Lactobacillus delbrueckii subsp. *bulgaricus* (*L. bulgaricus*) is a prominent species of lactic acid bacteria (LAB) widely recognized for its essential role in the production of yogurt and other fermented dairy products. This bacterium is notable for its probiotic properties, which contribute to various health benefits, making it a significant subject of study in both food

science and microbiology. *L. bulgaricus* is a rod-shaped, gram-positive bacterium that is homofermentative, meaning it primarily ferments sugars to produce lactic acid. This fermentation process is crucial in yogurt production, where it helps to lower the pH of milk, leading to coagulation and the development of yogurt's characteristic texture and flavour. Research has demonstrated that *L. bulgaricus* exhibits several probiotic characteristics. It can enhance gut health by promoting the growth of beneficial gut microbiota and inhibiting pathogenic bacteria. Studies indicate that consumption of yogurt containing this bacterium can alleviate symptoms of lactose intolerance and improve overall digestive health.

Recent research on *L. bulgaricus* has made significant strides in understanding its biological functions, health benefits, and applications in food technology. Notably, a study published explored the oleate hydratase enzyme in this bacterium, which is involved in synthesizing conjugated linoleic acid (CLA), a compound linked to various health benefits, including anti-cancer properties[104]. Additionally, research conducted demonstrated that *L. delbrueckii* subsp. *bulgaricus* could alleviate acute mountain sickness in hypoxic mice by mitigating tissue damage and oxidative stress[105]. Another literature published focused on the interactions between *L. bulgaricus* and *Streptococcus thermophilus* during milk fermentation, revealing how these interactions enhance the texture and flavor of fermented products through metabolomic analyses[106]. Despite these advancements, several research gaps remain regarding *L. bulgaricus*. There is limited exploration into its adaptation to non-dairy substrates, which could facilitate the development of alternative fermented products. Additionally, while some studies have highlighted its probiotic effects, a deeper understanding of the mechanisms by which it interacts with the human microbiome and immune system is needed. Longitudinal studies examining the long-term health effects of consuming products containing this bacterium are also lacking, particularly concerning chronic conditions like functional dyspepsia and metabolic syndrome. Furthermore, more extensive genomic studies are required to uncover genetic variations across different strains that confer specific health benefits or fermentation traits.

Apart from health-related applications, this species holds immense potential for the biosynthesis of industrially significant metabolites, including optically pure DLA. Literature reports are unavailable on metabolic engineering of *L. bulgaricus*, and only a few investigations have focused on developing molecular cloning tools for its genetic manipulation. Extensive research is required to investigate the native restriction endonucleases in this organism to enable modifications aimed at improving its transformation efficiency. Addressing these gaps

could enhance our understanding of *Lactobacillus delbrueckii* subsp. *bulgaricus* and lead to innovative probiotic products and therapeutic strategies and extend its application beyond this.

1.3.1 Currently available strains for research and applications: Advancements in engineering

Over the years, various strains of *L. bulgaricus* have been isolated, characterized, and made available for research purposes and applications. *Lactobacillus bulgaricus* ATCC 11842, one of the most extensively utilized and well-characterized strains, has been pivotal in various scientific studies [2]. Although a variety of *Lactobacillus bulgaricus* strains are available in different culture banks, only a select few are suitable for genetic engineering purposes. ATCC 11842 is one of the publicly available strains, known to exhibit low to moderate electroporation efficiency when using pLEM series vectors [107].

Among commercially significant strains apart from ATCC 11842, *Lactobacillus bulgaricus* 2038 is widely applied in yogurt fermentation processes due to its favorable fermentative characteristics along with other applications [108] [109]. Additionally, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 is primarily used in the dairy industry as a starter culture for yogurt production. This strain is capable of synthesizing exopolysaccharides (EPS), which are important for the texture and viscosity of fermented dairy products like yogurt[110]. Another recent published research includes wild strains including IDCC 3601 has demonstrated antibacterial activity against carbapenem-resistant *Enterobacteriaceae*, making it a beneficial probiotic for enhancing gut health [111]. *L. bulgaricus* strains L9-7 and L4-32-12 were used in another literature report to investigate the role of membrane lipid composition differences on spray drying survival [112]. Very few strains have been genetically modified including the *L. bulgaricus sp1.1*, which has been specifically employed in spray-drying research, highlighting its robustness and industrial relevance [113]. Similarly, the *L. bulgaricus ljj* wild type, originally isolated from yogurt, has been genetically engineered for knockout experiments, showcasing its utility in advanced genetic and metabolic studies [114]. Another literature report investigated the role of key *eps* genes in exopolysaccharide synthesis and their immunomodulatory effects in *L. bulgaricus* OLL1073R-1 where *eps* genes in Cluster I were deleted via conjugal transformation to understand their role in EPS synthesis [115]. Collectively, all these strains, including the publicly available *Lactobacillus bulgaricus* ATCC 11842, represent invaluable resources for advancing research in microbiology, biotechnology, and health sciences.

Recent advances in the engineering of *L. bulgaricus* have also focused on enhancing its functional properties and applications in food technology. Researchers have developed genetic tools and methodologies that allow for the precise modification of this bacterium, improving its metabolic pathways and fermentation characteristics. For instance, studies have explored the effects of gene deficiencies, such as the *ccpA* gene, under various conditions to understand their impact on protein expression and metabolism [116]. Additionally, the introduction of food-grade selection markers, like the nisin resistance gene, has paved the way for safer genetic modifications without compromising food safety standards. However, significant limitations remain, including the need for comprehensive studies on the long-term stability of engineered strains in commercial production settings and their interactions with other microbial species during fermentation. Most importantly, research is still needed to explore the potential of *L. bulgaricus* in non-dairy applications and its ability to produce novel metabolites that could enhance health benefits or sensory qualities in fermented foods.

1.3.2 Challenges associated and molecular cloning tool availability

Engineering *Lactobacillus bulgaricus* presents several challenges due to its unique physiological and genetic characteristics. One of the primary difficulties is the lack of efficient transformation methods [117]. Traditional methods like protoplast transformation and electroporation have shown limited success, with low reproducibility and efficiency. Additionally, *L. bulgaricus* strains harbour very few plasmids, and the replication mechanisms of these plasmids are not well understood. This makes it challenging to introduce and maintain foreign DNA in the bacterial cells. Another significant limitation is the strain dependency of transformation efficiency. Different strains of *L. bulgaricus* exhibit varying abilities to take up and express foreign DNA, which complicates the development of a universal transformation protocol. Moreover, the presence of restriction-modification systems in *L. bulgaricus* can inhibit the transformation with foreign DNA, further reducing the efficiency of genetic modifications[117].

To overcome these challenges, several strategies can be employed. Optimizing the electroporation conditions, such as the growth stage of the cells, the composition of the wash and electroporation buffers, and the parameters of the electrical pulse, can significantly improve transformation efficiency [118]. Using endogenous plasmids with native selectable markers can also enhance the success rate of genetic modifications. Additionally, developing and utilizing integrative vectors that can stably integrate into the *L. bulgaricus* chromosome can provide a reliable method for introducing foreign genes. Overall, while engineering *L.*

bulgaricus is challenging, advancements in transformation techniques and the development of new genetic tools hold promise for overcoming these limitations and enabling more efficient genetic modifications.

1.4 Dynamic metabolic engineering for enhanced biosynthesis of target metabolites

Dynamic metabolic engineering has emerged as a powerful strategy to optimize bioproduction by dynamically regulating cellular processes and minimizing metabolic burdens. This approach involves real-time modulation of metabolic pathways to achieve precise control of gene expression and metabolic fluxes in response to environmental and cellular changes [119]. One key aspect is the development of genetic circuits that sense and respond to intracellular metabolite levels. These circuits, based on transcription factors, riboswitches, or other regulatory elements, enable fine-tuning of metabolic pathways to balance cell growth with product synthesis. Feedback loops play a crucial role in dynamically adjusting pathway gene expression, thereby ensuring efficient resource allocation between growth and production phases.

Advanced strategies also focus on engineering dynamic control systems that regulate enzyme activity and cofactor availability. By designing cells to produce or regenerate essential cofactors, such as NADH and ATP, in response to metabolic demands, researchers can enhance biosynthetic pathway efficiency and improve overall yield. Additionally, optogenetic tools provide temporal control of gene expression by synchronizing metabolic activities with external stimuli like light.

Computational synthetic biology tools such as GEMs and COBRA frameworks as mentioned previously further enhance dynamic metabolic engineering as well. These models predict and simulate metabolic fluxes, identify pathway bottlenecks, and guide dynamic control strategies to minimize metabolic burdens and maximize productivity. The integration of real-time control mechanisms, computational modelling, and advanced genetic tools establishes dynamic metabolic engineering as a versatile and effective approach for developing robust microbial cell factories capable of producing high-value biochemicals with improved efficiency and sustainability.

1.4.1 Dynamic control modalities

Two-phase fermentations involve decoupling growth and production phases, often through chemical inducers. For example, microbial cultures can be grown to a specific cell density, after

which production pathways are induced [120]. This approach minimizes growth-related metabolic burdens and maximizes production efficiency. Open-loop control relies on predefined adjustments to optimize production, while closed-loop control incorporates real-time feedback to dynamically adjust fermentation parameters [121]. An open-loop system might be used in a fermentation setup where temperature and pH are maintained at fixed levels based on historical data. While this can work well under controlled conditions, it lacks flexibility in response to unexpected changes. Closed-loop systems are particularly advantageous in responding to process perturbations, ensuring stable and optimal production. Quorum sensing systems, such as the Lux system derived from *Vibrio fischeri*, utilize acyl-homoserine lactones (AHLs) as signalling molecules that bind to *LuxR* at high cell densities, activating gene expression for luminescence and biofilm formation [119]. AHL-based systems from various Gram-negative bacteria also support controlled gene expression in synthetic biology. Temperature-inducible systems, including the *cI/cro* system from bacteriophage λ, enable temperature-dependent gene regulation, where *cI* represses transcription at low temperatures but is degraded at elevated temperatures, allowing gene expression [122]. Similarly, heat shock promoters like *hsp*70 are activated under heat stress [123] and pH-inducible systems, facilitating survival and controlled gene expression in low-pH fermentations [124]. Light-inducible systems, like phytochrome-based systems and the *CRY2/CIB1* blue-light system, offer precise spatial and temporal control of gene expression using specific wavelengths of light [125]. Stress-inducible promoters can be used to control gene expression by activating the transcription of target genes in response to specific stress conditions. For example, in the study, hybrid plasmids were constructed with stress-inducible promoters from *Bacillus subtilis*, such as the SOS promoter P*dinC*, the methylation-specific response promoter P*alkA*, and the oxidative stress promoter P*mrgA* [126]. Metabolite-inducible systems, such as the *XylS* system activated by aromatic compounds and glycolytic promoters responding to metabolic intermediates, link gene expression to cellular metabolic states [127]. Synthetic inducible systems, including modified pLac or pBAD promoters and CRISPR-based technologies, provide tightly controlled, precise regulation. Finally, oxygen-inducible systems, such as the SoxRS regulon in *Escherichia coli* is activated by elevated dissolved oxygen concentrations, which can be used to induce the expression of recombinant proteins [128]. Using the product of interest as the inducer molecule for dynamic metabolic engineering applications is also an innovative strategy for autonomously regulating target metabolite biosynthesis as well.

1.5 Engineered strain for market deployment: A real challenge

While industrial biotechnology and microbial metabolic engineering hold great promise in addressing the increasing demand for sustainable, cost-effective commodity chemicals and natural products, the number of biochemicals that are commercially producible remains limited. Metabolic engineering has improved significantly in recent years. Its essence is the integration of analytical tools for quantifying fluxes and controlling fluxes with molecular biological procedures for implementing specified genetic alterations [129]. Metabolic engineering aims to improve the production of desired metabolites, but several crucial factors need careful consideration for successful large-scale commercialization in industries (*Fig.1.5*). These factors include the selection of appropriate feedstock, achieving high product yield, productivity, and titre, as well as cost-effectiveness of midstream and downstream processes. Metabolic engineering is essential for creating a robust industrial strain by designing pathways and optimizing flux to ensure genetic stability and strain resilience during actual fermentation. Limitations like plasmid curation can be avoided by genetic alteration in the main genome of host organism. Apart from the fact that industrial strains need to be stable through multiple generations in order to be scaled up to industrial grade, cost factor and environmental concerns needs to be considered as well. The utilization of inducer molecules and antibiotics primarily adds to the cost factor, making them less favorable for widespread acceptance. Recent advances in strain modification have preferred chromosomal pathway integration using CRISPR technology over plasmid-based expression if possible since it will get rid of apprehension of chances for plasmid curation. Use of inducer molecules and additives for overexpression of target gene promoters of wild strains and removing intracellular metabolites from host cells are possible ways to enhance production of metabolite of interest without molecular cloning techniques. Dynamic metabolic engineering strategies using the target product as inducer molecule are also in trend nowadays where foreign inducer molecules are not required reducing toxicity and cost. Biotechnology firms have already amassed a plethora of unpublished information's on scaling up processes for fulfilling societal demands, therefore academia-industry partnerships will definitely hasten successful scale-up processes in future.

Regulatory challenges significantly impact the market deployment of engineered microbial strains, as stringent safety, environmental, and compliance requirements must be met before commercialization. The use of genetically modified *Lactobacillus delbrueckii* subsp. *bulgaricus* for DLA production introduces additional regulatory considerations, particularly regarding GMO classification, biosafety, and market acceptance. Since *L. bulgaricus* is

traditionally associated with the dairy industry, regulatory scrutiny may be heightened to assess potential risks such as horizontal gene transfer, antibiotic resistance marker usage, and unintended ecological impacts. If applied in food or pharmaceutical sectors, approvals such as GRAS (Generally Recognized as Safe) status or Novel Food approval may be required. For industrial applications (e.g., bioplastics or biofuels), regulatory concerns may be lower, but biocontainment strategies are still necessary to prevent unintended environmental release. Compliance with regulatory standards adds to production costs, particularly when using antibiotics or chemical inducers, which may face restrictions due to toxicity and environmental concerns. Intellectual property disputes over genetic modifications and proprietary metabolic pathways also create barriers, especially for academia-industry collaborations. Additionally, consumer perception and labelling laws influence market acceptance, especially in regions with strict GMO regulations. Addressing these challenges requires strategic academia-industry partnerships, transparent regulatory navigation, and innovative approaches such as chromosomal pathway integration (instead of plasmid-based expression), antibiotic-free selection markers, and self-regulated metabolic circuits (e.g., DLA as an inducer molecule), aligning with safety, sustainability, and cost-effectiveness standards.

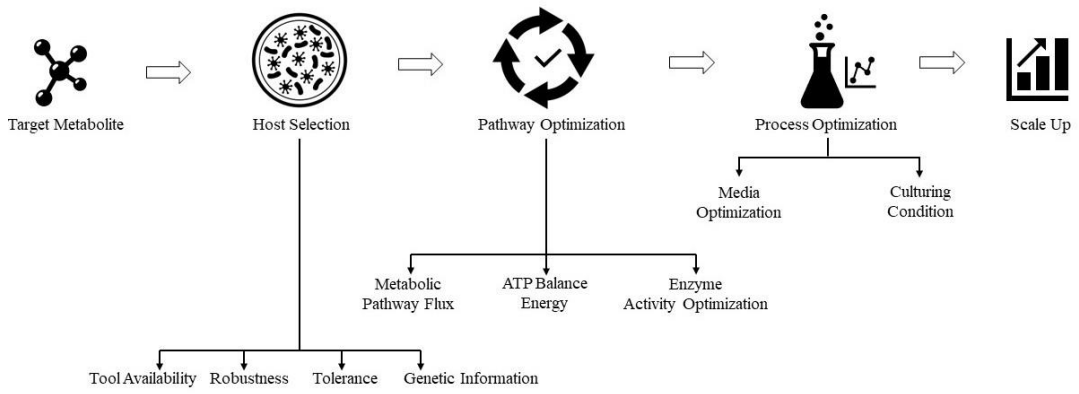
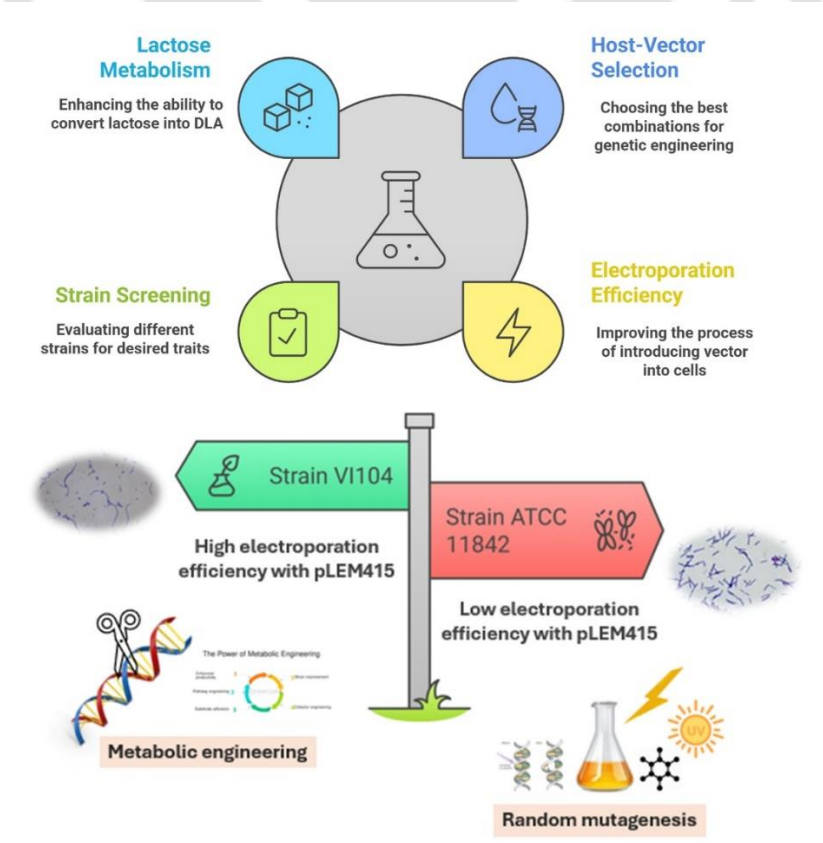


Figure 1.6 Steps involved for scale up and commercialization of metabolically engineered microbial strains producing value added compounds.

CHAPTER 2:

Selection of optimal *Lactobacillus delbrueckii* host strains for strain improvement in enhanced D-lactic acid biosynthesis with high optical purity



2.1 Summary

Lactic acid, particularly its optically pure D-enantiomer (DLA), is a critical precursor for producing biodegradable polymers such as PLA. This study focuses on optimizing host-vector selection and electroporation efficiency in *L. delbrueckii* to enhance optically pure DLA production through strain improvement strategies. Multiple *L. delbrueckii* strains, including *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*, were screened for their ability to metabolize lactose and produce DLA under static and shaking culture conditions. Strains *L. bulgaricus* ATCC 11842 and VI104 demonstrated superior lactose utilization and DLA yields, with VI104 achieving the highest DLA titre (1.98 g L^{-1}) and optical purity (99.09%) under shaking conditions in presence of 2% lactose-MRS media. Electroporation protocols were optimized for shuttle vector pLEM415 transformation, identifying critical parameters such as early exponential-phase cell harvesting and pulse voltages between 1.5–1.8 kV to improve transformation efficiency. The P_{ldhL} promoter from *L. sakei* was validated for robust recombinant protein expression in recombinant *L. bulgaricus*. Based on these results, strain VI104 was identified as the optimal host for metabolic engineering due to its superior electroporation efficiency, transformation reproducibility, and comparatively high DLA production capability. Conversely, strain ATCC 11842 exhibited significantly low electroporation efficiency and poor reproducibility, limiting its utility for direct genetic manipulation. Therefore, in this study, we selected two *L. bulgaricus* strains for further investigation: VI104 for targeted metabolic engineering to enhance DLA biosynthesis and ATCC 11842 for random mutagenesis to improve its DLA production capability. Additionally, this study discusses the limitations and research gaps associated with these two strains, providing a foundation for future advancements in their optimization and industrial applications.

2.2 Introduction

Lactic acid, a naturally occurring organic acid, plays a pivotal role in diverse industrial applications, ranging from food and pharmaceuticals to the production of biodegradable polymers such as PLA. The enantiomeric forms of lactic acid—DLA and LLA—are of particular importance due to their influence on the physical and chemical properties of PLA. For instance, the optical purity of DLA is a critical determinant in the development of PLA with desirable thermal stability and mechanical strength. This has driven substantial interest in

microbial production systems capable of synthesizing optically pure DLA with high efficiency and yield.

Among the various lactic acid-producing microorganisms, *Lactobacillus delbrueckii* is a promising host for DLA biosynthesis due to its metabolic versatility, robustness under industrial fermentation conditions, and natural ability to produce optically pure lactic acid. Its subspecies, such as *L. delbrueckii* subsp. *bulgaricus* and *L. delbrueckii* subsp. *lactis*, exhibit unique metabolic pathways that can be exploited to achieve high lactic acid yields with exceptional optical purity. These characteristics make *L. delbrueckii* an attractive candidate for exploring metabolic engineering and other strain improvement strategies aimed at enhancing its biosynthetic capabilities.

Lactobacillus delbrueckii, particularly its subspecies *bulgaricus* and *lactis*, has been the focus of numerous recent studies reflecting its significance in both the food industry and health sciences. Studies have explored the probiotic potential of *L. delbrueckii* strains in alleviating gastrointestinal issues [130]. A comprehensive genomic analysis revealed a highly conserved proteolytic system in *L. delbrueckii*, which is crucial for its growth in dairy environments[131]. Research has also focused on the fermentation characteristics of *L. delbrueckii* strains when combined with other bacteria, such as *Streptococcus thermophilus*, to optimize yogurt production [132]. Overall, these studies contribute to a deeper understanding of *L. delbrueckii*'s role in food technology and its potential health benefits, paving the way for further applications in probiotics and functional foods. Despite the industrial significance of *Lactobacillus delbrueckii* strains, their genetic and metabolic engineering remains an underexplored area, with limited reports available in the literature. As mentioned in previous chapter, unlike other LAB, such as *Lactococcus lactis* or *Escherichia coli*, which have well-established molecular tools and protocols, *L. delbrueckii* strains pose unique challenges for genetic manipulation due to their recalcitrant nature and low transformation efficiencies. Critical gaps exist in understanding the molecular mechanisms underlying lactose metabolism, substrate utilization, and the regulation of DLA biosynthesis in these strains. Furthermore, the lack of complete genome sequences for many *L. delbrueckii* strains hinders precise genetic modifications and pathway optimization. While a few studies have explored plasmid-based transformations, the reproducibility and efficiency of these methods remain suboptimal, especially for industrially relevant strains like *L. bulgaricus*. Additionally, random mutagenesis as an alternate strategy to enhance metabolic traits in *L. delbrueckii* has not been systematically applied or reported. Addressing these gaps is crucial to unlock the full potential of *L.*

delbrueckii strains as robust microbial cell factories for industrial-scale DLA production. Furthermore, selecting the appropriate *L. delbrueckii* host strain for specific metabolic engineering applications is a critical step, as it lays the foundation for developing high-performing production platforms tailored to industrial requirements.

Most LAB grow efficiently in presence of lactose instead of glucose. Glycolytic pathway being the major pathway in these strains, lactic acid production is directly dependent on the type and amount of carbon source available. This chapter explores the selection criteria and strategies for identifying the most suitable *Lactobacillus delbrueckii* host organism for strain improvement through metabolic engineering and strain improvement studies. This chapter also explores the lactose utilization efficiency of the selected *L. bulgaricus* strains, highlighting their limitations, research gaps, and potential areas for improvement. Furthermore, it delves into innovative strategies that can be employed to overcome these challenges, paving the way for expanding their applicability in industrial-scale production. Lastly, based on the preliminary results obtained and underlying research gaps the main objectives of this thesis work have been concisely outlined, providing a clear framework for addressing these gaps and advancing the development of *L. bulgaricus* strains for enhanced DLA biosynthesis.

2.3 Materials and methods

2.3.1 Bacterial strains, plasmids, media and culture conditions

The bacterial strains employed in this study and the plasmids details are listed in Table 2.1. All lactic acid bacteria strains including *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 and ATCC 11842 was cultivated in deMan Rogosa Sharpe (MRS) media. For preculture preparation, the strain was propagated in 10 mL MRS broth at 37°C shaking conditions prior to the culture entered its phase of exponential growth (~10^6 CFU mL^{-1}). MRS medium composition: lactose 20 g L^{-1}, beef extract 10 g L^{-1}, peptone 10 g L^{-1}, yeast extract 5 g L^{-1}, $CH_3COONa.3H_2O$ 5 g L^{-1}, K_2HPO_4 2 g L^{-1}, tri-ammonium citrate 2 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, $MnSO_4.4H_2O$ 0.5 g L^{-1} and Tween 80 1 g L^{-1}. A PVDF membrane filter with a 0.22-µm diameter was used to sterilise the fermentation medium. All LAB strains used in this study were grown in 37°C shaking conditions. All reagents and chemicals including antibiotics employed in this present study were purchased from M/s HiMedia Laboratory Chemicals (Bengaluru, India).

Table 2.1: List of bacterial strains and plasmids used in this study

Strain	Description	Source
***E. coli* TOP10F'**	Host for gene cloning, F′ [*lacI* q, Tn10(TetR)] *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(StrR) *end*A1 *nup*G	Invitrogen
***E. coli* BL21(DE3)**	F^- *ompT gal dcm lon hsd*S_B($r_B^-m_B^-$) *λ(DE3)*	Novagen
***Lactobacillus delbrueckii* subsp. *bulgaricus* VI104**	Selected LAB expression host in this study	A kind gift from Prof. Emmanuelle Maguin, French National Institute for Agriculture, Food, and Environment INRAE, France
Lactobacillus delbrueckii* subsp. *delbrueckii* *NBRC 3534	Screening of suitable LAB expression host	NITE (National Institute of Technology and Evaluation) Biological Research Center, Japan.
Lactobacillus delbrueckii* subsp. *lactis* *NBRC 3073	Screening of suitable LAB expression host	NITE (National Institute of Technology and Evaluation) Biological Research Center, Japan.
Lactobacillus delbrueckii* subsp. *lactis* *NBRC 102622	Screening of suitable LAB expression host	NITE (National Institute of Technology and Evaluation) Biological Research Center, Japan.
Lactobacillus delbrueckii* subsp.*bulgaricus* *ATCC 11842	Screening of suitable LAB expression host	American Type Culture Collection located

	in Manassas, Virginia, US

2.3.2 Electroporation protocols used for optimization

Plasmid pLEM415 was subsequently transformed into LAB via electroporation. LAB competent cells were prepared, and the transformation protocol was optimized based on previously published protocols, incorporating modifications to enhance efficiency [117]. After incubating overnight, the culture was harvested at the early stationary phase, indicated by an optical density at 600 nm (OD_{600}) of 1.7, and collected through centrifugation. The cell pellet were rinsed once with 100 ml of cold electroporation buffer (EB) (comprising 0.4 M sucrose, 1 mM $MgCl_2$, 5 mM KH_2PO_4; pH 6 as per reports by [117]) and then twice with 30 ml of cold EB. The cells were resuspended in EB to reach an OD_{600} of approximately 50. The cell suspension was incubated at 45°C for 20 minutes and subsequently placed on ice for 10 minutes. For electroporation, 80 µl of the cell suspension was combined with 0.3 to 2 µg of recombinant plasmid DNA. The mixture was then subjected to a 1.5-kV, 800-Ω, 25-µF electric pulse in electroporation cuvette using a Gene Pulser (0.2cm gap MicroPulser Electroporation cuvette, BioRAD). Immediately following electroporation, 2 ml of MRS medium (with 2% w/v glucose) was added, and the cells were incubated for 3 hours at 37°C before being plated on MRS-glucose (2% w/v) agar plates with erythromycin. The plates were incubated at 37°C for 3 days under anaerobic conditions using Gaspak (M/s Himedia, Bengaluru, India).

2.3.3 Screening of the LAB host

For screening the best LAB host, the preculture was transferred into 250 mL baffled flasks containing 100 mL of fermentation medium, adjusted to an initial optical density (OD_{600}) of approximately 0.05, and incubated at 37°C. For shaking conditions culture was agitated at 150 rpm. Cells were collected regularly at 1 hour time intervals, and cell pellet were harvested by centrifugation for biomass analysis while the supernatant was stored at −20°C for subsequent product and by-product analysis.

2.3.4 Analytical techniques

Supernatant collected at regular intervals for all shake-flask and static flask studies were further evaluated for metabolite quantification. Glucose, lactate, and other metabolites in the collected supernatant were assessed using Phenomenex Aminex HPX-87H column and Phenomenex guard column attached to HPLC-RID detector system (M/s. Shimadzu, Corp., Kyoto, Japan).

The isocratic elution procedure was adopted with a flow rate of 0.6 mL min^{-1} and a mobile phase of 5 mM H_2SO_4. Total lactic acid was evaluated using UV detector at 210nm. Cell growth was measured using a UV-Vis spectrophotometer (Spectronics 200, ThermoFisher, UK. All the analysis was performed in duplicates, and the metabolite concentrations were represented as mean value of duplicates. To specifically verify the presence of DLA, a *dldh* based enzyme kit (K-DATE, M/s Megazyme, Ireland) was used and L-lactic acid (LLA) was analysed using an L-lactate oxidase kit (K-LATE, M/s Megazyme, Ireland) based on manufacturers protocol in a multimode plate reader (VarioskanTM Lux, M/s ThermoFisher Scientific). Optical purity was estimated using the formula given in equation 1:

$$\left(\frac{(D(+)-Lactic\ acid - L(-)-Lactic\ acid)}{Total\ Lactic\ acid}\right) * 100 \quad \text{..... (Eq1)}$$

2.4 Results and discussion

Optimization of host-vector selection and electroporation efficiency in *Lactobacillus delbrueckii*

Different *Lactobacillus delbrueckii* strains were screened for their capacity to produce high yields of optically pure DLA using MRS-lactose as the carbon source, under both shaking and static culture conditions. *Lactobacillus delbrueckii* strains being a facultative anaerobe exhibit optimal growth under both static and shaking conditions, with shaking generally enhancing oxygen transfer and biomass yield, while static conditions can favour the production of certain metabolites [2]. *Lactobacillus delbrueckii* strains NBRC 3073, 3534, and 102622, along with ATCC 11842 and VI104, were screened for their production efficiency (Table 2.2 and 2.3). *Lactobacillus delbrueckii* strains produce maximum DLA when lactose and fructose are used as carbon sources, although lactose is the preferred carbon source where the growth parameters are enhanced including doubling time. Therefore, the aim of this chapter was to select a host with enhanced lactose utilization capability and further engineer it for complete lactose utilization by constructing a galactose utilization pathway so that overall yield of DLA from lactose is enhanced for future valorization applications. NBRC 3073 and NBRC 3534 strains showed less growth on MRS lactose media under both static and shaking conditions. *Lactobacillus delbrueckii* subsp. *delbrueckii* does possess the capability to metabolize glucose and lactose, although the efficiency and mechanisms can vary among different strains [133][134]. The complete genome sequence of NBRC 3073 is also unavailable, making it challenging to address the exact reasons for its growth failure on MRS lactose media.

Alternatively, even if lactose is transported into the cytoplasm, the absence of functional beta-galactosidase, which converts lactose into glucose and galactose, could be a limiting factor. The glucose produced could then support the growth of NBRC 3073 and NBRC 3534 on MRS lactose media. The strains NBRC 102622, ATCC 11842, and VI104, which demonstrated successful growth on MRS lactose media, were further evaluated. The assessment focused on their kinetic parameters to identify the most suitable host for producing high optically pure DLA.

The specific lactose utilization rate in *L. bulgaricus* strains ATCC 11842 and VI104 was significantly higher compared to *L. delbrueckii lactis* strain NBRC 102622, indicating a more efficient metabolic adaptation to lactose in these strains (Table 2.2 and 2.3). Under static conditions, NBRC 102622 exhibited a significant reduction in residual lactose, decreasing from 18.37 g L^{-1} to 3.12 g L^{-1}, with tandem increase in residual galactose concentration to 14.44 g L^{-1}. This indicates that the NBRC 102622 strain efficiently metabolizes and hydrolyses lactose under static conditions. Additionally, the optical purity of the DLA improved to 94.38%. In contrast, *L. delbrueckii bulgaricus* strains exhibited a lower initial residual lactose concentration of 1.80 g L^{-1} under shaking conditions, with substantial galactose (12.72 g L^{-1}) and maximum DLA titre achieved (1.81 g L^{-1}), with an optical purity of 99.08%. Under static conditions, residual lactose further diminished to 1.46 g L^{-1}, with galactose at 12.67 g L^{-1}, attaining maximum DLA titre of 1.36 g L^{-1}, with optical purity at 98.37%. Due to improved lactose utilization, rapid substrate dissociation, and high yields of optically pure DLA, strains ATCC 11842 and VI104 were selected as optimal hosts for electroporation optimization and advanced metabolic engineering, employing compatible molecular cloning tools.

L. bulgaricus efficiently metabolizes lactose to produce DLA, with optimal production observed under shaking conditions due to presence of *d-ldh* gene. Among the tested strains, the highest DLA titre of 1.98 g L^{-1} was achieved by *L. bulgaricus* VI104 under shaking conditions, accompanied by an exceptional optical purity of 99.09%, in shake flask experiments. ATCC 11842 and VI104 strains were utilized for optimization of electroporation protocol using shuttle vector pLEM415. While previous literature has demonstrated the successful transformation of the pLEM415 vector into *Lactobacillus bulgaricus* strain ATCC 11842 [107] , our study denoted that transformation reproducibility and efficiency was extremely low. These difficulties underscore the complexities inherent in metabolic engineering within this species, necessitating further exploration of alternative transformation methods to improve genetic manipulation outcomes [118]. High electroporation efficiency of the pLEM415 vector

in *Lactobacillus bulgaricus* strain VI104 strain was reported [117], but the successful protocol, albeit with slight modifications, indicated that harvesting cultures during the early exponential phase is crucial; otherwise, a significant reduction in electroporation efficiency was observed. Additionally, optimal pulse voltages between 1.5 kV and 1.8 kV were identified to enhance this efficiency. It was also observed that the P_{ldhL} promoter from *L. sakei* exhibits significantly enhanced activity, making it highly effective for driving heterologous recombinant protein production in *Lactobacillus bulgaricus.* Thus, VI104 strain was further selected as the optimum host with high efficiency of transformation and was further metabolically engineered for enhanced DLA biosynthesis in this study.

Table 2.2: Kinetic parameters for DLA biosynthesis using MRS-Lactose under shaking conditions (180rpm) in 100 ml flask volume

Strain No.	Strain	Residual lactose (g L^{-1})	Residual galactose (g L^{-1})	DLA titre (g L^{-1})	Specific Lactose utilization rate q_s (g g^{-1} h^{-1})	Optical purity (%)	Electroporation efficiency with pLEM415
NBRC 102622	*L. delbreuckii* subsp. *lactis*	18.37	2.78	1.34	0.011	90.09	Nil
NBRC 3534	*L. delbrueckii* subsp. *delbrueckii*	12.98	2.34	0.56	0.025	89.09	Nil
NBRC 3073	*L. delbreuckii* subsp. *lactis*	9.78	3.08	0.78	0.034	86.09	Nil
ATCC 11842	*L. delbrueckii* subsp. *bulgaricus*	1.83	13.98	1.78	0.334	99.07	Less
VI104	*L. delbrueckii* subsp. *bulgaricus*	0.45	14.78	1.98	0.398	99.09	Good

Table 2.3: Kinetic parameters for DLA biosynthesis using MRS-Lactose under static conditions in 100 ml flask volume

Strain No.	Strain	Residual lactose (g L^{-1})	Residual galactose (g L^{-1})	DLA titre (g L^{-1})	Specific Lactose utilization rate q_s (g g^{-1} h^{-1})	Optical purity (%)	Electroporation efficiency with pLEM415
NBRC 102622	*L. delbreuckii* subsp. *lactis*	3.12	14.44	1.67	0.123	94.38	Nil
NBRC 3534	*L. delbrueckii* subsp. *delbrueckii*	11.98	12.89	0.87	0.034	90.78	Nil
NBRC 3073	*L. delbreuckii* subsp. *lactis*	9.67	10.34	1.01	0.078	89.78	Nil
ATCC 11842	*L. delbrueckii* subsp. *bulgaricus*	2.89	10.09	1.89	0.323	99.00	Less
VI104	*L. delbrueckii* subsp. *bulgaricus*	1.56	13.98	1.88	0.287	99.08	Good

2.5 Conclusion and research gap to be addressed in this thesis

This chapter identified and optimized *Lactobacillus bulgaricus* strain VI104 as a superior host for producing optically pure DLA due to its efficient lactose metabolism, high DLA yield, optical purity and excellent electroporation efficiency. Optimized electroporation protocols and the use of the P_{ldhL} promoter from *L. sakei* further enhanced the strain's genetic manipulation potential. These findings establish a strong foundation for targeted metabolic engineering of strain VI104 for industrial-scale DLA production. Meanwhile, the challenges associated with strain ATCC 11842 highlight the need for alternative strategies such as random mutagenesis to enhance its utility. Overall, this chapter provides insights into strain selection, metabolic engineering, and genetic transformation methodologies for improving DLA biosynthesis in *Lactobacillus delbrueckii*.

The potential of *L. bulgaricus* extends well beyond its traditional role in dairy fermentation, yet significant research gaps hinder its broader industrial applications. One of the primary challenges facing *L. bulgaricus* is its genetic intractability, which severely limits strain improvement and metabolic engineering efforts. The lack of established constitutive/ inducible promoter-repressor systems, compatible RBS, spacer regions and effective electroporation protocols has made genetic manipulation of this species particularly difficult. Current methodologies for genetic modification are not reproducible or efficient, creating a barrier for researchers aiming to harness the organism's metabolic capabilities for novel applications. Furthermore, techniques such as mutagenesis and adaptive evolution have not been thoroughly explored in *L. bulgaricus*. These methods could potentially enhance the organism's metabolic pathways, allowing for increased production of desired compounds (including organic acids like acetic acid and lactic acid) from various renewable source of substrates. The absence of comprehensive studies utilizing these techniques limits the understanding of how to optimize *L. bulgaricus* for industrial processes.

In summary, while *Lactobacillus delbrueckii* subsp. *bulgaricus* possesses remarkable potential for industrial biotechnology, significant research gaps remain, particularly regarding its genetic manipulation and application beyond dairy fermentation and these bottlenecks will be investigated and resolved in this thesis work. The development of advanced genetic tools and methodologies is crucial to unlocking this potential and enabling the organism to serve as an effective platform for producing high-value organic compounds. Addressing these challenges will not only enhance our understanding of this species but also expand its utility in various biotechnological fields including probiotics and therapeutics.

2.6 Study of the wild *Lactobacillus bulgaricus* VI104 strain: Identifying research gaps and opportunities

Limited literature is available regarding the *L. bulgaricus* VI104 strain. However, a few studies have highlighted its potential in genetic manipulation and biotechnological applications. One study reported previously, utilized this strain to identify two thermosensitive plasmids and investigate the activity of various insertion sequences (IS)[135]. The research demonstrated that IS1223 and IS1201 could successfully transpose in the VI104 strain, showcasing its utility as a tool for genetic studies. Another investigation employed the VI104 strain as a host for heterologous expression. The study revealed that the *hlbA* gene promoter facilitated high-level expression and secretion of the staphylococcal nuclease reporter protein[136]. These findings

underscore the strain's potential for efficient protein production and secretion, making it a promising candidate for biotechnological applications. Additionally, the VI104 strain was used to develop an efficient in vivo system for random transposon mutagenesis [137]. This system enables the generation of random mutations, which is invaluable for functional genomic studies and the exploration of gene function. In another study, the VI104 strain was employed to characterize two *csp*-like genes, *cspA* and *cspB*, and to investigate their response to cold shock [138]. This research provided insights into the cold-shock response mechanism in *Lactobacillus delbrueckii* subsp. *bulgaricus*, further expanding the understanding of its physiological adaptations. These studies collectively highlight the unique applications of the VI104 strain in genetic, physiological, and biotechnological research.

Most notably, the VI104 strain stands out as the only *L. bulgaricus* strain with an optimized electroporation procedure using the pLEM415 vector, demonstrating remarkable efficiency. The pLEM415 plasmid, a shuttle vector derived from *Lactobacillus reuteri* and *Escherichia coli*, was employed to refine the electroporation process for the VI104 strain of *L. bulgaricus*. Under optimal conditions, the transformation efficiency achieved an impressive 10^4 transformants per µg of DNA, significantly enhancing the genetic manipulation capabilities of the VI104 strain[117]. Despite its demonstrated potential, this strain had never been explored for metabolic engineering purposes prior to this research. The current work addresses this critical gap by leveraging the unique features of the VI104 strain to expand its applications in metabolic engineering and synthetic biology for enhanced DLA biosynthesis.

2.7 Study of the wild *Lactobacillus bulgaricus* ATCC 11842 strain: Identifying research gaps and opportunities

Recent research on the *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 strain has focused on its applications in fermentation, probiotic properties, and potential health benefits. A study investigated the effect of various nitrogen sources on the growth of *L. bulgaricus* ATCC 11842 and its production of β-galactosidase when grown in deproteinized whey. The research showed that this strain could utilize a range of nitrogen sources, including yeast extract and peptone, which were found to be particularly effective for β-galactosidase production[139]. Research also focused on developing a selective medium (modified reinforced clostridial medium or mRCM) that enhanced the growth of *L. bulgaricus* ATCC 11842 compared to conventional media [140]. Studies have highlighted the probiotic potential of *L. bulgaricus* ATCC 11842, particularly its antibacterial activity against various pathogens.

For example, research indicated that this strain could suppress foodborne pathogens in vitro, suggesting its beneficial role in food safety and gut health [141].

Additionally, genomic studies have explored the presence of clustered regularly interspaced short palindromic repeats (CRISPRs) in *L. bulgaricus* strains, including ATCC 11842. The identification of CRISPR systems could provide insights into the strain's adaptive responses to environmental stresses and its genetic resilience [142]. These findings collectively underscore the significance of *L. bulgaricus* ATCC 11842 in both industrial applications and health-related research, paving the way for further exploration into its benefits as a probiotic organism and its role in fermentation technology.

The research gap concerning *L. bulgaricus* ATCC 11842 is significant, particularly in the context of metabolic engineering and genetic manipulation. Despite the strain's recognized probiotic properties and potential applications in food technology, there is a notable lack of literature addressing its engineering or metabolic engineering. One major barrier to advancing research in this area is the lack of compatible vectors and low efficiency of electroporation with recombinant vectors. Current studies primarily focus on its probiotic effects and antioxidative properties, but they do not explore the genetic modifications that could enhance these traits or introduce new functionalities. Moreover, the existing literature emphasizes the strain's health benefits and fermentation capabilities without delving into engineered strains that could potentially improve its performance in various applications. In the absence of established reproducible transformation protocol for *L. bulgaricus* ATCC 11842, exploring random mutagenesis techniques presents a viable alternative for enhancing the production efficiency of specific metabolites. This approach could facilitate the development of mutant strains with improved capabilities, such as increased lactic acid or other beneficial compound production, without the need for sophisticated genetic manipulation techniques. Notably, this line of study has not been thoroughly investigated in the context of *L. bulgaricus* ATCC 11842, representing a significant research gap. By employing random mutagenesis, researchers could potentially unlock new metabolic pathways and optimize the strain's performance in various applications, thereby contributing to both academic knowledge and industrial practices in fermentation technology.

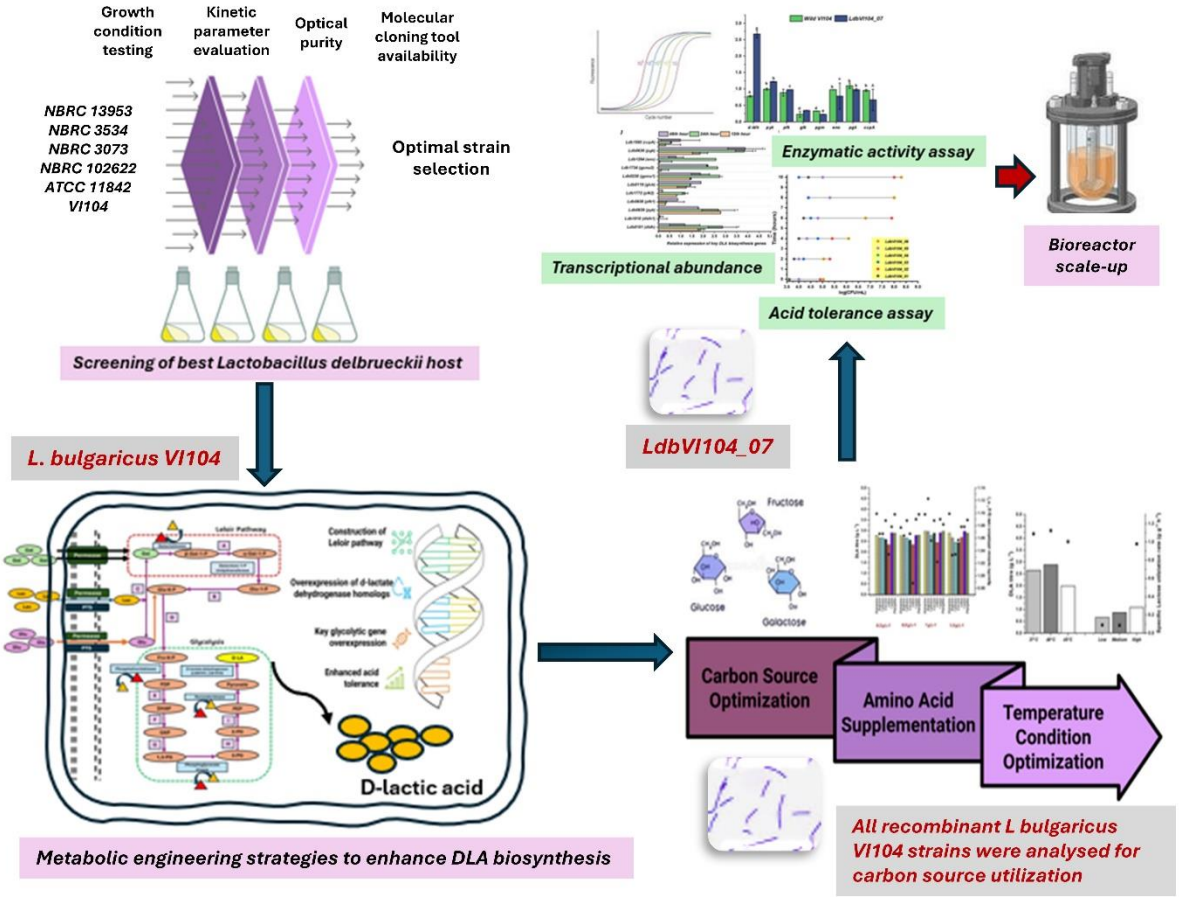
2.8 Major Objectives

1. Metabolic engineering of *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 as a cell factory through strategic pathway optimization for enhanced DLA biosynthesis.

2. Engineering the DLA responsive promoter/repressor system as dynamic metabolic engineering tool in *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 for controlled DLA biosynthesis.
3. Random mutagenesis and optimization studies for improved DLA biosynthesis from diverse carbon sources in mutant *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842.

CHAPTER 3:

Metabolic engineering of *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 as a cell factory through strategic pathway optimization for enhanced DLA biosynthesis



3.1 Summary

Lactobacillus delbrueckii subsp. *bulgaricus* is a homofermentative LAB renowned for producing optically pure DLA, essential for biodegradable polymer synthesis. However, its industrial applicability is impeded by inefficient lactose assimilation and genetic transformation. Galactose accumulation, a byproduct of lactose hydrolysis, was addressed by heterologous expression of galactokinase (*galK)* and galactose 1-phosphate uridyltransferase (*galT)*, constructing the complete Leloir pathway for improved DLA yield. ATP imbalance from redirected metabolic flux was mitigated through introduction of homologous pyruvate kinase (*pyk)* gene, conferring enhanced acid tolerance. Systematic overexpression of key glycolytic enzymes (*dldh* homologs, *pfk*, and *pgk*) boosted DLA titres. The engineered strain LdbVI104_07 produced 9.39 g L^{-1} DLA (240% improvement) with yield of 0.188 g g^{-1} and 273% increase in acid tolerance. Transcriptomics and enzymatic assays substantiated successful metabolic reprogramming in LdbVI104_07. This study explores metabolic engineering of *L. bulgaricus* VI104 to enhance DLA biosynthesis and establishes molecular cloning tools for prospective probiotic and therapeutic applications.

3.2 Introduction

LAB, renowned for their lactic acid biosynthesis capabilities, have been the subject of extensive scientific scrutiny and industrial exploitation. Engineered LAB, particularly *Lactobacillus plantarum* and *Lactococcus lactis*, are widely utilized for genetic modifications, supporting a diverse array of industrial applications [143][79]. This is primarily due to the availability of robust molecular cloning tools and established transformation protocols. Only a limited number of *Lactobacillus* species can be effectively engineered, primarily due to the absence of well-established molecular cloning tools. *L. delbrueckii* is another species within the LAB group that has gained attention for its potential in the biosynthesis of value-added products including lactic acid[144][145]. While other subspecies of *Lactobacillus delbrueckii* are primarily studied for their ability to produce high-value metabolites and bioproducts, the homofermentative probiotic *Lactobacillus delbrueckii* subsp. *bulgaricus* has been predominantly utilized in the dairy industry as a starter culture for yogurt production [146]. Recent advancements have broadened its role to encompass probiotic applications, owing to its health-enhancing attributes [147][148].

Intriguingly, *L. bulgaricus* exhibits the ability to synthesize optically pure DLA, a valuable organic acid in diverse industrial applications. Despite its potential, the industrial exploitation of *L. bulgaricus* for DLA production remains underexplored. *L. bulgaricus* lacks the ability to metabolize galactose. Previous studies have demonstrated the complete utilization of lactose through co-culture strategies, where *L. delbrueckii* was combined with genetically engineered *Lactococcus lactis* strains with enhanced galactose utilization capabilities [79]. This co-culture approach facilitated the efficient fermentation of lactose, leading to the high-volume production of DLA. Hence, the published literature records substantiate that limited exploration on the metabolic engineering of *L. delbrueckii* species [2] and this knowledge gap is further exacerbated by the absence of well-established molecular cloning tools and the challenges associated with electro-transformation techniques for this species which has been optimized in previous chapter [117]. Surmounting these obstacles could pave the way for novel industrial applications of *L. bulgaricus*, particularly in the sustainable production of value-added products including optically pure DLA.

DLA being an essential commodity in the chemical industry, serving as a monomer for the synthesis of PLA, a biodegradable polymer [1][5]. The demand for PLA is witnessing a rapid surge due to its applications in packaging, textiles, and biomedical devices [149]. Various LAB have been employed for DLA production using different substrates and fermentation methods. For instance, *Lactobacillus lactis* ATCC 4797 and *L. bulgaricus* LB-12 have been used in shake flask and bioreactor studies with casein WP and hydrolysate, yielding DLA titres ranging from 0.04 to 24.3 g L^{-1} with high optical purity (> 98%)[150]. Other LAB species like *Sporolactobacillus inulinus* and *Lactobacillus bulgaricus* CGMCC 1.6970 have also been used in bioreactor settings with whey protein hydrolysate and cheese whey powder, respectively, yielding high DLA titres and productivities, demonstrating the diversity and effectiveness of LAB for large-scale DLA production [151][152]. *L. bulgaricus* achieves up to 99.05% optical purity in batch fermentations, effectively converting agro-industrial residues like sugarcane molasses and orange peel waste into high-quality DLA [153][154]. Interestingly most strains employed for DLA production are wild type, with very few *Lactobacillus* strains having been genetically engineered to enhance the kinetics of DLA production. Due to the challenges in engineering *Lactobacillus* strains, several mutagenesis techniques have been employed to improve DLA biosynthesis [155][2].

Manufacturing of PLA with enhanced properties require high volume optically pure lactic acid isomers. However, the current production methodologies for DLA are inadequate to cater to

the escalating demand. *L. bulgaricus* is recognized for its proficient lactose and fructose metabolism, leading to the production of high optically pure DLA, other metabolites and biomass. Among the array of carbon sources that *L. bulgaricus* can effectively metabolize, galactose and sucrose remain unutilized by this microorganism [150] [2]. The published literature is bereft of comprehensive reports elucidating the reasons behind the inefficient uptake of sucrose by this organism. The non-utilization of galactose can be attributed to the absence of the complete galactose utilization pathway. Therefore, this thesis aims to overcome limitations in *Lactobacillus bulgaricus* by engineering the Leloir pathway for enhanced galactose utilization, enabling complete lactose uptake. *L. bulgaricus* VI104 was systematically engineered to optimize lactose metabolism, refine metabolic pathways, maintain optimal ATP levels, and increase DLA production. Advanced molecular cloning, pathway reconstruction, and fermentation optimization led to significant improvements in DLA titre, yield, and specific productivity. This present chapter not only demonstrates the potential of *L. bulgaricus* as a cell factory for sustainable DLA production but also provides a framework for futuristic genetic modifications and scale-up strategies, making it a robust candidate for industrial applications in bioplastics, probiotics, and therapeutic product development.

3.3 Materials and methods

3.3.1 Microbial strains, plasmids, media and growth conditions

The bacterial strains employed in this chapter and the plasmids details are listed in Table 3.1. All restriction and other genetic manipulation enzymes, including Q5 High-Fidelity DNA Polymerase and Phusion High-Fidelity DNA Polymerase, were procured from New England Biolabs (NEB). Oligonucleotide primers, as listed in Table 3.2, were synthesized by BioServe, India. The ribosome binding site (RBS) was uniformly employed across all constructs along with the spacer region in multiple gene constructs. *E. coli* TOP10F' and BL21 (DE3) cells were cultivated and maintained in Luria-Bertani (LB) broth and LB agar (LB broth containing 1.5% (w/v) agar). All LAB strains including *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 was cultivated in MRS media. For preculture preparation, the strain was propagated in 10 mL MRS broth at 37°C shaking conditions prior to the culture entered its phase of exponential growth (~10^6 CFU mL^{-1}). MRS medium composition: lactose 20 g L^{-1}, beef extract 10 g L^{-1}, peptone 10 g L^{-1}, yeast extract 5 g L^{1}, $CH_3COONa.3H_2O$ 5 g L^{-1}, K_2HPO_4 2 g L^{-1}, tri-ammonium citrate 2 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, $MnSO_4.4H_2O$ 0.5 g L^{-1} and Tween 80 1 g L^{-1}. A PVDF membrane filter with a 0.22-µm diameter was used to sterilise the fermentation medium. All wild and

recombinant strains used in this study were grown in 37°C shaking conditions. 100 µg mL^{-1} of ampicillin for *E. coli* and 25 µg mL^{-1} of erythromycin for *L. bulgaricus* were supplemented in the medium for the stable replication of recombinant plasmids. All reagents and chemicals including antibiotics employed in this present study were purchased from M/s HiMedia Laboratory Chemicals (Bengaluru, India).

Table 3.1: List of bacterial strains and plasmids used in this study

Strain	Description	Source
***E. coli* TOP10F'**	Host for gene cloning, F′ [*lacI* q, Tn10(TetR)] *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(StrR) *end*A1 *nup*G	Invitrogen
***E. coli* BL21(DE3)**	*F⁻ ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) *λ(DE3)*	Novagen
***Lactobacillus delbrueckii* subsp. *bulgaricus* VI104**	Selected LAB expression host in this study	A kind gift from Prof. Emmanuelle Maguin, French National Institute for Agriculture, Food, and Environment INRAE, France
LdbVI104_01 (LdbVI104-g*alK-galT)*	*Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 harbouring plasmid pLEM415_01 (pLEM415-g*alK-galT)*	This study
LdbVI104_02 (LdbVI104-*galK-galT-pyrk*)	*Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 harbouring plasmid pLEM415_02 (pLEM415- ***g****alK-galT-pyrk)*	This study
LdbVI104_03 (LdbVI104-*dldh)*	*Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 harbouring pLEM415_03 (plasmid pLEM415-*dldh)*	This study
LdbVI104_04 (LdbVI104-*ldb1010)*	*Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 harbouring plasmid pLEM415_04	This study

	(pLEM415-*ldb1010)*	
LdbVI104_05 (LdbVI104-*dldh+ldb1010)*	*Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 harbouring plasmid pLEM415_05 (pLEM415- *dldh+ldb1010)*	This study
LdbVI104_06 (LdbVI104-*dldh+pfk*)	*Lactobacillus delbrueckii* subsp.*bulgaricus* VI104 harbouring plasmid pLEM415_07 (pLEM415- *dldh+pfk)*	This study
LdbVI104_07 (LdbVI104-*dldh+pfk+pgk*)	*Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 harbouring plasmid pLEM415_07 (pLEM415-*dldh+pfk+pgk)*	This study
Plasmids	**Description**	**Source**
pLEM415-*ldhL-mRFP1*	Emr, Ampr; *E. coli-Lactobacillus* shuttle vector	Deposited by Dr.Sujin Bao (Addgene plasmid no.99842)
pLEM415_01 pLEM415-g*alK-galT*	Em^r, Amp^r; *galK* and galT genes from *L.helveticus* cloned downstream of P_{ldhL} promoter in pLEM415	This study
pLEM415_02 pLEM415- g*alK-galT-pyrK*	Em^r, Amp^r; *galK,* galT genes from *L.helveticus* and pyk gene from *L. bulgaricus* cloned downstream of P_{ldhL} promoter in pLEM415	This study
pLEM415_03 pLEM415-*dldh*	Em^r, Amp^r; *dldh* gene from *L. bulgaricus* cloned downstream of P_{ldhL} promoter in pLEM415	This study
pLEM415_04 pLEM415-*ldb1010*	Em^r, Amp^r; *dldh* homolog (ldb1010) gene from *L.bulgaricus* cloned downstream of P_{ldhL} promoter in pLEM415	This study
pLEM415_05 pLEM415- *dldh+ldb1010*	Em^r, Amp^r; both *dldh* homolog (*ldb0101+ldb1010*) gene from	This study

	L.bulgaricus cloned downstream of P_{ldhL} promoter in pLEM415	
pLEM415_06 pLEM415- *dldh+pfk*	Em^r, Amp^r; key *dldh* gene and *pfk* gene from *L. bulgaricus* cloned downstream of P_{ldhL} promoter in pLEM415	This study
pLEM415_07 pLEM415-*dldh+pfk+pgk*	Em^r, Amp^r; key *dldh, pfk* and *pgk* gene from *L. bulgaricus* cloned downstream of P_{ldhL} promoter in pLEM415	This study

Em^r, Erythromycin resistance; Amp^r, Ampicillin resistance

Table 3.2: List of primers used in this study

Name of the primer	**Sequence of the primer 5' to 3'**
galK_FP	cgtcggcgaaagcttgcatgcgaaaggagctgtacaatgaataaagaagaactact
galK_RP	cggccggctcctttcttaaattctcttagtaccgtcaacgatttctgc
galT_FP	gagaatttaagaaaggagctgtacaatgaaaattattgaaaaatttgc
galT_RP	agctcctttcttacttaatctctttagtcaaagctgcaataaacttatccc
pyk_FP	gattaagtaagaaaggagctgtacaatgaaaaaaacgaaga
pyk_RP	acttctcagtttacaggtttgaaacttcaccttggtaaat
Ldb0101_FP	agaggatccccgggtaccggtgaaaggagctgtacaaatgactaaaatttttgcttacgcaattc
Ldb0101_RP	ttcgcatgccctaggttagccaaccttaactggag
Ldb1010_FP	cctagggcatgcgaaaggagctgtacaatgactaaaattgccatgtat
Ldb1010_RP	ttcgatatcaagcttatcgatttacaggttaacgatgctt
dldh_FP	gaggatccccgggtaccggtgaaaggagctgtacaaatgactaaaatttttgcttacgcaattc
dldh_RP	tcctttcgcatgcttagccaaccttaactggagtttc
pfk_FP	ttggctaagcatgcgaaaggagggatccaatgaaacggattggtattttgactag
pfk_RP	ctcggaggaggccatcctaggttattactatcttgataaatctgcgttgagct
pgk_FP	caagatagtaataacctagggaaaggagggtaccgatggct
pgk_RP	tcggaggaggccatcctaggttattactacttgtctgaaacgcaagcgatacc

3.3.2 Construction of recombinant *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 strains

The recombinant plasmids were constructed using a Gibson assembly technique to facilitate the seamless integration of multiple target genes. For the construction of pLEM415_01, the *galK* and *galT* genes were PCR-amplified from the genomic DNA of *Lactobacillus helveticus* CNRZ32 (extracted using the Sigma Aldrich genomic DNA isolation kit) using gene-specific primers (*galK_FP*, *galK_RP*, *galT_FP*, and *galT_RP*). To construct pLEM415_02, the *pyk* gene was additionally amplified from the genomic DNA of *L. bulgaricus* VI104 using primers *pyk_FP* and *pyk_RP*. Similarly, the *dldh* (*ldb0101*), *ldb1010*, *pgk*, and *pfk* genes were PCR-amplified from *L. bulgaricus* genomic DNA, with primers *dldh_FP*, *dldh_RP*, *ldb1010_FP*, *ldb1010_RP*, *pgk_FP*, *pgk_RP*, *pfk_FP*, and *pfk_RP*. The PCR-amplified fragments were assembled using Gibson Assembly Master Mix (New England Biolabs), enabling efficient and accurate ligation of multiple DNA fragments. The ligation mix was transformed into cloning host *E. coli* TOP10 cells through the heat-shock method, and positive clones were screened via colony PCR to confirm the successful assembly of recombinant plasmids. The verified plasmids were subsequently transformed into *L. bulgaricus* VI104 via electroporation. *L. bulgaricus* competent cells were prepared, and the transformation protocol was optimized based on previously published protocols, incorporating modifications to enhance efficiency [117]. After incubating overnight, the culture was harvested at the early stationary phase, indicated by an optical density at 600 nm (OD_{600}) of 1.7, and collected through centrifugation. The cell pellet was rinsed once with 100 ml of cold electroporation buffer (EB) (comprising 0.4 M sucrose, 1 mM $MgCl_2$, 5 mM KH_2PO_4; pH 6 as per reports by [117]) and then twice with 30 ml of cold EB. The cells were resuspended in EB to reach an OD_{600} of approximately 50. The cell suspension was incubated at 45°C for 20 minutes and subsequently placed on ice for 10 minutes. For electroporation, 80 µl of the cell suspension was combined with 0.3 to 2 µg of recombinant plasmid DNA. The mixture was then subjected to a 1.5-kV, 800-Ω, 25-µF electric pulse in electroporation cuvette using a Gene Pulser (0.2cm gap MicroPulser Electroporation cuvette, BioRAD). Immediately following electroporation, 2 ml of MRS medium (with 2% w/v glucose) was added, and the cells were incubated for 3 hours at 37°C before being plated on MRS-glucose (2% w/v) agar plates with erythromycin. The plates were incubated at 37°C for 3 days under anaerobic conditions using Gaspak (M/s Himedia, Bengaluru, India).

3.3.3 Cell-free extract preparation, protein expression analysis and shake flask analysis

In order to assess the intracellular fraction for protein production, cell pellet was resuspended in 50 ml lysis buffer (Tris-50mM pH 6.5, Imidazole-10mM, NaCl-500mM, PMSF-1mM) and incubated at 37 °C for 15 minutes along with lysozyme (Sigma Aldrich, USA) followed by sonication at 33% amplitude (pulse 10 sec ON and 30 sec OFF) for 20 minutes. The lysed cells were centrifuged at 10,000 rpm at 4°C for 10 minutes, and the supernatant collected was loaded on 12% SDS PAGE.

The seed culture for both shake flask and bioreactor experiments was cultivated in MRS-Lactose (3% w/v) broth at 37°C for 16 hours, 150 rpm. For shake flask experiments, the preculture was transferred into 250 mL baffled flasks containing 100 mL of fermentation medium, adjusted to an initial optical density (OD_{600}) of approximately 0.05, and incubated at 37°C with agitation at 150 rpm. Cells were collected regularly at 1 hour time intervals, and cell pellet were harvested by centrifugation for biomass analysis while the supernatant was stored at −20°C for subsequent product analysis.

3.3.4 Transcriptional abundance and enzyme activity assay

The extraction of RNA from *L. bulgaricus* cells was conducted using the reported procedure [156]. cDNA was acquired by reverse transcription of 4 to 5 µg of DNA-free RNA with iScript™ cDNA Synthesis kit (Bio-RAD). q-RT PCR was conducted with iTaq™ Universal SYBR Green Supermix (Bio-RAD). Primers corresponding to specific genes, as indicated in Table 3.3, were formulated in accordance with the publicly available genome sequences of *L. bulgaricus* ATCC 11842 (GenBank accession number: NC_008054.1). The quantification of these genes was executed using 16s ribosomal RNA (16S rRNA) as the internal standard. The real-time PCR was conducted in a LightCycler instrument (ABI PRISM® 7500 Real-Time PCR System, Applied Biosystems, USA) following the manufacturer's guidelines. The samples underwent a 30 s denaturation at 95 °C, followed by 40 cycles of 5 s at 95°C and 34 s at 60 °C. The data were analysed using the ABI PRISM 7500 System Sequence Detection software. Three independent experiments were performed to compute relative gene expression, which was calculated using the $2^{-\Delta\Delta CT}$ method [157].

To measure d-lactate dehydrogenase (*dldh*) activity, the reaction mixture (1 mL) contained 100 mM potassium phosphate buffer (pH 7.5), 1.34 mM D-lactate, 2 mM NAD^+, and an appropriate amount of cell lysate. The increase in absorbance at 340 nm, corresponding to NADH formation,

was monitored continuously for 5 minutes at 37°C using a Shimadzu UV-1800 spectrophotometer. Enzyme activity was calculated using the molar extinction coefficient of NADH (ε340 = 6.22 mM^{-1} cm^{-1}). One unit (U) of *d-ldh* activity was defined as the amount of enzyme catalyzing the formation of 1 µmol of NADH per minute under the assay conditions[158]. Protein concentration in the cell lysates was determined using the Bradford method with bovine serum albumin as the standard. Specific enzyme activity was expressed as units per milligram of protein (U mg^{-1} protein). All measurements were performed in duplicates, and the results are presented as mean ± standard deviation. Enzymatic activity for each of the key glycolytic enzymes were measured at 340 nm by monitoring the decline or rise of NAD (phosphate) [NAD(P)H or $NAD(P)^{+}$] using methods as described in published literature [81].

Table 3.3: Oligonucleotide pairs used for determining gene expression of LdbVI104_07

Primers	Protein/gene	Amplicon size (bp)	Tm (°C)
Sense: ATCGGAAACTGTCATTCTTG Antisense: CTAATCCTGTTCGCTACCC	16s rRNA	157 bp	52
Sense:CATCCTGCGTCAAGACAAG Antisense:TAAGCGATAACCTTAGCGC	*Ldb0101*	174 bp	53
Sense: TTCCGCTACCGTATGGAC Antisense: CACGTCATAGGCGATGAC	*Ldb1010*	165 bp	50
Sense: TAACTTCATCTTTGCTTC Antisense: CACCACGGGCAACCATCA	*Ldb0839*	179 bp	55
Sense: TGTTGTCGTTATTGGTG Antisense:CGCGTCCATTGCCGTCATG	*Ldb0838*	160 bp	52
Sense:GAAGCTCAACCTGGACG Antisense:ATCCAGGTATCTGGTGGCA	*Ldb1772*	184 bp	48
Sense:GCTGACTGGCCTGGAC Antisense:CGGAAGCCCCAAAGGC	*Ldb0119*	179 bp	50
Sense:TTACACTTCAGTTTTG Antisense:TCAGCAGTTTCAGC	*Ldb0230*	149 bp	45
Sense:GATTCACACTTCCCTTTTG	*Ldb1734*	150 bp	50

Antisense:CTGGACACGTCCTTGTCC			
Sense: ACTTCATTCCGTGACGG Antisense:CACGTTCAATAGCTTCG	*Ldb1294*	170 bp	51
Sense:ACGGCCATGAAGGCTG Antisense: GTGATAACGCCGATC	*Ldb0636*	152 bp	55
Sense:AGTCGTTTTGGCCGGGACTGT Antisense:TCAAAGGCCGGAATCC	*Ldb1593*	180 bp	62

3.3.5 Acid tolerance assay

Acid tolerance assay was done as per protocol described in previous reports [159]. All recombinant strains were cultured at an initial pH of 6.5 and harvested during the mid-exponential growth phase (OD_{600} ~2.0). To assess acid tolerance, the cells were centrifuged, washed, and resuspended in modified MRS medium adjusted to pH 3.3 using 25 % (w/v) lactic acid. At various time intervals, 1 mL samples were taken, centrifuged at 10,000 × g for 2 minutes, and washed with saline to remove residual acidic medium. The cells were then serially diluted, plated on MRS agar, and incubated at 37°C for 48 h. Cell viability was expressed as the log of colony forming units (CFU mL^{-1}) with increase in fermentation time.

3.3.6 Evaluation of the optimal carbon source and media optimization for enhanced DLA biosynthesis

All constructed recombinant strains were cultured in shake flask experiments using MRS media supplemented with 3 % (w/v) lactose, galactose, mannose, and fructose to evaluate their growth performance, substrate utilization, and DLA production. The strain exhibiting the highest DLA titre and substrate conversion efficiency was identified and selected for further optimization using the One-Factor-at-a-Time (OFAT) approach. The optimization process involved evaluating the effects of varying incubation temperatures (37°C, 40°C, and 45°C) on DLA production and substrate metabolic efficiency. Additionally, the impact of amino acid supplementation on DLA titre and substrate utilization rate was assessed by supplementing the media with different concentrations (0.3, 0.5, 1, and 1.5 g L^{-1}) of glutamine, arginine, cysteine, leucine, valine, lysine, and phenylalanine (selected based on literature reports). The buffering effect of calcium carbonate ($CaCO_3$) was also investigated by incorporating it into the media

at different ratios to lactose (w/w). All experiments were conducted in duplicates, and the averaged data were analysed to determine the optimal medium ingredients facilitating maximum DLA production and substrate utilization.

3.3.7 Bioreactor experiment

Bioreactor experiments were conducted using optimized medium obtained from shake flask experimental results reported in section 3.3.6. Inoculum was cultured for 6 hour in 100 mL MRS media supplemented with 10 g L^{-1} lactose. DLA fermentation was performed in a 1.5 L autoclavable bench-scale bioreactor (Minifors 2, Infors AG) containing 30 % (w/v) lactose supplemented with required amino acids at optimal amounts. The bioreactor operating conditions viz., pH, temperature, stirring speed were maintained at 6.5, 40°C and 150 rpm respectively. Dissolved oxygen tension and air flow rate value was maintained at 20 % and 0.2 vvm respectively [150] . pH was maintained by automatic feeding of 4 N KOH. All experiments were conducted in duplicates, and the mean outcomes of the experimental data were provided.

3.3.8 Metabolite analysis and DLA optical purity

Supernatant collected at regular intervals for all shake-flask and bioreactor studies were further evaluated for metabolite quantification. Glucose, lactate, and other metabolites in the collected supernatant were assessed using Phemomenex Aminex HPX-87H column and Phenomenex guard column attached to HPLC-RID detector system (M/s. Shimadzu, Corp., Kyoto, Japan). The isocratic elution procedure was adopted with a flow rate of 0.6 mL min^{-1} and a mobile phase of 5 mM H_2SO_4. Total lactic acid were evaluated using UV detector at 210nm. Cell growth was measured using a UV-Vis spectrophotometer (Spectronics 200, ThermoFisher, UK. All the analysis was performed in duplicates, and the metabolite concentrations were represented as mean value of duplicates. To specifically verify the presence of DLA, a *dldh* based enzyme kit (K-DATE, M/s Megazyme, Ireland) was used and LLA was analysed using an L-lactate oxidase kit (K-LATE, M/s Megazyme, Ireland) based on manufacturers protocol in a multimode plate reader (VarioskanTM Lux, M/s ThermoFisher Scientific). Optical purity was measured using the formula given in eq1.

3.3.9 Statistical analysis

All the shake flask study and bioreactor experiments in this present study were carried out in duplicates and the results were expressed as mean values. Experiments including transcriptional abundance and enzymatic activity were carried out in triplicates and the results expressed as

mean values ± standard deviations. Graphs were plotted using Origin2021 (OriginLab Corporation, USA) software for statistical analysis and evaluated using analysis of variance (ANOVA) and Tukey Post-hoc test. Student's t-test was performed using Microsoft Excel (Microsoft, Redmond, WA, USA). Statistical analysis was conducted with a p-value of <0.05 considered indicative of statistical significance.

3.4 Results and discussion

3.4.1 Construction of Leloir pathway and ATP balance optimization for complete lactose utilization in recombinant *L. bulgaricus* VI104

Lactobacillus delbrueckii subsp. *bulgaricus* is known for its inability to utilize galactose, which is evident from the presence of galactose in fermentation media expelled during fermentation [79]. Galactose utilization in bacteria typically occurs through one of two pathways: the Leloir pathway or the Tagatose-6-phosphate pathway. The major genes involved in the Leloir pathway include Galatokinase (*galK)*, Galactose-1-phosphase uridyltransferase (*galT)*, and Galactose mutarotase (*galM)* [160]. It has been observed that *galM* gene is already present in the *L. bulgaricus* genome, although its role was previously unknown[161]. The presence of these genes raises questions about their functionality and potential for galactose metabolism. Previous reports also states that partial galactose utilization in *L. bulgaricus* can occur at higher lactose concentrations, suggesting a possible conditional activation of these pathways [79]. *L. bulgaricus* lacks a complete Leloir pathway for galactose metabolism[162]. While it possesses some of the genes for Leloir pathway, they are often not expressed or functional under normal conditions. This metabolic characteristic contributes to the accumulation of galactose in fermented dairy products like yogurt [163].

Highest DLA titre was observed for initial lactose concentration of 30 g L^{-1} from shake flask level media optimization experiments and was used as optimal concentration for all subsequent shake flask fermentations, since higher lactose concentration led to substrate inhibition [79]. In order to enhance DLA yield from lactose, the incomplete galactose utilization pathway in *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 was addressed by reconstructing the complete Leloir pathway through heterologous expression of *galK* and *galT* from *Lactobacillus helveticus* (*Fig 3.1*). SDS-PAGE analysis confirmed the successful expression of *galK* and *galT* in the engineered recombinant strain (*Fig. 3.2b*). Enzymatic assays further demonstrated significantly elevated activity levels in the recombinant strain, with *galK* and *galT* activities

reaching 58 U mg^{-1} and 38 U mg^{-1}, respectively, in the whole-cell lysate. In contrast, no detectable activity was observed in the wild-type strain.

Complete consumption of lactose was observed within 27 hours for the wild-type *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 strain, resulting in a maximum DLA titre of 1.98 g L^{-1}, alongside a peak biomass concentration of 1.0 g L^{-1} (*Fig 3.2d*). Lactose was completely hydrolysed into glucose and galactose, and the glucose moiety was completely consumed within 35 hours of fermentation and galactose remained completely unutilized for wild VI104 (*Fig 3.2d*). Notably, DLA was not produced from galactose metabolism, indicating a metabolic limitation in galactose assimilation and its subsequent conversion into DLA within the native metabolic framework of this strain [164]. The lactose consumption rate was measured at 1.3 g g^{-1} h^{-1}, while the specific DLA productivity reached 0.0707 g g^{-1} h^{-1} of biomass. The specific growth rate (μ) for wild VI104 was estimated as 0.076 h^{-1}. For recombinant strain LdbVI104_01 (*galK-galT* heterologous gene overexpression), the amount of galactose expelled out into media was 5.23 g L^{-1} which was utilized back by the recombinant strain after 27 hours (*Fig 3.2e*). Overall galactose in media remaining by 40 hour was 1.89 g L^{-1} with maximum DLA titre achieved was 1.89 g L^{-1}, slightly less compared to wild VI104. Notably, the lactose utilization rate decreased by 2.18-fold compared to the wild strain, while galactose metabolism was markedly enhanced. Although earlier studies have suggested that *L. bulgaricus* stores some galactose as a macromolecule [165], it is hypothesized that a significant portion of galactose in recombinant strain LdbVI104_02 is actively utilized through the engineered Leloir pathway. The observation that the recombinant strain LdbVI104_01 exhibits enhanced galactose metabolism while experiencing a reduced lactose utilization rate aligns with findings from previous studies on metabolic engineering in LAB. For instance, research on *Lactococcus lactis* demonstrated that the introduction of the Leloir pathway genes significantly improved galactose utilization, although it sometimes resulted in decreased lactose fermentation efficiency due to resource allocation shifts within the metabolic network [166]. Similarly, a study involving *L. plantarum* highlighted the potential of engineered strains to effectively metabolize both lactose and galactose yet also noted a tendency for residual lactose when competing pathways were activated, which suggests that optimizing one metabolic pathway can inadvertently hinder another [167].

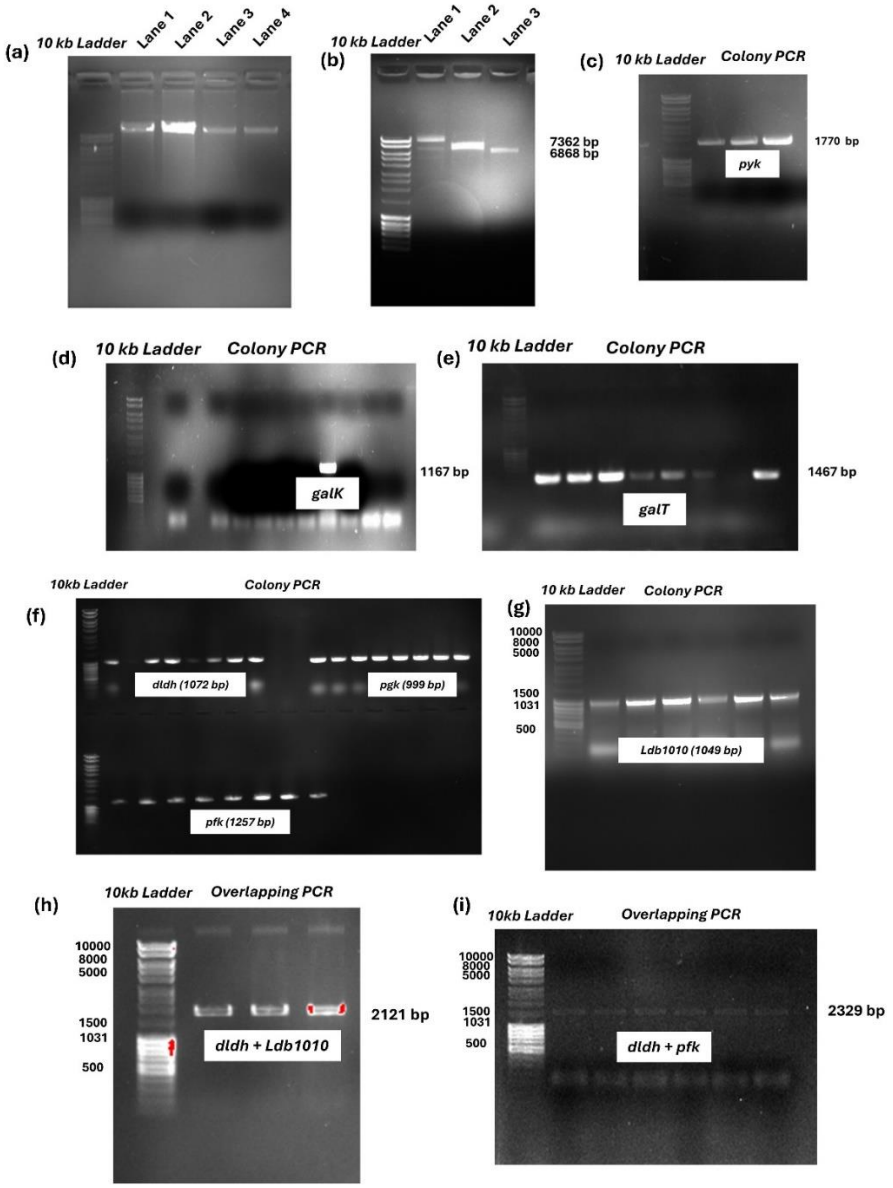


Figure 3.1 Agarose gel images for construction of recombinant VI104 clones LdbVI104_01,02, 03, 04, 05, 06 and 07; (a): Ladder (1st well), Lane 1: genomic DNA of *L. bulgaricus*, Lane 2: genomic DNA of *L. bulgaricus*, Lane 3 genomic DNA of *L. helveticus*, Lane 4: genomic DNA of *L. helveticus*; (b) Ladder (1st well), Lane 1: native plasmid pLEM415, Lane 2: single digested pLEM415 with *SphI* (7362 bp), Lane 3: double digested pLEM415 with *SphI* and *ClaI* (6868 bp (visible) and 891 bp (not visible in gel)); (c) Colony PCR of pyruvate kinase gene (band at 1770 bp); (d) Colony PCR of galactokinase gene (band at 1167 bp); (e) Colony PCR of galactose 5-phosphate uridyltransferase gene (band at 1464 bp); (f) Colony PCR of d-lactate dehydrogenase (1072 bp), phosphoglycerate kinase (999 bp) and phosphofructokinase gene (1257 bp); (g) Colony PCR of Ldb1010 homolog gene (band at 1049 bp); (h) Overlapping PCR of *dldh* homologs (Ldb0101+Ldb1010) (band at 2121 bp); (i) Overlapping PCR of *dldh* +*pfk* (band at 2329 bp).

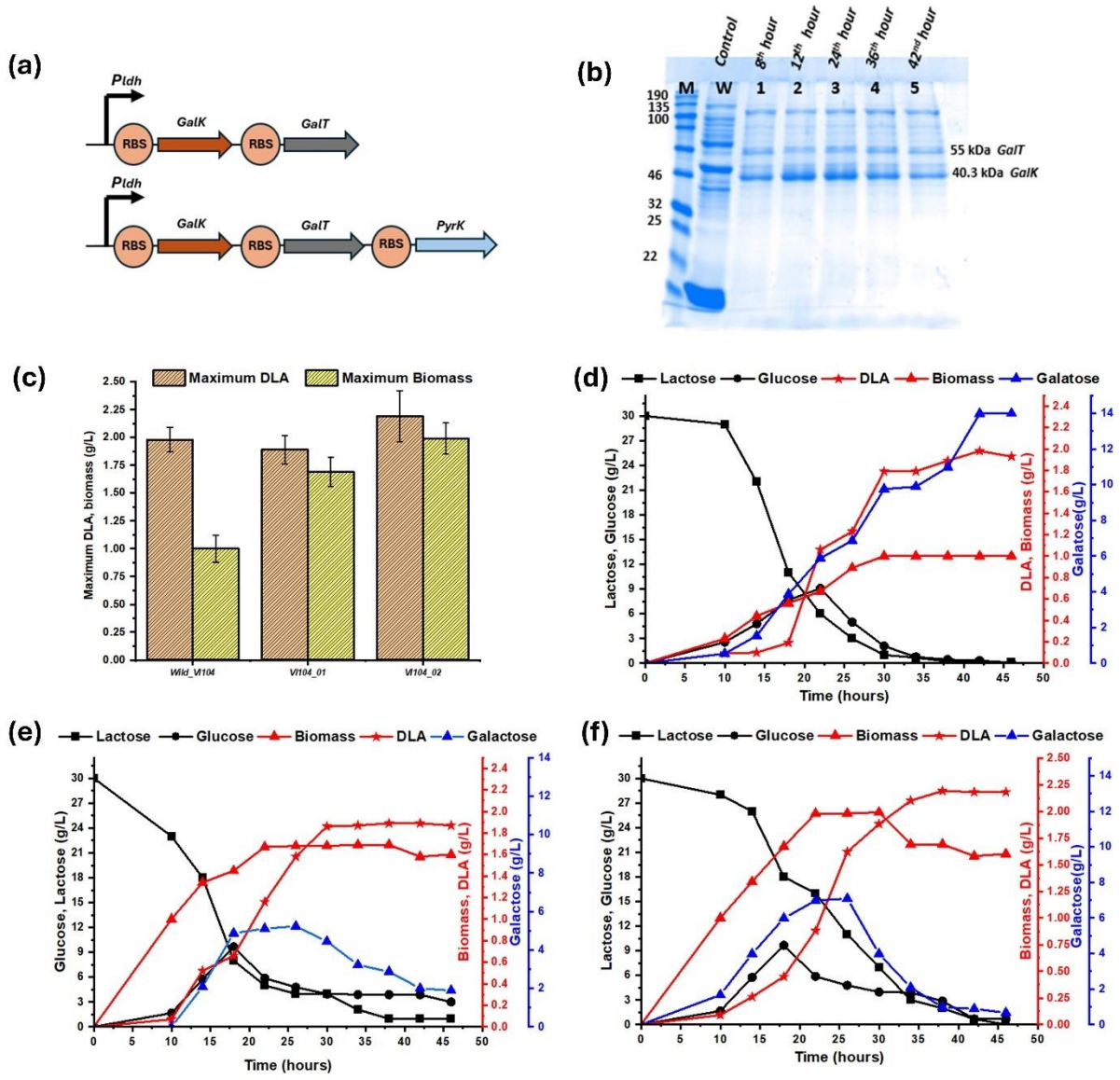


Figure 3.2 Engineering and analysis of recombinant LdbVI104 strains, including gene constructs, protein expression, and dynamic profiles for DLA production and biomass comparison, (a) Designing multiple gene constructs for the Leloir pathway, conferring ATP balance and acid tolerance; (b) SDS-PAGE analysis of whole-cell lysates from recombinant LdbVI104_01 strains, illustrating protein expression profiles. Lane 1: Molecular weight marker; Lane 2: Control (wild VI104); Lane 3: whole cell lysate collected at 8th h; Lane 4: whole cell lysate collected at 12th h; Lane 5: whole cell lysate collected at 24th h, Lane 6: whole cell lysate collected at 36th h, Lane 7: whole cell lysate collected at 42nd h, *galK* and *galT* protein expression is visible in all lanes at 40.3 kDa and 55 kDa respectively; (c)Comparison of the maximum DLA and biomass obtained of recombinant LdbVI104_01 and 02 with wild VI104; (d) Dynamic profile of wild VI104 in presence of MRS-Lactose (3% w/v); (e) Dynamic profile of recombinant LdbVI104_01; (f) Dynamic profile of recombinant LdbVI104_02

It was hypothesized that LdbVI104_01 revealed a significant reduction in intracellular ATP levels relative to the wild-type, potentially attributable to the cloned *galT* gene, which utilizes ATP to produce ADP, thus shifting metabolic flux towards ATP-generating pathways and other by-products. Moreover, the metabolic byproduct profile demonstrated a substantial increase in acetic acid production, further distinguishing the recombinant strain LdbVI104_01 from the wild type by having different metabolite profiles. In order to address the intracellular ATP imbalance, the pyruvate kinase (*pyk*) gene was cloned downstream of *galK* and *galT*, under the control of the constitutive P_{ldhL} promoter, generating the LdbVI104_02 strain (*Fig 3.1*). *Pyk*, catalysing the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, facilitates the formation of ATP and pyruvate in the final step of glycolysis. The *pyk* gene derived from *L. bulgaricus* has been shown to confer acid tolerance, in addition to its role in ATP generation, potentially alleviating ATP scarcity in recombinant cells while enhancing acid tolerance—an essential trait given that product inhibition from accumulated DLA is a major bottleneck in DLA biosynthesis [168].

The recombinant LdbVI104_02 strain demonstrated improved biomass production; however, the maximum DLA titre achieved was 2.19 g L^{-1} (1.1-fold improvement than wild) (*Fig 3.2c*). The lactose hydrolysis rate further decreased, with residual galactose concentrations in the medium reaching 7.09 g L^{-1} at 26 h, which were subsequently metabolized to 0.67 g L^{-1} in the later stages of fermentation (*Fig 3.2f*). The major by-product acetic acid was also enhanced when compared to LdbVI104_01 and wild VI104 (data not shown). This shift is attributed to the role of pyruvate kinase in glycolysis, where the increased conversion of PEP to pyruvate may enhance the flux towards acetate production pathways. The yield of DLA from lactose was overall enhanced by 1.36 folds and 2.34 folds for recombinant strains LdbVI104_01 and LdbVI104_02 respectively. Similarly, the yield of biomass from lactose obtained for recombinant strains were also enhanced compared to wild. Although the glucose utilization rate remained unchanged, the galactose utilization rate improved for both recombinant strains, reaching a maximum of 0.255 h^{-1}, demonstrating the successful integration of the Leloir pathway. The specific growth rate for recombinant strains decreased to 0.029 and 0.056 h^{-1} for LdbVI104_01 and LdbVI104_02 respectively symbolizing metabolic burden due to external plasmid expression system. These findings align with prior studies, demonstrating that *pyk* gene expression in *Lactobacillus bulgaricus* enhances glycolytic flux and redirects pyruvate metabolism toward fatty acid biosynthesis due to which there wasn't a significant enhancement in DLA. This metabolic shift reduces lactic acid production, strengthens acid tolerance, and

optimizes sugar utilization and biomass yield [168]. It has also been reported that overexpressing the *pyk* gene enhances growth and biomass yield by increasing glycolytic activity, which supplies additional energy and essential metabolic intermediates for cell growth and division. Consequently, the *pyk* gene has been shown to not only enhance sugar metabolism but also significantly boost overall biomass production in *Lactobacillus casei*, similar to the findings in this study [169].

However, the use of a single constitutive promoter to drive multiple gene expressions resulted in suboptimal expression of genes located downstream in the construct, such as *galT* and *pyk*, in the LdbVI104_01 and LdbVI104_02 strains respectively (*Fig 3.2a*). This reduced gene expression efficiency underscores the importance of promoter engineering or operon optimization for achieving balanced expression and improving metabolic outputs. The presence of these *Gal* genes and their potential conditional activity highlights the complex nature of sugar metabolism in *L. bulgaricus* and suggests that the organism may have retained some vestigial capacity for galactose utilization, even though it is not typically observed under standard fermentation conditions [170][171].

Table 3.4: Kinetic parameters of the recombinant *L. bulgaricus* VI104 strains with constructed Leloir pathway

Strain	Yield (DLA/ lactose)	Yield (Biomass/ Lactose)	Specific substrate utilization rate q_s (Glucose) (g g^{-1} h^{-1})	Specific substrate utilization rate *qs* (Galactose) (g g^{-1} h^{-1})	Specific productivity q_p (g g^{-1} h^{-1})	Specific growth rate μ (h^{-1})	Yield (DLA/ Galactose) (g g^{-1})	Specific substrate utilization rate (q_s)(Lactose) (g g^{-1} h^{-1})
Wild VI104	0.041	0.042	1.89	0	0.070	0.076	0	1.3
LdbVI104_01	0.086	0.05	1.98	0.255	0.028	0.029	0.082	1.14
LdbVI104_02	0.076	0.123	1.81	0.212	0.032	0.056	0.155	0.607

3.4.2 Effect of overexpression of key *d-lactate dehydrogenase* homologs systematically for enhanced DLA biosynthesis

Previous literature has elucidated the major *dldh* gene and its homologs to delineate their roles in DLA biosynthesis [172]. *Ldb0101* is the principal *dldh* gene, extensively characterized for

its structural and functional attributes, and has been employed in metabolic engineering across various other organisms [173]. Conversely, *Ldb1010* is another pivotal gene implicated in DLA biosynthesis under specific aerobic conditions, unlike *Ldb0101* which functions efficiently under anaerobic conditions [172]. The objective of this secion was to overexpress both genes in *L. bulgaricus* and assess their functionality and efficacy in DLA production under optimized fermentation parameters, both individually and synergistically (*Fig 3.3b*). DLA titres and biomass achieved for all recombinant strains under anaerobic (static) conditions were reduced compared to aerobic (shaking) conditions, therefore aerobic conditions were applied for all investigations in this study.

It was observed that recombinant LdbVI104_03 (*Ldb0101)* exhibited higher efficiency in DLA biosynthesis compared to LdbVI104_04 (*Ldb1010)*, achieving a maximum DLA titre of 2.81 g L^{-1}, whereas the maximum DLA titre for LdbVI104_04 obtained was 0.98 g L^{-1} under aerobic conditions in flask level with 3% initial lactose (Table 3.5). The maximum biomass concentration attained for LdbVI104_03 was enhanced compared to wild which signifies the efficient expression of *dldh* (*Ldb0101*) gene in recombinant *L. bulgaricus* with high metabolic burden assimilation. The recombinant LdbVI104_04 strain showed less expression of *Ldb1010* with lessened overall biomass formation reaching 1.13 g L^{-1}, which corroborates with literature [172] that has characterized *Ldb0101* as the principal *dldh* gene, suggesting that its structural and functional attributes are well-suited for DLA biosynthesis compared to the homologs. When both genes were co-expressed, the maximum DLA titre achieved for LdbVI104_05 (*Ldb0101+Ldb1010)* was 2.20 g L^{-1} with superior kinetic parameters when compared to wild VI104. The yield, productivity and substrate utilization rate were much more efficient when *Ldb0101* was expressed solely with high optical purity of DLA (> 99%). However, LdbVI104_05 cultured under static conditions did not produce a high DLA titre, with metabolic flux diverted towards other by-products, primarily acetic acid. In contrast, *Ldb1010* was reported to produce DLA in an *E. coli* expression host under both aerobic and anaerobic conditions [172].

The obtained data from this study highlights that while *Ldb0101(dldh)* significantly contributes to enhanced DLA biosynthesis, *Ldb1010* has a more nuanced role, perhaps involved in condition-specific metabolic functions that may extend beyond direct DLA production as reported in previous literature[172]. The significantly lower DLA yield and biomass achieved by *Ldb1010* under similar conditions supports the hypothesis that *Ldb1010* may fulfil alternative metabolic roles or regulatory functions that do not directly prioritize DLA

production. Additionally, the increase in substrate utilization rate observed in the co-expressed strain LdbVI104_05 may be linked to an increased cellular demand for metabolic intermediates to accommodate both catalytic pathways, thus leading to less efficient DLA production per unit of lactose.

3.4.3 Overexpression of major glycolytic genes for shifting carbon flux towards enhanced DLA biosynthesis

Glycolysis is key pathway to DLA production, converting glucose to pyruvate, which *d-ldh* reduces to DLA using NADH as a cofactor. This enzyme-driven step is crucial in shaping fermentation outcomes [1]. The enhancement of lactic acid production, particularly DLA, has been linked to the regulation and expression of several key genes involved in glycolysis, including phosphofructokinase (*pfk*), pyruvate kinase (*pyk*), phosphoglycerate kinase (*pgk*), triosephosphate isomerase (*tpi*) and glucokinase (*glk*)[174]. *Pfk* regulates glycolysis by converting fructose-6-phosphate to fructose-1,6-bisphosphate, boosting glycolytic flux and pyruvate levels to enhance lactic acid production. Overexpressing *pfk* in strains like *Corynebacterium* sp. increases lactic acid yields by channelling glucose through glycolysis and sustaining DLA production during fermentation. Similarly, elevated *pgk* activity improves ATP generation, enhancing pyruvate conversion to lactic acid. In homofermentative lactic acid bacteria, ATP primarily derives from sugar metabolism via the Embden–Meyerhof pathway [175][176].

To enhance DLA biosynthesis, *L. bulgaricus* strains were engineered to coexpress *Ldb0101* (the major *dldh* gene), *pfk*, and *pgk* in a sequential manner, resulting in the construction of recombinant strains LdbVI104_06 and LdbVI104_07 (*Fig 3.3b*). These strains exhibited significant improvements in DLA titre, yield, and metabolic efficiency compared to the wild-type strain VI104, thereby validating the effectiveness of multiple gene overexpression strategies. The recombinant strain LdbVI104_06, harbouring pLEM415_06 (*dldh+pfk)*, displayed a slight enhancement in DLA titre to 2 g L^{-1} (1-fold enhancement from wild-type), coupled with a maximum biomass to 1.83 g L^{-1}. However, despite the established role of *pfk* in enhancing DLA production in other microbial systems, its overexpression in *L. bulgaricus* did not result in a pronounced improvement in DLA titre but lead to enhanced biomass formation. Contrastingly, strain LdbVI104_07, expressing all three genes (*dldh, pfk, and pgk*), demonstrated the most pronounced metabolic enhancement, achieving the highest DLA titre of 2.65 g L^{-1} (99.09% optical purity) alongside a biomass of 1.57 g L^{-1}. Specific lactose utilization

rates decreased across all recombinant strains due to metabolic reprogramming; however, the overall DLA yield from lactose was increased by an average of 7-folds compared to wild-type (Table 3.5). Notably, LdbVI104_03 (*dldh*) and LdbVI104_07 (*dldh+pfk+pgk*) achieved the highest productivity rates of 0.094 g L^{-1} h^{-1} and 0.089 g L^{-1} h^{-1}, respectively (Table 3.5). This improvement is attributed to the coexpression of the *pgk* gene, which enhances ATP regeneration by catalyzing the conversion of ADP to ATP, thereby compensating for the increased energy demand and also enhancing overall DLA biosynthesis. Similarly, the glucose utilization rates for LdbVI104_03 and LdbVI104_07 enhanced by 33% and 58% respectively. These findings align with previous reports showing that overexpression of glycolytic genes such as glucokinase (*gck*), glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), *pfk*, triosephosphate isomerase (*tpi)*, and enolase (*eno*) led to a 146% increase in LLA and a 56% increase in DLA concentrations along with sugar utilization capabilities [177].

Further analysis of enzymatic performance revealed robust overexpression of *dldh* gene for all the recombinant strains when expressed under the P_{ldhL} promoter [107]. SDS-PAGE profiling confirmed elevated *dldh* expression levels along with other genes (*Fig 3.3c*), and enzymatic activity assays measured from whole-cell lysates during the exponential growth phase indicated a 76% increase in *dldh* activity relative to the wild-type. Strain LdbVI104_07, expressing all three genes, achieved the highest DLA titre using lower cell biomass, indicating a synergistic effect of multiple gene overexpression on metabolic efficiency similar to previous reports [178][177]. This concludes that overexpressing *pfk* boosts glycolytic flux, while increased *pgk* activity enhances ATP generation, both critical for DLA biosynthesis in *L. bulgaricus*.

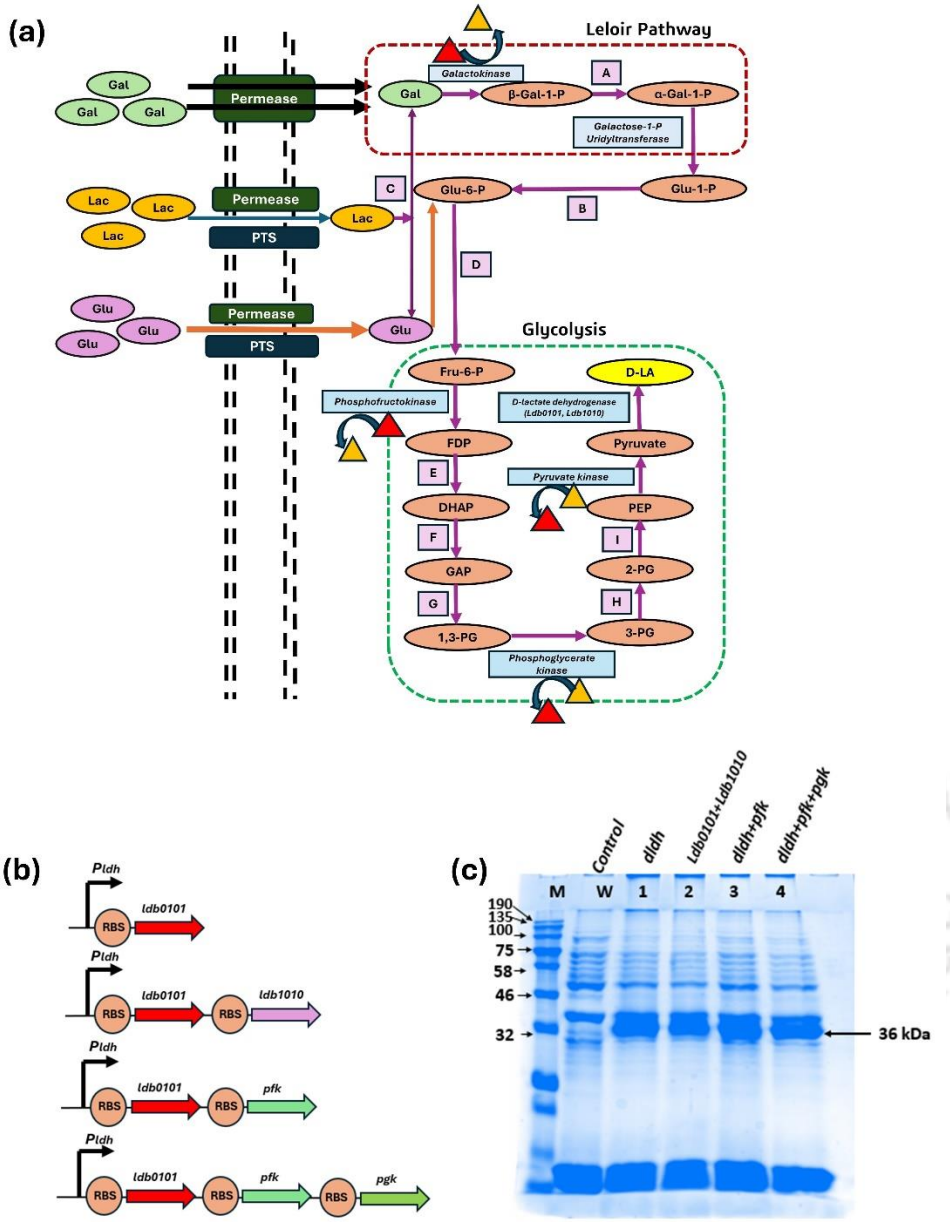


Figure 3.3 Metabolic pathway schematics, gene constructs, and SDS-PAGE analysis demonstrating overexpression of glycolytic genes and successful *dldh* protein expression in recombinant *L. bulgaricus* strains; (a)Schematic representation of the metabolic pathways for glucose, lactose and galactose utilization in lactic acid bacteria. Enzymes mentioned in blue box are the target genes expressed in this study with an hypothesis to enhance DLA production. β-Gal-1-P refers to β-galactose-1-phosphate, α-Gal-1-P refers to α-galactose-1-phosphate, Glu-1-P refers to glucose-1-phosphate, Glu-6-P refers to glucose-6-phosphate, Fru-6-P refers to fructose-6-phosphate, FDP refers to fructose-1,6-bisphosphate, DHAP to dihydroxyacetone phosphate, GAP refers to glyceraldehyde-3-phosphate dehydrogenase, 1,3-PG refers to 1,3-bisphosphoglycerate, 3-PG to 3-phosphoglycerate, 2-PG refers to 2-phosphoglycerate, 2-PG refers to 2-phosphoglycerate, PEP refers to phosphoenolpyruvate and D-LA refers to D-lactic acid. The alphabets

denoted the enzymes involved for the respective reactions. (A) Galactose mutarotase, (B) α-phosphoglucomutase, (C)β-galactosidase, (D)Phosphoglucose isomerase, (E)Aldolase, (F) triose phosphate isomerase, (G) Glyceraldehyde- 3-phosphate dehydrogenase, (H) Phosphoglycerate mutase, (I) Enolase; (b) Multiple gene construct designs for overexpression of major glycolytic genes in systematic order ; (c) SDS-PAGE analysis of whole-cell lysates from recombinant *L.bulgaricus* strains, illustrating protein expression profiles. Lane 1: Molecular weight marker; Lane 2: Control (wild VI104); Lane 3: LdbVI104_03; Lane 4: LdbVI104_05; Lane 5: LdbVI104_06; Lane 6: LdbVI104_07. A prominent protein band corresponding to ~36 kDa, representing the *dldh* gene product, is observed in Lanes 3–6. This indicates successful expression of the proteins driven by the P_{ldhL} promoter.

Table 3.5: Kinetic parameters of the recombinant *L.bulgaricus* VI104 strains with systematic overexpression of key homologous glycolytic genes for enhanced DLA biosynthesis in MRS-lactose (3% w/v)

Strain	Specific growth rate (μ) h^{-1}	Max DLA titre $g\ L^{-1}$	Specific Substrate Utilization Rate (q_s) $g\ g^{-1}\ h^{-1}$	Yield ($Y_{DLA/S}$) $g\ g^{-1}$	Yield ($Y_{X/S}$) $g\ g^{-1}$	Yield ($Y_{DLA/X}$) $g\ g^{-1}$	Productivity (q_p) $g\ g^{-1}\ h^{-1}$	Max Biomass conc. $g\ L^{-1}$
Wild VI104	0.076	1.98	1.3	0.042	0.041	0.93	0.070	1.00
LdbVI104_03 (*dldh*)	0.0469	2.81	1.259	0.293	0.175	1.51	0.094	1.65
LdbVI104_04 (*Ldb1010*)	0.0489	0.98	1.047	0.036	0.132	0.76	0.012	1.13
LdbVI104_05 (*Ldb0101+Ldb1010*)	0.0528	2.20	1.064	0.148	0.125	1.16	0.081	1.78
LdbVI104_06 (*dldh+pfk*)	0.0469	2.00	1.069	0.255	0.225	1.26	0.044	1.83
LdbVI104_07 (*dldh+pfk+pgk*)	0.0772	2.65	1.089	0.264	0.095	1.24	0.089	1.57

3.4.4 Optimization for enhanced DLA production in engineered *L. bulgaricus* VI104

DLA biosynthesis, as a strictly carbon source-dependent metabolic pathway, relies entirely on the substrate glucose [2]. Consequently, both the type and molar concentration of carbon source exert a pivotal influence on DLA production efficiency [150]. Lactose has been recognized as the optimal carbon substrate for *Lactobacillus delbrueckii* subsp. *bulgaricus*, although fructose has also been shown to support substantial growth and metabolic activity in this bacterium [179]. Sucrose was identified as a non-metabolizable carbon source for *L. bulgaricus* (as confirmed in assays with the wild-type strain, where sucrose utilization was absent), while lactose facilitated maximal DLA titres and it corroborated with reported literature [2]. To potentiate DLA biosynthetic output in the recombinant strains, a comprehensive carbon source screening was executed to elucidate kinetic parameters associated with substrate-specific metabolite flux and carbon partitioning. Doubling time of *L bulgaricus* also significantly varies under different carbon sources[179].

It was observed that all recombinant *L. bulgaricus* produced relatively high DLA titres in presence of lactose and fructose compared to glucose and mannose. Highest DLA titres achieved in flask level was 2.81 g L^{-1} for recombinant LdbVI104_03 and 2.65 g L^{-1} for recombinant strain LdbVI104_07 in presence of lactose (*Fig 3.4a*). The wild-type strain VI104 exhibited a baseline DLA production of 1.98 g L^{-1} from lactose, 1.67 g L^{-1} from fructose, and 0.23 g L^{-1} from mannose, with no production observed from galactose. Among the mutant strains, LdbVI104_03 (*dldh*) produced the highest DLA titre from lactose (2.81 g L^{-1}), representing a 42 % increase over the wild-type. This strain also achieved improved titres from fructose (1.34 g L^{-1}) and mannose (0.34 g L^{-1}). Notably, galactose was effectively utilized only by strain LdbVI104_01 (*galK*+*galT*), which produced 0.38 g L^{-1} DLA. Strain LdbVI104_07 (*dldh*+*pfk*+*pgk*) exhibited a marked improvement in fructose utilization, achieving the highest titre of 2.45 g L^{-1}, corresponding to a 46% increase relative to VI104. Similarly, LdbVI104_06 (*dldh*+*pfk*) achieved 2.1 g L^{-1} DLA from fructose, underscoring the synergistic effect of metabolic engineering for improved hexose catabolism. A strong correlation was observed between DLA productivity and titre across substrates, with lactose as the preferred carbon source for most strains. For instance, LdbVI104_03 exhibited the highest lactose productivity of 0.094 g L^{-1} h^{-1}, consistent with its superior titre (2.81 g L^{-1}) (*Fig 3.4d*). Similarly, VI104_07 achieved a fructose productivity of 0.045 g L^{-1} h^{-1}, aligning with its high titre (2.45 g L^{-1}).

Interestingly, LdbVI104_06, which showed balanced improvements across substrates, demonstrated comparable productivities from lactose (0.0444 g L^{-1} h^{-1}) and fructose (0.045 g L^{-1} h^{-1}), further supporting the link between substrate assimilation optimization and DLA production efficiency. Biomass concentrations were also affected by carbon source and genetic modifications (*Fig 3.4b*). Strain LdbVI104_02 (*galK+galT+pyk*) achieved the highest biomass concentration from lactose (2.01 g L^{-1}), which likely contributed to its relatively high titre (2.19 g L^{-1}) and productivity (0.032 g L^{-1} h^{-1}) since lactic acid is a growth associated product. In contrast, strain LdbVI104_03 showed a trade-off between biomass concentration and DLA titre and despite achieving moderate biomass (1.65 g L^{-1}) on lactose, it outperformed all other strains in DLA titre (2.81 g L^{-1}) and productivity (0.094 g L^{-1} h^{-1}), suggesting a metabolic shift favouring DLA production overgrowth. Although DLA titres from galactose remained lower than from hexoses, these findings substantiate the scope for application of metabolic engineering to expand substrate range. Strain LdbVI104_07 displayed the most consistent performance across substrates, achieving high titres and productivity from lactose (2.65 g L^{-1}, 0.081 g L^{-1} h^{-1}), fructose (2.45 g L^{-1}, 0.078 g L^{-1} h^{-1}), and mannose (0.65 g L^{-1}, 0.023 g L^{-1} h^{-1}), with comparatively lower biomass production underscoring the effectiveness of combining *dldh*, *pfk*, and *pgk*. The results of this study underscore the importance of optimal substrate selection and metabolic engineering in enhancing DLA production in *Lactobacillus bulgaricus*. The observed preference for lactose and fructose over glucose and mannose aligns with previous findings that demonstrate how specific carbon sources can significantly influence metabolite yields [2] [180]. This results also corroborates with previous reports where engineered strains exhibited improved production of lactic acid when utilizing alternative sugars, emphasizing the role of substrate choice in metabolic efficiency [177].

The recombinant strain LdbVI104_07 exhibited elevated DLA titre utilizing low cell biomass concentration (high specific DLA productivity) and favourable kinetic profiles using 3% (w/v) lactose-MRS, was further selected for optimization studies. The influence of amino acid supplementation on DLA production and specific lactose utilization rate (q_S) in strain LdbVI104_07 was assessed across varying concentrations (0.3, 0.5, 1, and 1.5 g L^{-1}) (*Fig 3.4e*). The study revealed that DLA production and lactose utilization (q_S) were significantly influenced by amino acid type and concentration similar to previous literature reports [181]. At 0.3 g L^{-1}, lysine and phenylalanine achieved the highest DLA titres (2.9 g L^{-1} and 2.89 g L^{-1}, respectively), with glutamine and phenylalanine showing superior q_S values (1.099 and 1.098 g g^{-1} h^{-1}). At 0.5 g L^{-1} concentration, phenylalanine and lysine maintained the highest titres,

while glutamine and phenylalanine optimized q_S (1.077–1.098 g g^{-1} h^{-1}). At 1 g L^{-1}, glutamine and phenylalanine yielded high DLA production (2.98 g L^{-1}); high q_S (1.098 and 1.123 g g^{-1} h^{-1}) was observed for glutamine and arginine. At 1.5 g L^{-1}, lysine had the highest titre (2.98 g L^{-1}), followed by glutamine and phenylalanine (2.89 g L^{-1}), which also showed optimal q_S. Glutamine and phenylalanine consistently enhanced DLA production and q_S, supporting their role in optimizing amino acid supplementation for improved industrial DLA yields in recombinant *L. bulgaricus* LdbVI104_07.

Similar to previous reports [182]., the addition of $CaCO_3$ to the culture was evaluated to determine its effect on DLA production. It is well-established that $CaCO_3$ sequesters lactic acid as calcium lactate, thereby buffering the culture pH (*Fig 3.4f*). To investigate its effect, $CaCO_3$ was supplemented at varying ratios to lactose as the carbon source: 1:4 (low), 1:2 (medium), and 3:4 (high) (w/w) [182]. However, these conditions did not significantly improve the DLA titre or the specific lactose utilization rate in shake flask cultures (*Fig 3.4f*). The limited impact of $CaCO_3$ in this setup can likely be attributed to the small culture volumes, where lactic acid production may not sufficiently lower the pH, while other metabolites, such as acetic acid, might contribute to greater pH reductions. Nevertheless, the buffering capacity of $CaCO_3$ holds potential for large-scale bioreactor applications, where higher lactic acid accumulation could result in more significant pH fluctuations [183]. In addition to buffering, the impact of temperature on DLA production was assessed. Among the tested conditions, 40°C yielded the highest DLA titre for strain VI104_07 (*Fig 3.4f*), highlighting the importance of optimizing temperature for enhanced metabolic performance of *L.bulgaricus* [184]. These findings provide insights into the optimal growth conditions for DLA biosynthesis in *L.bulgaricus* and underscore the need for scaling-up experiments to fully evaluate the utility of $CaCO_3$ supplementation [185] [2].

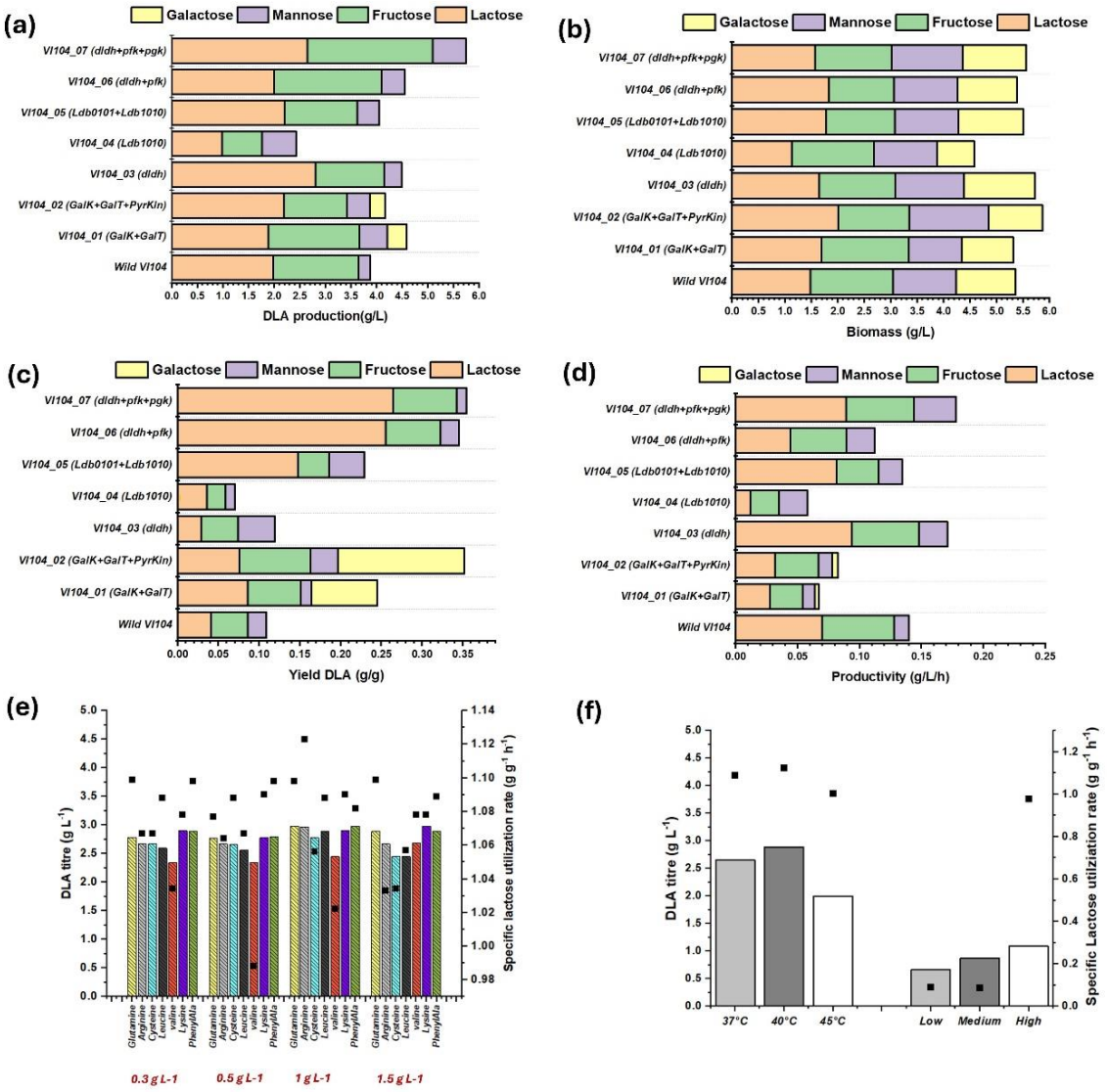


Figure 3.4 DLA production, biomass, yields, and productivity of recombinant L. bulgaricus strains across carbon sources, with optimization studies on LdbVI104_07 for lactose utilization, temperature, and $CaCO_3$ concentration; (a) DLA titres achieved by all *L. bulgaricus* recombinant strains in presence of 3% lactose, galactose, mannose and fructose; (b) Maximum biomass achieved by all *L. bulgaricus* recombinant strains in presence of 3% lactose, galactose, mannose and fructose; (c) DLA yield from respective carbon source calculated for all recombinant *L.bulgaricus* strains; (d) DLA productivity calculated for all recombinant *L.bulgaricus* strains in presence of 3% lactose, galactose, mannose and fructose; (e) DLA titres and specific lactose utilization rates (*qs*) calculated for recombinant strain LdbVI104_07 cultures in 100 ml MRS-Lactose(3% w/v) supplemented with amino acids; (f) Optimization of DLA biosynthesis by recombinant strain LdbVI104_07 at varying temperatures and $CaCO_3$ concentrations

3.4.5 Transcript-level analysis, enzymatic activity, and acid tolerance analysis of recombinant LdbVI104_07

The acid tolerance of the constructed *Lactobacillus delbrueckii* subsp. *bulgaricus* recombinant strains were assessed by measuring the log of CFU mL^{-1} over time in extremely low pH media [159]. The results demonstrated significant variability in acid tolerance among the different recombinant strains. At initial time point (0 hours), the log of CFU mL^{-1} values ranged from 3.6 to 5.03 (*Fig 3.5b*). Over the course of 10 hours, the recombinant strains exhibited different growth patterns. LdbVI104_02 showed the highest acid tolerance, with CFU mL^{-1} increasing from 4.9 at 0 hours to 8.0 at 10 h. The acid tolerance of LdbVI104_02, was enhanced compared to LdbVI104_01, corroborating the hypothesis that *pyk* improves stress resistance [168]. Recombinant strains LdbVI104_06 and LdbVI104_07 exhibited significantly high acid tolerance, evidenced by a significant increase in CFU mL^{-1} during fermentation, underscoring the critical roles of *pgk* and *pfk* genes in acid stress response [168]. In contrast, strains LdbVI104_01, LdbVI104_03, LdbVI104_04, and LdbVI104_05 displayed moderate tolerance, with CFU mL^{-1} values ranging from 4.0 to 5.3 at 10 hours (*Fig 3.5b*). High superior performance of by LdbVI104_06 and LdbVI104_07 enumerates their potential future use for industrial applications requiring robust growth under acidic conditions. This observation aligns with the fact that enhanced glycolysis can lead to increased production of specific intermediate metabolites, which may redirect the metabolic flux towards fatty acid biosynthesis. This shift can contribute to improved acid tolerance in the recombinant strains. Previous studies suggest that sucrose supplementation enhances stress tolerance in *L. bulgaricus*, supporting the current findings that genetic modifications boosting specific pathways may similarly improve stress responses [186]. Similarly, a link between strong growth and acid tolerance in *L. delbrueckii* subsp. *bulgaricus* strains was reported. This aligns with the acquired findings in recombinant strains LdbVI104_06 and LdbVI104_07, highlighting improved growth under acidic conditions as a trait of robust strains [187].

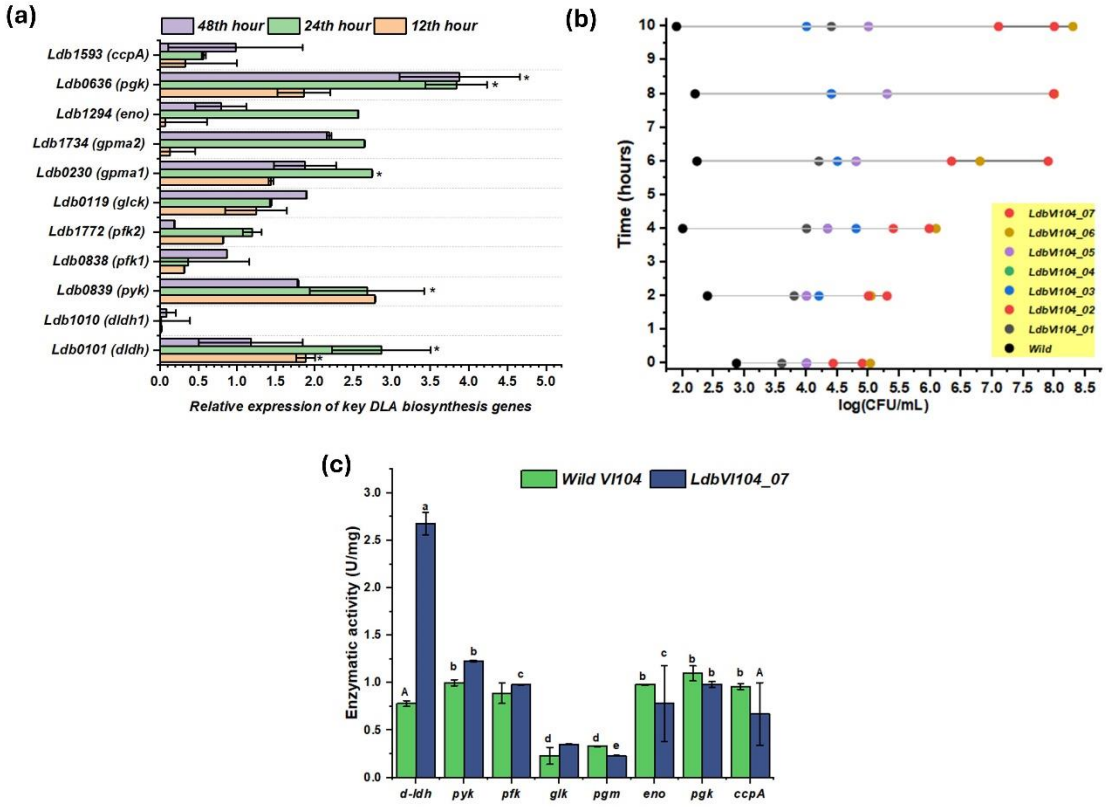


Figure 3.5. Transcript analysis of key DLA biosynthesis enzymes, acid tolerance assay, and enzymatic activity of glycolytic genes in recombinant LdbVI104_07; (a) Transcript level analysis indicating relative expression of key enzymes responsible for DLA biosynthesis. Results are mean ± standard deviation: *p < 0.05 indicate significant difference between wild VI104 and recombinant LdbVI104_07, analysed by Student's t-test. Each experiment included three biological replicates. (*Ldb0101: dldh, Ldb1010: dldh1, Ldb 0838: pfk1, Ldb0839: pyk, Ldb1772: pfk2, Ldb0119: glck, Ldb0230: gpma1, Ldb1734: gpma2, Ldb1294: eno, Ldb0636: pgk, Ldb1593: ccpA*); (b) Acid tolerance assay showing the acid resistance levels of recombinant strains over the fermentation time; (c) Enzymatic activity of the key glycolytic genes responsible for enhanced DLA production in LdbVI104_07. The same letter on the bars denotes insignificant variations among the levels of the factors (p > 0.05)

The transcript level analysis and enzymatic activity data demonstrate a coordinated regulation of genes and enzymes involved in glycolysis and DLA biosynthesis in recombinant strain LdbVI104_07. The robust expression of *dldh* in the engineered strain (*Ldb0101*) at 24 hour (2.87 ± 0.64) aligns with its 3.4-fold increase in enzymatic activity (2.68 ± 0.12 U mg^{-1}), driving enhanced DLA production compared to wild-type (*Fig 3.5a and c*). In contrast, *dldh* homolog (*Ldb1010*) showed minimal expression and negligible activity, corroborating with low DLA titres (in previous section) highlighting its limited role in DLA biosynthesis in *L.bulgaricus*.

The high expression of *pyk* (*Ldb0839*) at 12 hour (2.79 ± 0.003) corresponds to its 1.2-fold rise in activity (1.23 ± 0.009 U mg^{-1}), ensuring sufficient pyruvate supply for DLA biosynthesis. Similarly, *pfk1* (*Ldb0838*) exhibited a 2.7-fold transcript increase by 48 hour, while *pfk2* (isoform of *pfk*) peaked at 24 hour (1.20 ± 0.12), reflecting moderate enzymatic activity increases (0.98 ± 0.003 U mg^{-1}), supporting efficient glycolytic flux. Increased expression of *glk* (*Ldb0119*) from 12 to 48 hours (52% rise) aligns with its 1.5-fold activity increase (0.35 ± 0.003 U mg^{-1}), facilitating glucose utilization for enhanced DLA production. Enhanced expression of *gpma1* and *gpma2* at 24 hours (2.75 ± 0.003 and 2.65 ± 0.003, respectively) contrasts with decreased *pgm* activity, suggesting a flux shift favoring DLA production. The striking 35-fold rise in *eno* expression from 12 to 24 hours (2.57 ± 0.003) contrasts with its reduced activity, indicating a regulatory bottleneck. Meanwhile, *pgk* transcript levels showed a 2.6-fold increase by 48 hours (3.88 ± 0.78), supporting sustained enzymatic activity (0.98 ± 0.03 U mg^{-1}), crucial for glycolysis and ATP production. Although the *ccpA* gene has been reported to regulate stress responses, in this study, its moderate expression peaking at 48 hours (0.98 ± 0.87) and reduced enzymatic activity in LdbVI104_07 suggest a shift in metabolic priorities, indication of reprogramming to favor DLA biosynthesis with minimal stress formation [116]. Similar findings were previously reported in a mutant strain of *L. bulgaricus*, where significant upregulation of key glycolytic genes (*dldh*, *pgk*, *glk*, *pfk*, and *pyk*), coupled with decreased expression of *ccpA*, resulted in enhanced DLA production [2]. The integration of transcript-level analysis and enzymatic activity profiling demonstrates successful metabolic reprogramming in LdbVI104_07, providing valuable insights into glycolytic regulation and its optimization for future upscaling biosynthesis of optically pure DLA in *L.bulgaricus*.

3.4.6 Batch fermentation in bioreactor using efficient recombinant *L. bulgaricus* strain

The recombinant LdbVI104_07 strain was selected for scale-up experiments due to its superior performance, characterized by consistent high DLA titres achieved with minimal biomass, coupled with enhanced acid tolerance. Pure lactose (99.09% purity) in MRS-lactose 3% (w/v) medium supplemented with amino acids glutamine and phenylalanine (0.15% w/v) was used for batch fermentation using the efficient recombinant strain LdbVI104_07. It was observed that lactose was consumed efficiently over the course of fermentation, decreasing from 30 g L^{-1} at the start to negligible levels (<0.001 g L^{-1}) by 58 hours. Concurrently, DLA production peaked at 9.39 g L^{-1}(99.09% optical purity), achieving a high yield of 0.1888 g g^{-1} based on

lactose conversion. The specific productivity of DLA was calculated to be 0.035 g g^{-1} h^{-1}, demonstrating the metabolic efficiency of the LdbVI104_07 strain over the wild. The volumetric productivity was 0.152 g L^{-1} h^{-1}, and the specific substrate uptake rate (*qs*) was determined to be 1.108 g g^{-1} h^{-1}. Cellular biomass levels increased steadily during the initial phase of fermentation, reaching a maximum of 2.9 g L^{-1}at 62 hours, which indicates efficient conversion of lactose into cellular biomass and metabolites (including DLA). Interestingly, the yield of DLA per unit biomass ($Y_{DLA/X}$) was observed to be 4.64 g g^{-1}, demonstrating the strain's capability for high metabolic flux toward DLA biosynthesis rather than excessive growth. Glucose and galactose, the hydrolysis products of lactose, were also monitored and their accumulation reached peak values of 8 g L^{-1} and 11.89 g L^{-1}, respectively. But glucose and galactose were subsequently depleted by the end of fermentation, reflecting the dual-substrate utilization capability of the strain (*Fig 3.6*). Notably, residual glucose was nearly exhausted by 55 h, while galactose persisted longer, suggesting differential substrate preference and lack of galactose utilization pathway in LdbVI104_07.

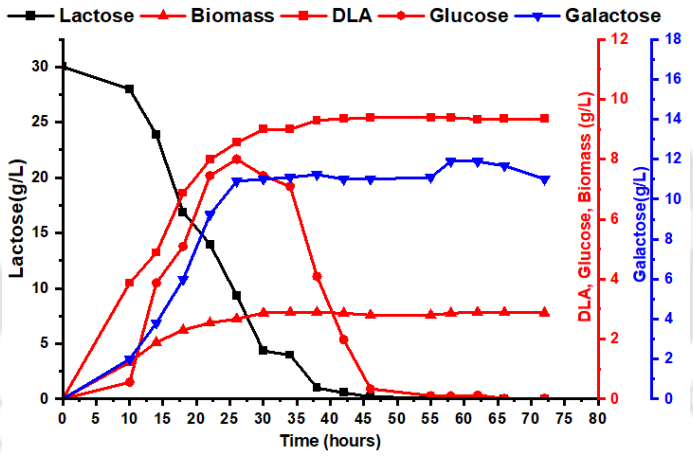


Figure 3.6 Dynamic batch fermentation profile of recombinant LdbVI104_07 in MRS-Lactose (3% w/v) supplemented with a 1 g L^{-1} amino acid mixture

This chapter represents a pioneering investigation into the biosynthesis of DLA in *Lactobacillus bulgaricus* VI104, marking the first exploration of this metabolic pathway in the strain. Various strains of *L. bulgaricus* demonstrate the ability to produce DLA at varying levels [188][150]. For instance, *L. bulgaricus* T15 exhibited remarkable DLA production through continuous fermentation using immobilized cells, significantly surpassing yields achieved with free cells, as noted in previous reports [189]. Additionally, previous reports indicate that immobilized *L. bulgaricus* strains achieved a DLA yield of 2.68 g $L^{-1}h^{-1}$ over extended fermentation periods, outperforming free-cell systems [190]. This highlights the

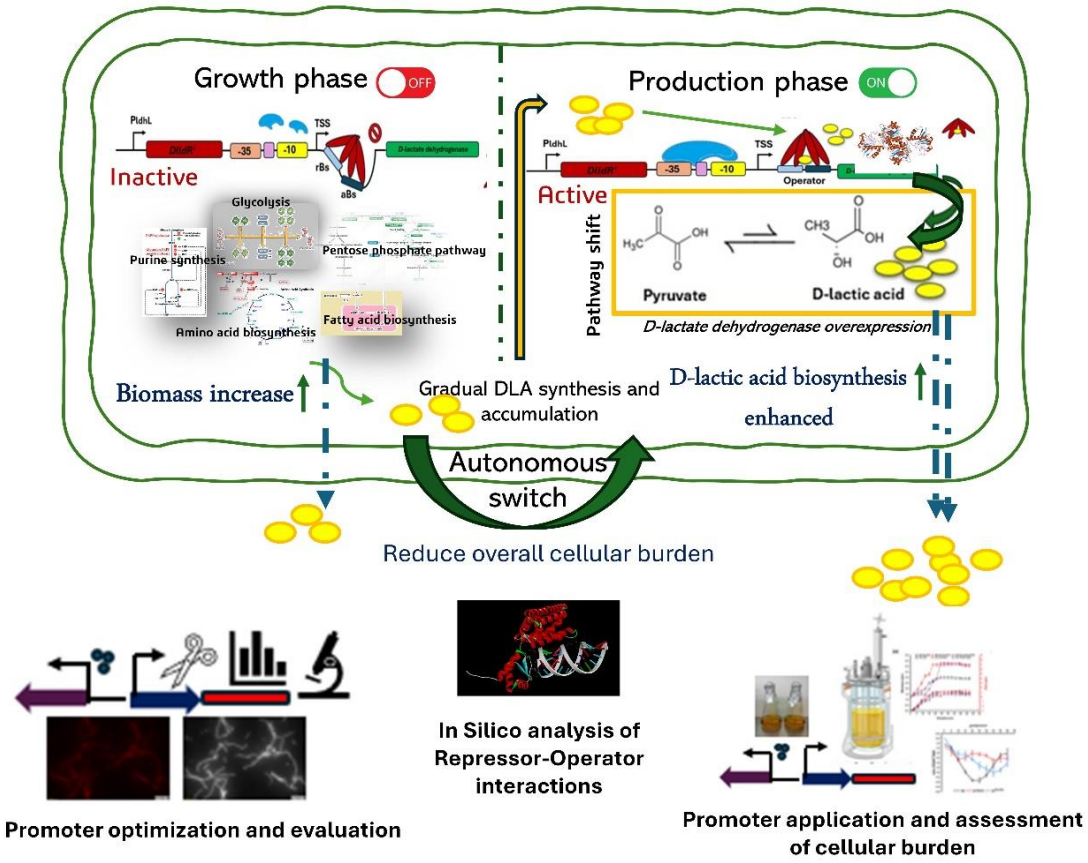
potential of immobilization techniques to enhance both productivity and stability, akin to the metabolic efficiency observed in the recombinant strain LdbVI104_07. In this chapter, the recombinant strain LdbVI104_07 further demonstrated efficient lactose conversion into DLA, through the successful overexpression of major glycolytic genes. The high specific productivity and yield of DLA from cellular biomass observed in this study suggest that the recombinant strain LdbVI104_07 excels in channelling metabolic flux towards DLA biosynthesis rather than biomass accumulation. These characteristics can be further leveraged in industrial applications through advanced bioprocessing strategies like continuous fermentation and cell recycling [191] [192]. Such approaches, as demonstrated in previous reports, have proven to enhance titre and productivity, reinforcing the efficiency and scalability of DLA fermentation systems in *L.bulgaricus* VI104 could be explored in future investigations.

3.5 Conclusion

Overall, chapter 3 represents the first-of-its-kind to demonstrate enhanced DLA biosynthesis in engineered *Lactobacillus bulgaricus* VI104 through innovative metabolic engineering approaches combined with fermentation optimization. Not all *Lactobacillus bulgaricus* strains exhibit high transformation efficiency, but *L. bulgaricus* VI104 stands out as one of the strains with notably high transformation efficiency and is also relatively underexplored. By reconstructing the galactose utilization pathway, balancing ATP flux, and overexpressing key glycolytic genes alongside *dldh* homologs, DLA titre were markedly improved by 240%. The engineered strain LdbVI104_07, with co-expression of major glycolytic genes, achieved superior DLA production of 9.39 g L^{-1} in bioreactor batch fermentation with exceptional optical purity of 99.09%. Further optimization of fermentation parameters, including amino acid supplementation, enhanced metabolic efficiency and DLA production ability. More importantly, the core research work of this present thesis establishes molecular cloning tools and transformation protocols tailored to engineering *L. bulgaricus*, offering a foundation for advanced genetic manipulations. These tools not only enable fine regulation of DLA biosynthesis but also pave the way for genetic manipulation of *L. bulgaricus* for future applications in probiotic and therapeutic industries.

CHAPTER 4:

Engineering the DLA responsive promoter/repressor system as dynamic metabolic engineering tool in *Lactobacillus delbrueckii* subsp. *bulgaricus* for controlled DLA biosynthesis



4.1 Summary

Dynamic metabolic engineering integrates synthetic logic circuits into cellular systems, optimizing metabolic fluxes and augmenting biosynthesis of target metabolites. This study evaluated a DLA-responsive promoter-repressor system from *Pseudomonas fluorescens* A506, re-engineered for heightened sensitivity and functional efficacy in *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104. The codon-optimized regulatory architecture exhibited peak performance at DLA inducer concentration range of 60–100 mM, validated by fluorometry and microscopy. As an application, overexpression of D-lactate dehydrogenase (*dldh*) downstream of the engineered promoter repressor system enabled finely tuned modulation of DLA biosynthesis, autonomously regulating the transition between growth and production phases, thereby attenuating overall metabolic load. Cross-species compatibility was confirmed by excising regulatory elements from the promoter-repressor system and functionally assessing them in recombinant *L. bulgaricus*. Molecular docking elucidated critical noncovalent interactions between D-*LldR* repressor and operator nucleotide sequence in absence of inducer DLA. The engineered promoter construct with high efficiency effectively elevated DLA biosynthesis by 1.63-folds and expanded the overall fermentation time relative to constitutive systems, attaining maximum DLA titre of 9.02 g L^{-1} in bioreactor setup. These results substantially broaden the molecular cloning toolkit available for *L. bulgaricus*, fostering potential future applications in biotherapeutics and probiotics.

4.2 Introduction

The main aim of metabolic engineering is to facilitate the biosynthesis of pharmaceuticals, biofuels, chemicals, and other valuable compounds in an efficient and sustainable manner where we optimize the productivity, concentration, and yield of desired compounds in microbial hosts, thereby ensuring economic viability. However, this goal is challenging due to the complex regulatory networks that maintain cellular homeostasis, which span transcriptional, translational, post-translational, metabolic, signalling, and epigenetic levels [193]. In genetic modifications within metabolic engineering, careful balance in achieving the desired results while avoiding unintended consequences due to cellular burden remains challenging [194][193]. Especially, in cases where manipulations are overly aggressive or excessive, deleterious effects can occur due to unexpected disturbances in the host's inherent regulatory mechanisms, leading to suboptimal production outputs [195]. An emerging strategy to enhance microbial synthesis efficiency is controlling biosynthetic pathways within tightly regulated cellular environments. Approaches

like gene overexpression, knockouts, promoter/RBS engineering, protein engineering, and enzyme co-localization have proven effective in optimizing enzyme expression and function [196][197][198]. However, continuous transcription throughout the fermentation process imposes constraints on the expression of foreign proteins, which can be potentially toxic to the host cell or create an excessive metabolic burden [199]. In contrast, dynamic control mimics natural biological processes, enabling organisms to adapt their metabolic states to real-time fluctuations in intracellular and environmental circumstances [200]. Dynamic metabolic engineering has emerged as a preeminent approach to potentiate the production of desired metabolites in microbial cell factories [201]. This paradigm dynamically regulates metabolic pathways based on environmental or intracellular cues, enhancing host robustness, reducing metabolic burden, and improving yields [193].

Bacterial promoters are key regulatory sequences that initiate transcription, critically shaping gene expression and enabling diverse applications in biotechnology and synthetic biology [202] [203] [193]. Promoters also play a crucial role in facilitating interspecies bacterial interactions through various mechanisms, including synthetic biosensors and quorum sensing systems [204]. Characterization of a heat-shock protein promoter (P_{hsp}) from *Enterococcus faecium* and its cloning into a lactococcal vector to create a stress-inducible shuttle vector for *Lactobacillus* and *Lactococcus* species was reported [205]. Researchers recently engineered 'LactoSpanks,' a collection of IPTG-inducible promoters derived from the *Bacillus subtilis* $P_{hyper\text{-}spank}$ promoter, for *Lactobacillus gasseri*, achieving varied expression levels with reduced basal leakiness [97]. Similarly, improvements to the classic lac and tet inducible promoter systems to enhance their strength, control, and portability across Gram-negative bacteria was reported [206]. The authors modified the promoter architecture to include consensus sequences for strong RNA polymerase binding and an optimized ribosome binding site. Two inducible promoters, the xylose-inducible promoter P_{xylA} from *Lactobacillus brevis* and the nisin-inducible promoter P_{nisA} from *Lactococcus lactis* were used as valuable tools for regulating gene expression in the probiotic bacterium *L. plantarum* [199]. Similarly, the P_{srfA} promoter derived from *Bacillus subtilis* was employed to develop a versatile inducible expression system in various LAB hosts, including *Lactobacillus plantarum, Lactococcus lactis*, and *Pediococcus acidilactici* is described [207]. Hence, the broad-host-range promoter enabled high-level recombinant protein production across multiple LAB species, providing a flexible biotechnology tool.

Recently, research studies are focussed on developing target metabolite-dependent promoter-repressor systems. Autonomous dynamic regulation (ADR) enables microorganisms to internally

adjust metabolic flux for biomass or metabolite accumulation, using the product itself as an inducer to regulate gene expression at specific fermentation stages [208]. The previously reported muconic acid-inducible promoter, an advanced tool in dynamic metabolic engineering, adjusts gene expression based on muconic acid levels to balance metabolic flux, enhancing yield and efficiency through precise, real-time pathway regulation [193]. Lactate-inducible promoters are a relatively underexplored area in molecular biology, with very few literatures reports available to date. Identification and characterization of lactate-inducible systems from various bacterial species, including *Escherichia coli* and *Cupriavidus necator* was reported recently [209]. In this present study, a novel DLA-inducible promoter-repressor system from *Pseudomonas* species was meticulously evaluated in *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 to achieve dynamic metabolic control for enhanced and regulated DLA biosynthesis. The DLA-inducible promoter modulated by the GntR-family repressor protein D-*lldR* from *Pseudomonas fluorescens* was previously reported to exhibit high specificity for DLA and robust inducibility [210]. *L. bulgaricus* is a LAB extensively employed as a starter culture in the production of yogurt and cheese [211]. Research on *L. bulgaricus* have focused on the genetic manipulation to enhance its applications as probiotics and understand its metabolic processes for health benefits [212] [213] [214]. Despite its industrial relevance, research on the metabolic engineering of *L. bulgaricus* for producing value-added compounds remains limited due to the scarcity of molecular cloning tools and challenges with electroporation which we have addressed in previous chapters. DNA uptake and integration in *L. bulgaricus* are hindered by native plasmids and restriction-modification systems [117]. DLA, a stereoisomer of lactic acid, is valued for its role in synthesizing PLA, a biodegradable thermoplastic from renewable resources, with high-purity DLA biosynthesis by *Lactobacillus delbrueckii* subsp. *bulgaricus* enhancing the sustainability and efficiency of PLA production[2]. In the current study, the D-*lldR*-P_{lldP} promoter-repressor system was codon-optimized, engineered, and validated in *L. bulgaricus* through a prototype dynamic control network regulating fluorescent protein expression thereby facilitating controlled and enhanced DLA biosynthesis followed by an application where the efficient promoter screened was further evaluated for dynamic control by substituting fluorescent protein with a key DLA biosynthetic gene.

4.3 Material and methods

4.3.1 Enzymes, primers and gene synthesis

All restriction and other genetic manipulation enzymes, including Q5 High-Fidelity DNA Polymerase and Phusion High-Fidelity DNA Polymerase, were procured from New England Biolabs (NEB). Oligonucleotide primers, as listed in Table 4.1, were synthesized by BioServe, India. The ribosome binding site (RBS) was uniformly employed across all constructs along with the spacer region. The D-*lldR* gene region was codon-optimized for enhanced protein expression in *L. bulgaricus* and subsequently synthesized by GenScript, USA, in tandem with the P_{lldP} promoter. The sequence for this optimized D-*lldR'* gene is provided in the Fig 4.1.

Table 4.1: List of primers used in this study

Name of the primer	Sequence of the primer 5' to 3'
GSP_FP	ctagaggatccccgggtaccggtgaaaggagctgtacaaatggggtttgatcaggtgcgtc
GSP_RP	ctcggaggaggccatcctaggtatggggtggcccctgattg
F1_FP	tagaggatccccgggtaccggtgaaaggagctgtacaaatggggtttgatcaggtgcgtcag
F1_RP	caacttctcagtggatcctttgccgcctgcgaaactttggtcg
F2_FP	caggcggcaaaggatccactgagaagttgctctcccc
F2_RP	ggggtgtgtcaagaattccatgcaagctttcgccg
F3_FP	aaagcttgcatggaattcttgacacaccctgcccagg
F3_RP	ctcggaggaggccatcctaggcatatggggtggcccctgattg
F4_FP	cctcgtcggcgaaagcttgcatggaattcgcccacag
F4_RP	acgtaccttaagcgggtgtcgctgacatttaaccataatggt
F5_FP	aaccaaccaagctcaccaaacaacaa
F5_RP	tacagtcgctgtgggctagattaagagttggttcgagtgg
F6_FP	tggactaataaaactcactctccacc
F6_RP	accggtacccggggatcctctagagtcgacctgattattttg
F7_FP	cccgatcttgtacagctcctttcaccg
F7_RP	gtttggtgagcttggttacagtcgctgtgggctagat
F8_FP	gtgagaggtaccggtacccggggatcctctag
F8_RP	aaccaagctcaccaaacaa
F9_FP	tacagtcgctgtgggctagatgtcagcgaca
F9_RP	acagtcgctgtgggctagaacatgtcg
F10_FP	tgaaaggagctgtacaaatgactaaaatttttgcttacgcaattcgt
F10_RP	atcaagcttatcgatttagccaaccttaactggagtttcagc
*dldh*_FP	cctaggatgactaaaatttttgcttacgcaattcgt
*dldh*_RP	atcgatttagccaaccttaactggagtttcagc

4.3.2 Bacterial strains, plasmids and cultivating conditions

The bacterial strains employed in this study and the plasmids details are listed in Table 4.2. *E. coli* TOP10F' and BL21 (DE3) cells were cultivated and maintained in Luria-Bertani (LB) broth and LB agar (LB broth containing 1.5% (w/v) agar). *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104 and ATCC 11842 was cultivated in deMan Rogosa Sharpe (MRS) media. For preculture preparation, the strain was propagated in 10 mL MRS broth at 37°C shaking conditions prior to the culture entered its phase of exponential growth (~10^6 CFU mL $^{-1}$). MRS medium composition: glucose 20 g L^{-1}, beef extract 10 g L $^{-1}$, peptone 10 g L^{-1}, yeast extract 5 g L^{1}, $CH_3COONa.3H_2O$ 5 g L^{-1}, K_2HPO_4 2 g L^{-1}, tri-ammonium citrate 2 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, $MnSO_4.4H_2O$ 0.5 g L^{-1} and Tween 80 1 g L^{-1}. A PVDF membrane filter with a 0.22-µm diameter was used to sterilise the fermentation medium. All wild and recombinant strains used in this study were grown in 37°C shaking conditions. 100 µg mL^{-1} of ampicillin for *E. coli* and 25 µg mL^{-1} of erythromycin for *L. bulgaricus* were supplemented in the medium for the stable replication of recombinant plasmids. All reagents and chemicals including antibiotics employed in this present study were purchased from M/s HiMedia Laboratory Chemicals (Bengaluru, India).

Table 4.2: List of bacterial strains and plasmids used in this study

Strain	Description	Source
E. coli TOP10F'	Host for gene cloning, F′ [*lacI* q, Tn10(TetR)] *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(StrR) *end*A1 *nup*G	Invitrogen
E. coli BL21(DE3)	*F⁻ ompT gal dcm lon hsdS*$_B$($r_B^- m_B^-$) *λ(D*	Novagen
Lactobacillus delbrueckii subsp.*bulgaricus* VI104	LAB expression host	A kind gift from Prof. Emmanuelle Maguin, French National Institute for Agriculture, Food, and Environment INRAE, France

LdbVI104-P_{GSP}	*Lactobacillus delbrueckii* subsp.*bulgaricus* VI104 harbouring plasmid pLEM415-D-*lldR'*-P_{lldP}-*mRFP1* (P_{GSP})	This study
LdbVI104-P_{Hyb}	*Lactobacillus delbrueckii* subsp.*bulgaricus* VI104 harbouring plasmid pLEM415-D-*lldR'*-P_{ldhL}-*PlldP-mRFP1* (P_{Hyb})	This study
LdbVI104-P_{Mut}	*Lactobacillus delbrueckii* subsp.*bulgaricus* VI104 harbouring plasmid pLEM415-D-*lldR'*-P_{ldhL}-*mRFP1* (P_{Mut})	This study
LdbVI104-$P_{GSP\text{-}dldh}$	*Lactobacillus delbrueckii* subsp.*bulgaricus* VI104 harbouring plasmid pLEM415-D*lldR'-PlldP-dldh* ($P_{GSP\text{-}dldh}$)	This study
LdbVI104-$P_{GSP\Delta Op}$	*Lactobacillus delbrueckii* subsp.*bulgaricus* VI104 harbouring plasmid pLEM415-D*lldR'*-P_{lldP}-Δop-*mRFP1* (P_{GSP}-Δop)	This study
LdbVI104-$P_{GSP\Delta Rep}$	*Lactobacillus delbrueckii* subsp.*bulgaricus* VI104 harbouring plasmid pLEM415-D*lldR'*-P_{lldP}-Δop-*mRFP1* (P_{GSP}-Δop)	This study
LdbVI104-$P_{GSP\Delta Op\Delta Rep}$	*Lactobacillus delbrueckii* subsp.*bulgaricus* VI104 harbouring plasmid pLEM415-*DlldR'-PlldP*-ΔopΔrep (P_{GSP}-Δop Δrep)	This study
Plasmids	**Description**	**Source**
pLEM415-*ldhL-mRFP1*	Em^r, Amp^r; *E. coli-Lactobacillus* shuttle vector	Deposited by Dr.Sujin Bao (Addgene plasmid no.99842)
pLEM415-D-*lldR'*-P_{lldP}-*mRFP1* (P_{GSP})	Em^r, Amp^r; The codon optimized repressor protein expressed under P_{ldhL} of pLEM415 along with P_{lldP} native promoter sequence from *Pseudomonas fluorescense* present downstream.	This study

pLEM415-D-*lldR'*-P_{ldhL}-P_{lldP}-*mRFP1* (P_{Hyb})	Em^r, Amp^r; The P_{GSP} construct harbouring the P_{ldhL} constitutive promoter sequence, strategically positioned between the codon-optimized repressor gene and the native P_{lldP} promoter sequence from *Pseudomonas* sp.	This study
pLEM415-D-*lldR'*-P_{ldhL}-*mRFP1* (P_{Mut})	Em^r, Amp^r; P_{GSP} promoter harbouring the P_{ldhL} constitutive promoter sequence, and devoid of native P_{lldP} promoter sequence.	This study
pLEM415-D-*lldR'*-P_{lldP}-*dldh* ($P_{GSP\text{-}dldh}$)	Em^r, Amp^r; P_{GSP} promoter harbouring *dldh* gene from *L. bulgaricus* replacing *mRFP1*.	This study
pLEM415-D-*lldR'*-P_{lldP}-Δop-*mRFP1* (P_{GSP}-Δop)	Em^r, Amp^r; P_{GSP} promoter devoid of operator sequence.	This study
pLEM415-D-*lldR'*-P_{lldP}-Δrep-*mRFP1* Δrep (P_{GSP}-Δrep)	Em^r, Amp^r; P_{GSP} promoter devoid of repressor sequence.	This study
pLEM415-D-*lldR'*-P_{lldP}-ΔopΔrep (P_{GSP}-Δop Δrep)	Em^r, Amp^r; P_{GSP} promoter devoid of operator and repressor sequence.	This study

Em^r, Erythromycin resistance; Amp^r, Ampicillin resistance

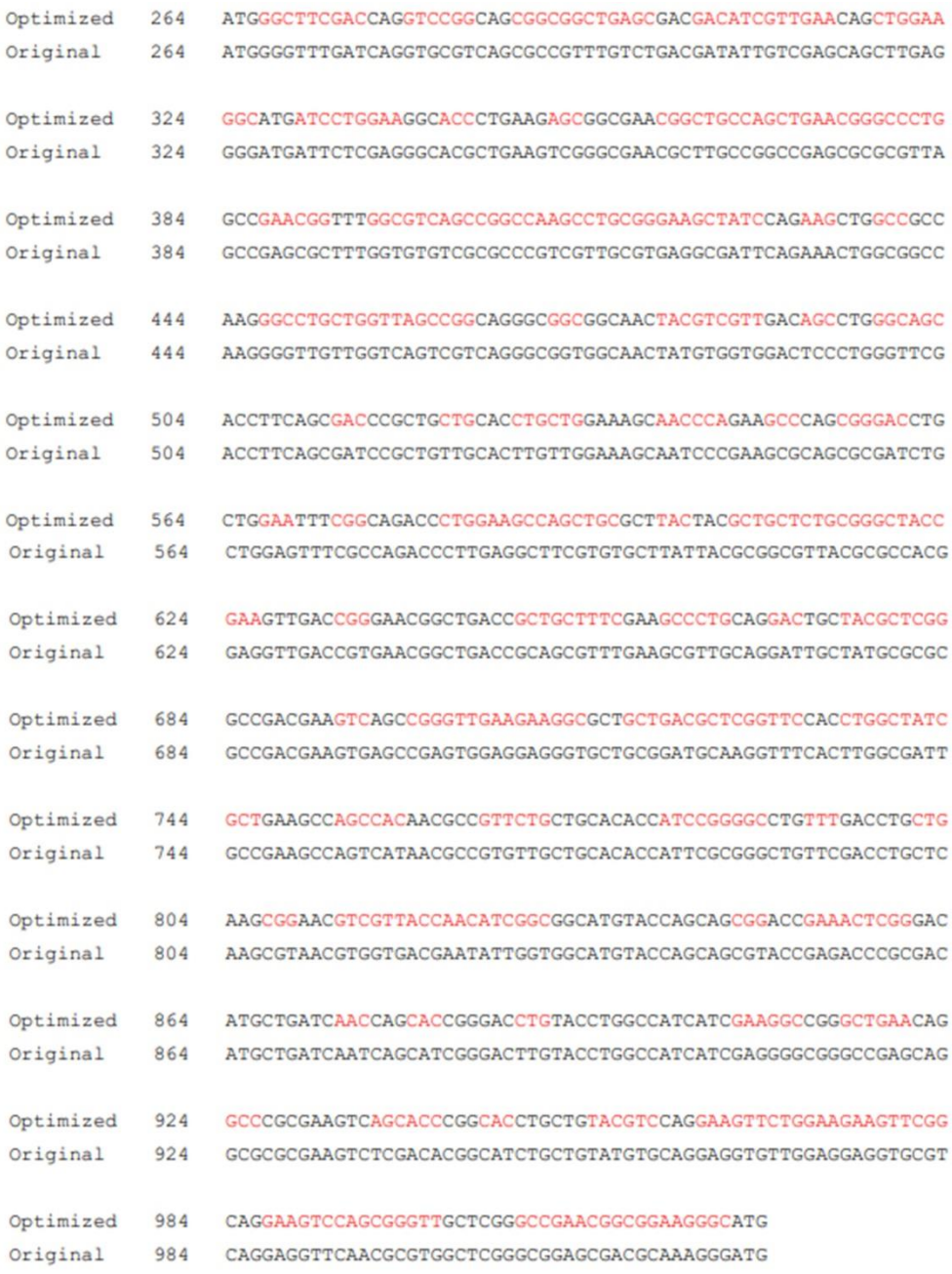


Figure 4.1 Comparison of the native and codon-optimized *DlldR'* gene sequences for *Lactobacillus delbrueckii* subsp. *bulgaricus*

4.3.3 Recombinant plasmids construction

The plasmid pLEM415 containing ori site for both *E .coli* and *Lactobacillus* sp. was used to develop the DLA responsive promoter-repressor system in this study [107]. DLA-inducible promoter from *Pseudomonas fluorescens* A506, regulating the D-*lldR* and *lldP_dld*-II operons was used in this study [210]. This promoter region comprises -10 and -35 elements along with a palindromic operator site. To construct the recombinant P_{GSP} plasmid, the codon-optimized (denoted as D-*lldR'*) repressor protein along with P_{lldP} promoter sequence (synthesized from

GenScript, USA) was cloned upstream of the *mRFP1* gene in the pLEM415 plasmid. P_{GSP} construction was achieved using a conventional cloning method, employing *AgeI* and *AvrII* restriction sites along with primers GSP_FP and GSP_RP. To construct the hybrid promoter system (P_{Hyb}), the P_{ldhL} promoter from pLEM415 was PCR-amplified using primers F2_FP and F2_RP. The amplified fragment was then ligated between the D-*lldR*' repressor gene and the -35 region of the native P_{lldP} promoter via Gibson assembly, employing primers F1_FP, F1_RP, F3_FP, and F3_RP, where all fragments were amplified separately with 15 bp overlapping sequences between each fragment. Similarly, the P_{Mut} construct was generated by PCR amplification of the entire P_{Hyb} promoter, excluding the native -35 and -11 regions (P_{lldP}), using primers F4_FP and F4_RP through Gibson assembly. For autonomous dynamic regulation (ADR) application, the pivotal gene responsible for DLA production, *dldh* was amplified from the genomic DNA of *L. bulgaricus* ATCC 11842, utilizing the Sigma Aldrich genomic DNA isolation kit. The *dldh* gene was subsequently ligated downstream of the P_{GSP} construct, replacing the *mRFP1* reporter gene using primers (*dldh*_FP and *dldh*_RP) carrying restriction enzymes *ClaI* and *AvrII* (conventional cloning method). Following ligation, all constructs were transformed into *E. coli* TOP10F' cells via heat shock method, with successful constructs verified via colony PCR. Verified constructs underwent sequence validation through Sanger sequencing (Bioserve, India) and were transformed into *E. coli* BL21 cells and also electroporated into *L. bulgaricus* VI104 for functional characterization analysis. To assess the functional roles of the operator and repressor regions, specific plasmid constructs were engineered using Gibson assembly. The recombinant plasmid P_{GSP} was used as the template for these modifications. For excision of the operator region, primers F5_FP and F5_RP were employed to amplify the entire plasmid, excluding the operator sequence. Similarly, the repressor region was excised using primers F6_FP and F6_RP. To generate a construct devoid of both the operator and repressor regions, the P_{GSP} plasmid served as the template, and the respective fragments were amplified independently using primer pairs F7_FP, F7_RP, F8_FP, F8_RP, F9_FP and F9_RP, followed by their assembly via Gibson assembly. For construction of the (Control) constitutive promoter system, a similar strategy was employed and *dldh* gene was cloned downstream of P_{ldhL} promoter in pLEM415 vector using primers F10_FP and F10_RP.

4.3.4 Electroporation of *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104

The *L. bulgaricus* competent cells were prepared as described by [117] . A freshly cultivated *L. bulgaricus* VI104 sample was serially diluted and inoculated into 100 ml of MRS medium, which was subsequently grown at 42°C. After incubating overnight, the culture was harvested at the

early stationary phase, indicated by an optical density at 600 nm (OD_{600}) of 1.7, and collected through centrifugation. The bacteria were rinsed once with 100 ml of cold electroporation buffer (EB) (comprising 0.4 M sucrose, 1 mM $MgCl_2$, 5 mM KH_2PO_4; pH 6 as per reports by [117]) and then twice with 30 ml of cold EB. The cells were resuspended in EB to reach an OD_{600} of approximately 50. The cell suspension was incubated at 45°C for 20 minutes and subsequently placed on ice for 10 minutes. For electroporation, 80 µl of the cell suspension was combined with 0.3 to 2 µg of recombinant plasmid DNA. The mixture was then subjected to a 1.5-kV, 800-Ω, 25-µF electric pulse in a 0.2-cm cuvette using a Gene Pulser (0.2cm gap MicroPulser Electroporation cuvette, BioRAD). Immediately following electroporation, 2 ml of MRS medium (with 2% glucose) was added, and the cells were incubated for 3 hours at 37°C before being plated on MRS agar plates with erythromycin. The plates were incubated at 37°C for 3 days under anaerobic conditions using Gaspak (Himedia, India).

4.3.5 Fluorescence assay and microscopy

To conduct the fluorescence assay/dynamic responses of the constructed biosensors, the recombinant VI104 strains were cultured in 5 ml preculture overnight, followed by transfer into 50 ml main culture in presence of erythromycin (25µg mL^{-1}), 37 °C, shaking condition 200 rpm. Recombinant VI104 cultures (containing P_{GSP}, P_{Hyb}, P_{Mut}) were allowed to grow for 3 hours followed by induction with pure DLA (99.09% purity) at varying concentrations (10mM to 100 mM). Samples were collected after 3 hours interval to check the normalized fluorescence intensity in plate reader. For the fluorescence assays, cell optical density was determined by measuring absorbance at 600 nm. A volume of 100 µL from the main culture was transferred to a black-wall, clear bottom 96-well plate (Corning, USA) and fluorescence was measured with a microplate reader (Varioskan™LUX, ThermoScientific) at excitation and emission maxima at 587 nm/625nm. Fluorescence values were normalized to the optical density at 600 nm (OD_{600}) to calculate the Relative Fluorescence Units (RFU) using the formula RFU = Fluorescence/OD_{600}. The same procedure was applied to *E. coli* BL21(DE3) engineered strains, which were grown in LB medium supplemented with 100 µg mL^{-1} ampicillin at 37 °C with shaking at 250 rpm. All experiments were performed in triplicate on three separate days. On 1.5 mm thick glass slides (Paul Marienfeld GmbH, Germany), a 10-µL aliquot of the 1000-µL resuspended mixture (PBS with modified bacteria) was deposited on glass slides (Paul Marienfeld GmbH, Germany), and 1.5 H glass coverslips (Carl Roth GmbH, Germany) were placed on top. The samples were then observed using inverted fluorescence microscope (CKX53SF-OLYMPUS, Tokyo, Japan). The *mRFP* signal was captured using a 561 nm laser

and a 575nm long-pass emission filter. Images were adjusted to identical brightness and contrast settings and processed using ImageJ software.

4.3.6 Optimization of fluorescence intensity via One-Factor-at-a-Time (OFAT) approach

To optimize the promoter activity for the constructed promoter library (P_{GSP}, P_{Hyb}, P_{Mut}), a series of experiments were conducted in shake flask cultures. Variables such as induction time, agitation speed (rpm), temperature, inducer molecule, and initial pH were systematically optimized using One-Factor-at-a-Time (OFAT) strategy. Recombinant strains were cultivated in a similar way as detailed in Section 2.5, followed by induction at different time intervals (3, 6, 9, 12, 15, and 18 hours). The normalized fluorescence intensity was measured, and the highest values were recorded and plotted to identify optimal induction timing. Inducer specificity was assessed by testing various molecules, including pyruvate, 3-hydroxypropionate, glyoxylate, lactic acid, and 99% pure DLA (selected based on previous reports) of their ability to activate maximum promoter activity. The recombinant VI104 strains were grown under varying agitation speeds (static, 100 rpm, 200 rpm, and 300 rpm), and normalized fluorescence were denoted as in previous section. Temperature optimization experiments were performed by incubating cultures at 30°C, 35°C, 37°C, 40°C, and 42°C to determine the temperature that maximized promoter activity. The pH of the culture medium was systematically adjusted across a range from 2.5 to 9.5 prior inoculation to evaluate its effect on promoter activity and identify the optimal pH conditions.

4.3.7 Cell free extract preparation, protein expression analysis and enzymatic activity assay

In order to assess the intracellular fraction for protein production, cell pellet was resuspended in 50 mL lysis buffer (Tris-50mM pH 6.5, Imidazole-10mM, NaCl-500mM, PMSF-1mM) and incubated at 37 °C for 15 minutes along with lysozyme (Sigma Aldrich, USA) followed by sonication at 33% amplitude (pulse 10 sec ON and 30 sec OFF) for 20 minutes. The lysed cells were centrifuged at 10,000 rpm at 4°C for 10 minutes, and the supernatant collected was loaded on 12% SDS PAGE.

The reaction mixture (1 mL) contained 100 mM potassium phosphate buffer (pH 7.5), 1.34 mM D-lactate, 2 mM NAD^+, and an appropriate amount of cell lysate. The increase in absorbance at 340 nm, corresponding to NADH formation, was monitored continuously for 5 minutes at 37°C using a Shimadzu UV-1800 spectrophotometer. Enzyme activity was calculated using the molar

extinction coefficient of NADH (ε340 = 6.22 mM^{-1} cm^{-1}). One unit (U) of *d-ldh* activity was defined as the amount of enzyme catalyzing the formation of 1 µmol of NADH per minute under the assay conditions[158]. Protein concentration in the cell lysates was determined using the Bradford method with bovine serum albumin as the standard. Specific enzyme activity was expressed as units per milligram of protein (U mg^{-1} protein). All measurements were performed in triplicate, and the results are presented as mean ± standard deviation.

4.3.8 Bioreactor fermentation and cellular burden assay

Inoculum was cultured for 6 hour in 100 mL MRS media supplemented with 20 g L^{-1} lactose instead of glucose as *Lactobacillus delbrueckii* subsp. *bulgaricus* exhibits enhanced metabolic activity in the presence of lactose compared to glucose [215][216]. DLA production was performed in a 1.5 L bench-scale bioreactor (Minifors 2, Infors AG) containing 20% (w/v) lactose supplemented MRS media. The bioreactor operating conditions, including pH (4.5), temperature (37°C), and stirring speed (200 rpm), were determined based on previously optimized experimental parameters. Dissolved oxygen and air flow rate was maintained at 20% and 0.2 vvm [150]. pH was maintained by automatic feeding of 4 N KOH. All experiments were conducted in in triplicate, and the results are presented as mean ± standard deviation. The intracellular levels of NADH and NAD^+ were quantified following the method previously described [217] [218].

4.3.9 Analytical methods

Glucose, lactate, and other metabolites in the collected supernatant were assessed using Phemomenex Aminex HPX-87H column and Phenomenex guard column attached to HPLC-RID detector system (M/s. Shimadzu, Corp., Kyoto, Japan). The isocratic elution procedure was adopted with a flow rate of 0.6 mL $minute^{-1}$ and a mobile phase of 5 mM H_2SO_4. Lactic acid were evaluated using UV detector at 210nm. Cell growth (OD_{600}) was measured using a UV-Vis spectrophotometer (Spectronics 200, ThermoFisher, UK. Cell pellets collected after centrifugation were washed twice with deionized water to remove any salts present and dried at 80ºC for dry cell weight (DCW) determination. Absorbance can be converted to DCW (in g L^{-1}) values by the OD vs DCW correlation (where 1 OD = 0.38*DCW for wild VI104; 1 OD = 0.69*DCW for Control and 1 OD = 0.47*DCW for dynamic system). All the analysis were performed in duplicates, and the metabolite concentrations were represented as mean of duplicates. To specifically verify the presence of DLA, a *dldh* based enzyme kit (K-DATE, M/s Megazyme, Ireland) was used and L-lactic acid (LLA) was analysed using an L-lactate oxidase kit (K-LATE, M/s Megazyme, Ireland) based on manufacturers protocol in a multimode plate

reader (VarioskanTM Lux, M/s ThermoFisher Scientific). Optical purity was measured using the formula given in Eq 1.

4.3.10 Bioinformatics and statistical analysis

Protein (D-*lldR*) model was generated using AlphaFold Server powered by AlphaFold 3. The ligand (DLA) was downloaded in sdf format from PubChem (CID: 61503). D-*lldR* (protein)-DLA (ligand) docking was done using JAMDA docking tool in ProteinsPlus [219]. The binding pocket was selected according to the binding residues identified by [210]. The model 1 with JAMDA Score (-1.47561) was selected for (D-*lldR*)-Nucleotide binding analysis. For nucleotide-protein docking, the operator sequence (AATTGGTATTACCAATT) was created in pdb format using Biovia Discovery Studio 2019. DLA bound D-*lldR* in pdb format was docked with operator sequence DNA using HDock server (http://hdock.phys.hust.edu.cn/) and further analyzed in detail using PLIP online tool[220].

All the experiments in this present study were carried out in triplicates and the results expressed as mean values ± standard deviations. Graphs were plotted using Origin2021 (OriginLab Corporation, USA) software for statistical analysis and evaluated using analysis of variance (ANOVA) and Tukey Post-hoc test for promoter functionality confirmation. Statistical analysis was conducted with a p-value of <0.05 considered indicative of statistical significance.

4.4 Results and discussion

4.4.1 Engineering of recombinant *Lactobacillus* strains harboring P_{GSP}, P_{Hyb}, and P_{Mut} promoter-repressor systems

The novel DLA-inducible promoter regulated by the GntR-family protein D-*lldR,* specifically responds to DLA, where D-*lldR* acts as a repressor that is deactivated upon DLA addition, activating the *lldP-dld-II* operon. Notably, the DLA-inducible *dldh* gene within this operon in *Pseudomonas* species coordinates DLA metabolism, enhancing the bacterium's efficiency in utilizing DLA for growth and adaptation in DLA-rich environments [210]. In the current chapter, the same DLA-inducible promoter-repressor system has been harnessed as a sophisticated dynamic metabolic engineering tool in *L. bulgaricus*, facilitating the precise regulation of DLA biosynthesis. To optimize compatibility and performance, the repressor protein was codon-optimized and successfully cloned alongside the P_{lldP} promoter along with LAB compatible RBS sequences (*Fig 4.2a*). The *mRFP* gene, encoded within the pLEM415 plasmid, has been previously demonstrated to exhibit robust expression in *Lactobacillus delbrueckii* subsp. *bulgaricus*, underscoring its genetic compatibility and functionality within

this host system [107]. To further potentiate the efficacy of the promoter, a hybrid promoter strategy was implemented, seamlessly integrating a *Lactobacillus sp*. compatible promoter P_{ldhL} from *Lactobacillus sakei* (pre-existing within the pLEM415 plasmid acquired from Addgene) along with the innate P_{lldP} promoter (gene synthesized from Genscript) from *Pseudomonas sp*., thereby synergistically augmenting the transcriptional output similar to previous published literature reports [221]. This innovative approach combines elements from disparate sources to forge a more efficient and controllable gene expression system in target expression host (*Fig 4.2b*) (agarose gel images for construction of promoter libraries P_{GSP}, P_{Hyb} and P_{Mut} provided in *Fig 4.3*). A *Lactobacillus delbrueckii* subsp. *bulgaricus*-compatible ribosome binding site (denoted as RBS') sequence, GAAAGGAG, accompanied by an 8 bp randomized spacer region, was strategically cloned upstream of the *mRFP* fluorescent protein, in conjunction with the native RBS sequence of the P_{lldP} promoter across all constructs [222]. For construction of P_{Mut} variant, the native P_{lldP} promoter sequence was excised from the P_{Hyb} construct through a combination of overlapping PCR and Gibson assembly approach (*Fig 4.2c*). This modification aimed to assess the functional significance and compatibility of the native promoter within *Lactobacillus* strains.

In *Pseudomonas* species, particularly *Pseudomonas putida* and *Pseudomonas aeruginosa*, have been extensively characterized regarding their promoter systems [223][224]. The σ70 family of sigma factors, which are responsible for recognizing promoter sequences, plays a pivotal role in the transcriptional regulation of genes in these organisms [225]. Promoters bearing the consensus −10 and −35 sequences have demonstrated broad activity across multiple hosts, including *E. coli* and other *Pseudomonas* strains, indicating a level of compatibility that can be exploited for heterologous gene expression [226]. The findings from this chapter is the first of its kind to demonstrate the functionality of a *Pseudomonas sp.* promoter when tested in a *Lactobacillus sp.* context. The comparative analysis of promoter sequences between *Lactobacillus* and *Pseudomonas* highlights both the conservation and divergence of transcriptional regulatory mechanisms. While both genera utilize similar core promoter motifs, the context and efficiency of these sequences can vary significantly due to differences in their genomic architecture and environmental adaptations [227][228].

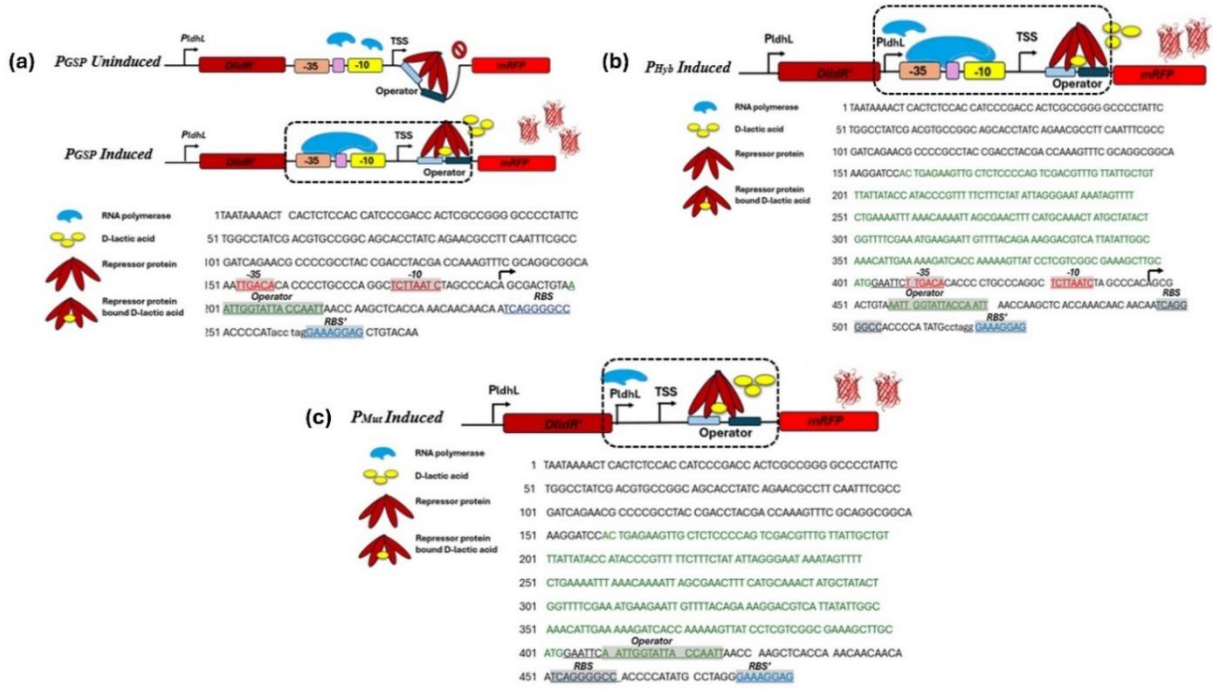


Figure 4.2. Structure, sequence, and mechanism of the engineered promoter library under induced and uninduced conditions with DLA inducer; (a) P_{GSP} promoter: Comprising a codon-optimized D-*lldR* repressor protein driven by the constitutive P_{ldhL} promoter, alongside an operator region and the native P_{lldP} sequence, including the -10 and -35 regions, transcription start site (TSS), and both native and *Lactobacillus*-optimized ribosome binding sites (RBS and RBS', respectively), (b) P_{Hyb} promoter: Modified by inserting the constitutive P_{ldhL} promoter between the D-*lldR* repressor protein and the native P_{lldP} sequence from P_{GSP}, (c) P_{Mut} promoter: Created by fully excising the native P_{lldP} sequence from the P_{Hyb} promoter. (Fig b and c depict promoter activity under induced conditions only).

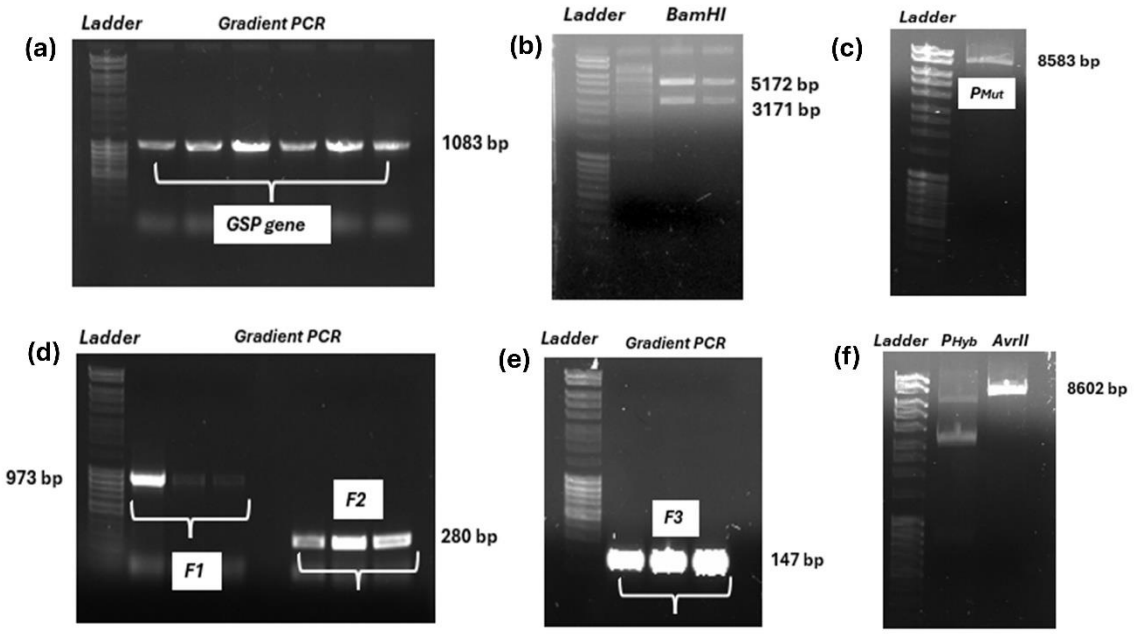


Figure 4.3 Construction of promoter libraries P_{GSP}, P_{Hyb} and P_{Mut}; (a) Gradient PCR of *GSP* gene (promoter repressor system codon optimized and synthesized), Lanes from left to right: Ladder, 1083 bp gene *GSP* PCR amplified for rest of the lanes; (b) Clone confirmation for P_{GSP}, Lanes from left to right: Ladder1, Ladder 2, Single digested with *BamHI* (5172 bp, and 3171 bp) for next two lanes; (c) PCR amplification of P_{Mut} excising out P_{lldP}, Ladder, 8583 bp; (d) Gradient PCR for Fragment F1 (973 bp) and F2 (280 bp) for constructing P_{Hyb} promoter, (e) Gradient PCR for fragment F3 (147 bp); (f) P_{Hyb} clone confirmation Ladder Lanes from left to right: Recombinant plasmid P_{Hyb}, P_{Hyb} single digested with *AvrII* (8602 bp)

4.4.2 Functional characterization of engineered promoter-repressor systems in *Escherichia coli* and *Lactobacillus delbrueckii* subsp. *bulgaricus* expression host

To characterize the functionality of the engineered DLA inducible promoter-regulator system, the promoter libraries were evaluated in both *E. coli* BL21 and *L. bulgaricus* VI104 expression host strain using pLEM415 shuttle vector. The performance of the constructed inducible promoters, P_{GSP}, P_{Mut}, and P_{Hyb}, was evaluated in comparison to a control strain expressing *mRFP* under constitutive promoter (P_{ldhL}). In *L. bulgaricus* VI104, the engineered P_{GSP} promoter exhibited the highest *mRFP* fluorescent protein expression levels (6388 normalized *mRFP*) when induced with 100 mM DLA (*Fig 4.5d*). Maximum normalized *mRFP* detected for P_{Hyb} was 4987 (induced with 70mM DLA) and for P_{Mut} was 2899 (induced by 100mM DLA) (*Fig 4.5e* and *Fig 4.5f*). Under high DLA induction, the P_{GSP} promoter-repressor system

achieved a 1.49-fold increase in *mRFP* expression over the P_{Hyb} promoter and a 2.37-fold increase over the P_{Mut} promoter. Stable *mRFP* expression was maintained with DLA inducer concentrations between 60 to 100 mM, while lower concentrations led to a decline in expression after 15-20 hours of fermentation (after induction), suggesting a threshold DLA concentration is necessary for repressor release and subsequent *mRFP* expression. A similar observation was reported in previous studies [210], where an increase in fluorescent protein expression was correlated with enhanced DLA production, with the concentration of DLA carefully maintained below 100 mM to prevent potential product inhibition that could negatively impact the system's overall efficiency [210]. The recombinant strain VI104-P_{GSP} reached stationary phase in 12^{th} to 17^{th} hours, faster than the wild-type and control strains. Cell growth plateaued, and fluorescence expression stabilized, balancing with the dilution effect of halted cell division. Between the 10^{th} and 35^{th} hours, the P_{GSP} system (induced at 60-100 mM DLA) showed stable fluorescence intensity, indicating that the inducer concentration was sufficient to maintain gene expression and that cells had reached a metabolic limit, where additional inducer did not further increase fluorescence. At lower concentrations (below 60 mM), the inducer might not reach the threshold required to fully alleviate repression or sufficiently activate transcription. However, as the concentration of DLA increases, the repressor protein becomes progressively inactivated, allowing for greater sequestration of the repressor protein from operator region. This results in a more robust transcriptional response and consequently higher *mRFP* expression, correlating with the increasing DLA inducer concentration. Under uninduced conditions (no exogenously supplied DLA), the P_{GSP} promoter showed a fluorescence profile where it gradually increased to a maximum normalized intensity of around 4000. This rise in *mRFP* expression was due to natural DLA biosynthesis by the wild-type *L. bulgaricus* VI104 strain, which activated the P_{GSP} promoter as endogenous DLA levels increased. Negligible or no fluorescence activity was detected for the P_{Hyb} and P_{Mut} promoters under uninduced conditions. The control system utilizing the P_{ldhL} constitutive promoter, recognized as a robust promoter in *Lactobacillus* species, exhibited the highest fluorescence intensity in comparison to engineered inducible promoters.

The P_{GSP} system exhibited minimal basal expression under uninduced conditions, suggesting slight promoter leakiness, but this had a negligible impact on system dynamics. In contrast, the control displayed a continuous increase in fluorescence with externally induced DLA. Notably, fluorescence intensity rose significantly with higher DLA concentrations across all promoter systems, despite similar growth pattern (growth curve profiles for recombinant VI104 strains

provided in *Fig 4.4*). These results underscore the enhanced sensitivity and precise inducible control of the codon-optimized P_{GSP} promoter-repressor system in *Lactobacillus* to DLA further validated by fluorescence microscopy *(Fig.4.6*). The hybrid promoter system showed significantly reduced activity compared to P_{GSP} in *Lactobacillus*, with *mRFP* expression remaining constant across DLA concentrations from 10 mM to 60 mM. However, responsiveness increased when DLA concentrations exceeded 70 mM. Similarly, the mutant promoter exhibited low activity in both *E. coli* and *L. bulgaricus*, emphasizing the importance of the native promoter's -10 and -35 sequences for effective regulation. The fluorescence intensity of P_{Mut} was very low, barely detectable under fluorescence microscopy (*Fig 4.6c*). When expressed in *E. coli* BL21(DE3), all the constructs showed reduced fluorescence intensity, likely due to incompatibility between the pLEM415 plasmid and its genetic regulatory elements, including the repressor gene. Fluorescence intensity increased with DLA concentration, peaking at 70 mM, while the constitutively expressed control system maintained high fluorescence. This trend was consistent across both P_{GSP} and P_{Hyb} promoter systems, highlighting their functional compatibility across species (*Fig 4.5a* and *Fig 4.5b*).

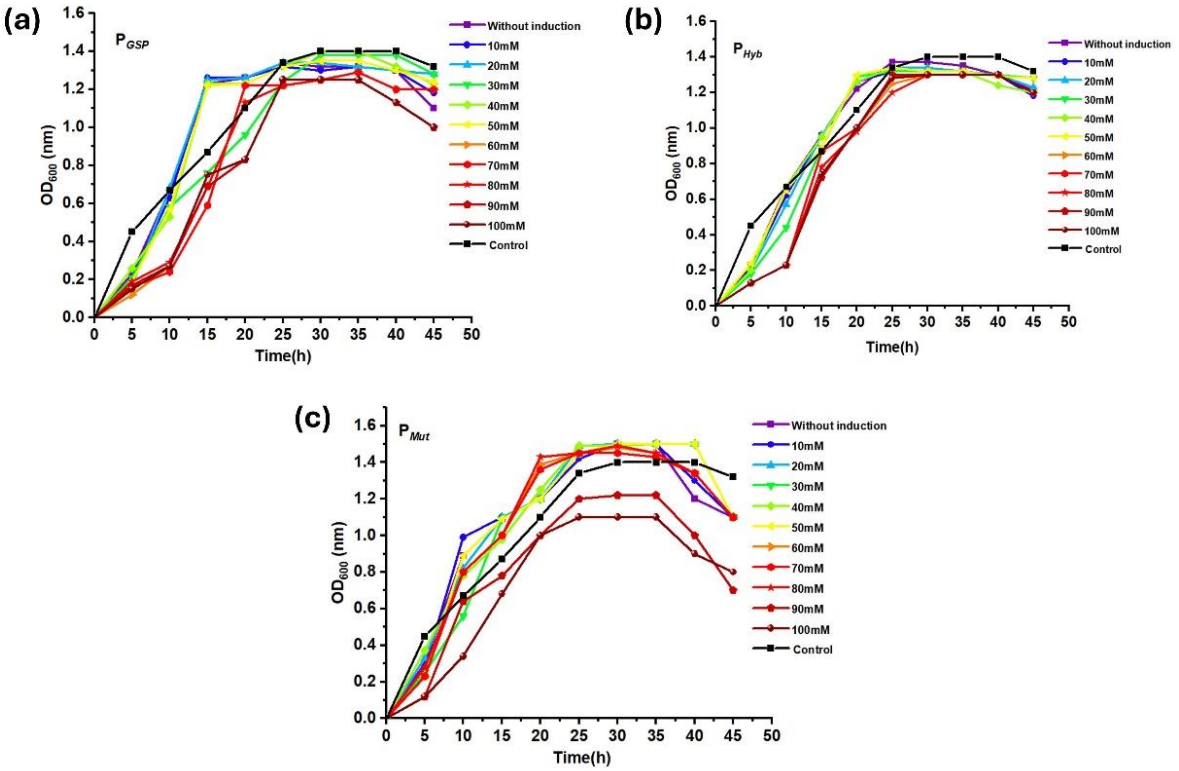


Figure 4.4 Growth curve profiles of recombinant P_{GSP}-*mRFP*, P_{Hyb}-*mRFP* and P_{Mut}-*mRFP* when induced with varying concentration of DLA.

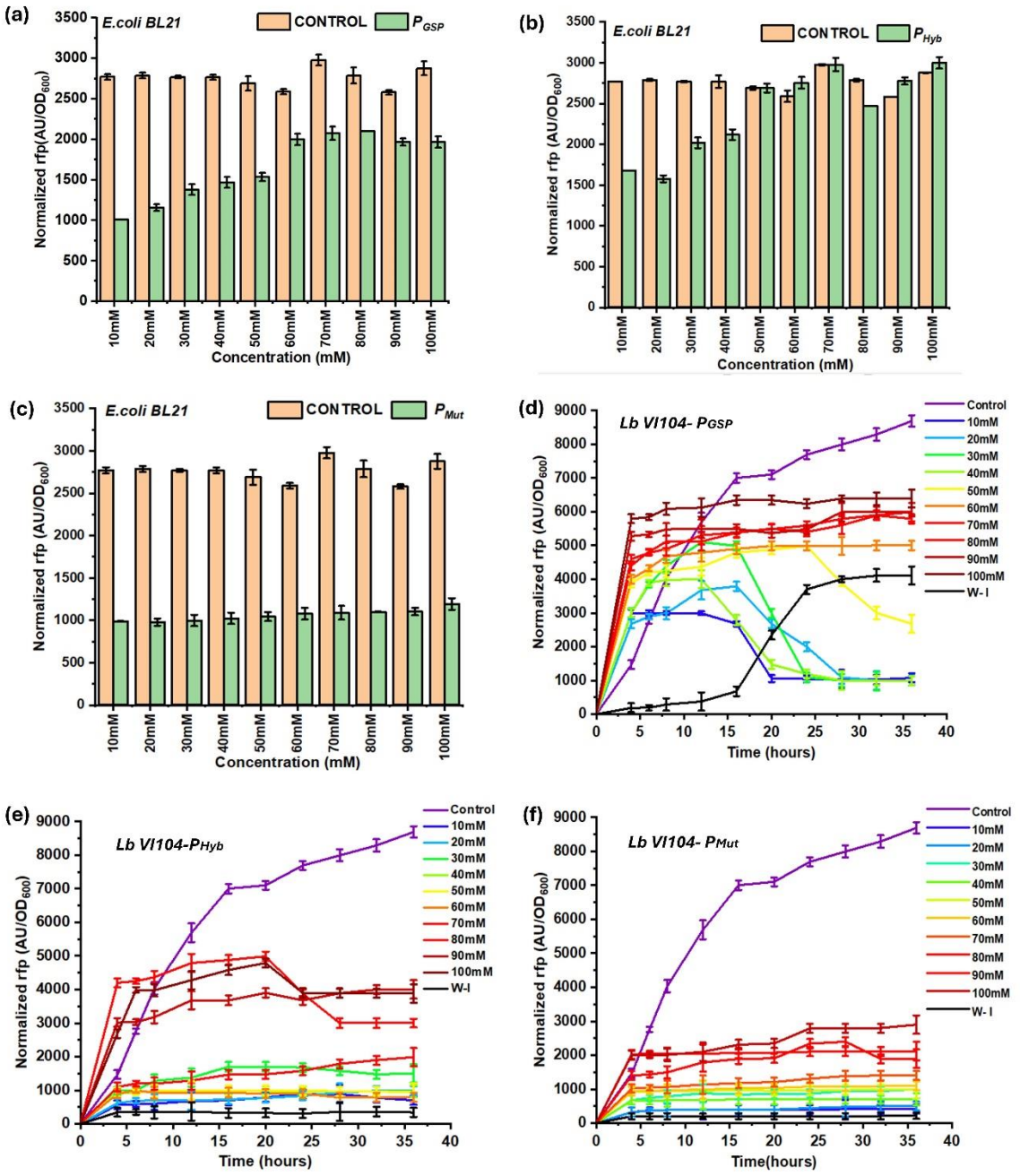


Figure 4.5 Functional characterization of the engineered promoter library (P_{GSP}, P_{Hyb}, and P_{Mut}); (a) Maximum normalized fluorescence (AU/OD_{600}) observed for P_{GSP} in *E. coli* BL21(DE3) following external induction with varying concentrations of DLA (10 mM to 100 mM) after 3 hours of growth; (b) Maximum normalized fluorescence (AU/OD_{600}) for P_{Hyb} in *E. coli* BL21(DE3) under the same DLA induction range and time frame; (c) Maximum normalized fluorescence (AU/OD_{600}) for P_{Mut} in *E. coli* BL21(DE3) with DLA induction across the same concentration range after 3 hours of growth; (d) Dynamic fluorescence profile (AU/OD_{600}) for P_{GSP} in *Lactobacillus delbrueckii* subsp. *bulgaricus* VI104, induced with varying DLA concentrations; (e) Dynamic fluorescence profile (AU/OD_{600}) for P_{Hyb} in *L. delbrueckii* VI104 under DLA induction at different concentrations; (f) Dynamic fluorescence profile (AU/OD_{600}) for P_{Mut} in *L. delbrueckii*

VI104, induced by DLA at varying concentrations (W-I refers to without induction); Error bars represent standard deviations from triplicate measurements of a single sample.

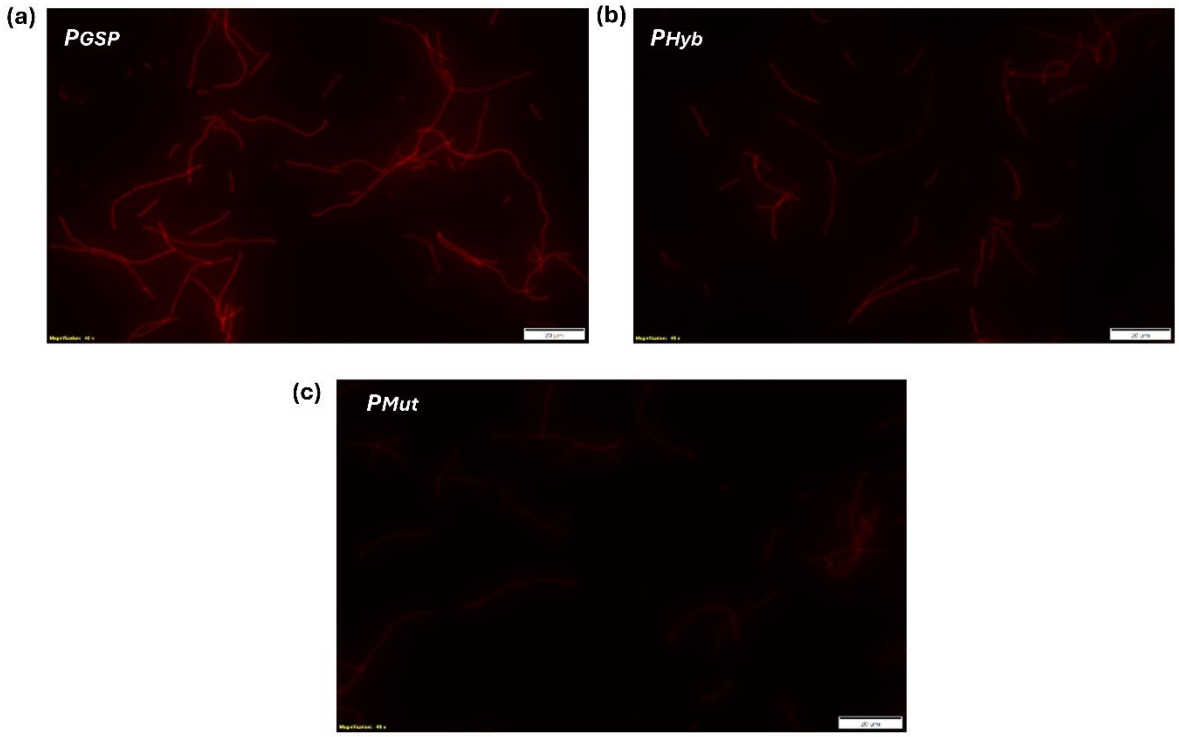


Figure 4.6 (a-c) Fluorescence microscopy images of recombinant *L. delbrueckii* VI104 expressing *mRFP* under the control of (a) P_{GSP}, (b) P_{Hyb} , and (c) P_{Mut} promoters, showing promoter-driven *mRFP* expression.

This study demonstrates that the native *Pseudomonas* sp. promoter is optimally expressed in *Lactobacillus*; however, the unoptimized promoter-repressor system was not included. Codon optimization of the repressor is essential for proper formation and binding of the repressor protein in *Lactobacillus bulgaricus*. The requirement for DLA concentrations above 60 mM to sustain *mRFP* expression suggests the repressor has a high binding affinity for DLA, necessitating elevated concentrations to displace the repressor and initiate transcription, consistent with previous observations [210]. Alterations to -10 and -35 regions in hybrid and mutant promoter constructs likely disrupted these processes, leading to lower *mRFP* expression. It is also stated that repressors are expected to attenuate *mRFP* expression by at least 70% without exerting any inhibitory effects on bacterial growth [229]. All inducible systems showed accelerated growth, with a later and prolonged stationary phase. Low to moderate inducer concentrations effectively dissociate repressors, enhancing transcription. However, at higher concentrations, saturation occurs, leading to a plateau in expression.

Excessive inducer concentrations may cause leaky expression, reducing system responsiveness and gene expression efficiency. High inducer levels can also exert cytotoxic effects, imposing a metabolic burden that compromises cellular health, highlighting the balance needed for optimal gene regulation [230][231]. In contrast to previous reports, this study found that higher DLA inducer concentrations were more effective than lower concentrations for the D*lldR*'-P_{lldP} system, although the cells reached stationary phase much more rapidly than the control.

While promoter sequences in *E. coli* are well-studied, the functionality of promoters in gram-positive bacteria, like *Lactobacillus*, is less understood, with conserved −35 and −10 regions and the TG motif upstream of −10 found in about 26% of *Lactobacillus* promoters, though its frequency varies across species [232]. These findings highlight the role of the −35, −10, and TG motifs in promoter efficacy in *Lactobacillus*. Modifying these can enhance gene expression, with low GC content promoting broad host activity and high GC content enabling selective activation aligned with host GC content [233]. The P_{lldP} promoter from *Pseudomonas* sp. in this study, with a GC content of 28.6%, demonstrates broad-spectrum functionality, enabling effective expression across diverse hosts such as *E. coli* and *Lactobacillus*. These insights enhance our understanding of *Lactobacillus* promoter architecture and provide a framework for optimizing gene expression in gram-positive bacteria.

4.4.3 Elucidating the role and mechanism of promoter regulatory sequences in *Lactobacillus delbrueckii* subsp. *bulgaricus*

The codon-optimized D-*lldR*' protein depends on the operator sequence for the DNA-binding domain (DBD) to induce repression in *Lactobacillus*, with native repressor and operator sequences previously identified for biosensor applications [210]. To evaluate this system in *Lactobacillus delbrueckii*, we excised the operator, repressor, and both regions from the recombinant plasmid and tested their effectiveness. This methodology facilitated an in-depth exploration of the influence of specific genetic elements on the performance of the engineered system within *Lactobacillus* species similar to literature reports [234]. It was observed that the developed constructs, devoid of operator and repressor regions, exhibited a significant enhancement in *mRFP* expression (*Fig 4.7a*). Interestingly, the native P_{GSP} promoter, even without induction (W-I), demonstrated a certain degree of *mRFP* expression, hinting at a potential leakiness in the system as mentioned in previous section. However, upon induction, the *mRFP* expression amplified by a factor of 4.2. Intriguingly, the mutant libraries constructed exhibited an equal amount of *mRFP* expression in both induced and uninduced states. Compared to control, the P_{GSP} inducible promoter system exhibited a 1.45-fold reduction in

activity, highlighting the importance of synergistic alignment of regulatory domains in *Lactobacillus delbrueckii*, which could be applied in future metabolic engineering and synthetic biology applications. Sigma factor compatibility is crucial for promoter expression across bacterial species, as sigma factors from one species can activate transcription from another's promoters, influencing gene expression [235][236]. For example, sigma factors from *Bacillus subtilis* have been shown to initiate transcription from *E. coli* promoters, indicating cross-species promoter recognition [237][238][239]. Literature suggests similarities between *Lactobacillus* and *Pseudomonas* sigma factors, especially within the σ70 family, which regulates growth and stress response genes [240] [241]. Phylogenetic tree analysis shows that despite the genetic distance between *Pseudomonas* and *Lactobacillus*, conserved regulatory elements enable successful *mRFP* expression in *Lactobacillus*, highlighting the potential for cross-species metabolic engineering and the use of regulatory sequences from distant organisms to enhance production in target species (*Fig 4.7d*).

Studies indicate that gene repression depends not only on repressor-operator binding affinity, as operators with weaker affinities can still achieve higher repression, suggesting operator sequences modulate both repressor occupancy and interaction with transcriptional machinery [242] [243]. Previous molecular docking studies analyzed the DBD and interactions between the modelled D-*lldR* protein and D(+)-lactate, offering insights into their interaction dynamics [210]. In this study, the D-*lldR* repressor protein model was computationally docked with operator sequence (DNA), both in the absence and presence of DLA, utilizing the HDock tool to analyze the interaction mechanism [244][245]. The analysis revealed key interactions, including hydrophobic, hydrogen bonds, salt bridges, pi-stacking, and pi-cation, essential for protein-DNA complex stability and specificity. Presence of DLA reduced protein-DNA interactions, while docking without DLA showed numerous interactions. Multiple hydrogen bonds interactions and complex network of interactions were identified between operator region (Operator region denoted as: dT-dT-dA-dA-dC-dC-dA-dT-dA-dA-dT-dG-dG-dT-dT-dA-dA where d refers to deoxyribose) and D-*lldR* protein (*Fig 4.7b*). ALA133 formed a hydrophobic interaction with DA7 at 3.53 Å. ARG33 in the DBD established two hydrogen bonds with DT1 at 2.88 Å and 3.21 Å, while ASP136 formed two bonds with DA7 at 3.60 Å and 3.24 Å. ARG155 interacted with DC5 and DC6 at 3.35 Å and 2.82 Å, respectively. Salt bridges were observed between ARG140 and DA7 at 4.75 Å, LYS29 with DG13 and DT14 at 3.52 Å and 2.79 Å, ARG67 with DA17 at 4.02 Å, ARG126 with DG13 at 4.98 Å, and ARG174 with DT14 at 4.91 Å. Pi-stacking interactions occurred between TYR73 and DA17 at 4.81 Å and 4.70 Å, and a pi-cation interaction between ARG155 and DT14 at 5.92 Å. The hydrophobic

interaction between ALA133 and DA7 suggests that hydrophobic forces play a significant role in the initial docking and stabilization of the protein on the DNA (*not shown in fig*). Hydrogen bonds, particularly those involving ARG33, ASP136, and ARG155, are critical for the precise positioning of the protein relative to the DNA, facilitating specific recognition and binding (*Fig 4.7 b*). Salt bridges, particularly involving ARG43 and ARG140, play a key role in electrostatic stabilization, enhancing binding affinity and specificity. Pi-stacking with TYR73 and pi-cation interactions with ARG155 emphasize the importance of aromatic and electrostatic interactions in DNA recognition, stabilizing the complex and facilitating specific nucleotide sequence recognition (*Fig 4.7 b*).

Conversely, the results for D-*lldR* docked with operator region in presence of DLA revealed few interactions (*Fig 4.7c*). Specifically, hydrophobic interactions were observed between ARG67 and DC12 at a distance of 3.86 Å. Multiple salt bridges were detected, notably between ARG155 and DG5 with a distance of 4.29 Å. Hydrogen bonds were prominent, with ARG33 of chain A forming bonds with DA8, DG6, and DT7, and additional interactions were observed involving ASP76, ASP84, HIS88, and ARG182. Overall, in the presence of inducer molecule DLA, additional hydrophobic interactions and disruptions to existing hydrogen bonds and salt bridges were observed, particularly affecting arginine residues, thus destabilizing the protein-DNA complex. Disruption of hydrogen bonds is evidenced by the absence of pi-stacking and pi-cation interactions in the ligand-bound state. DLA-induced perturbations likely trigger a conformational change in the repressor protein, reducing its DNA affinity and causing dissociation from the operator, enabling gene transcription. Comparative analysis highlights the balance of noncovalent interactions in D-*lldR* repressor function and DLA's role in modulating them, providing insights into gene regulation and potential therapeutic strategies.

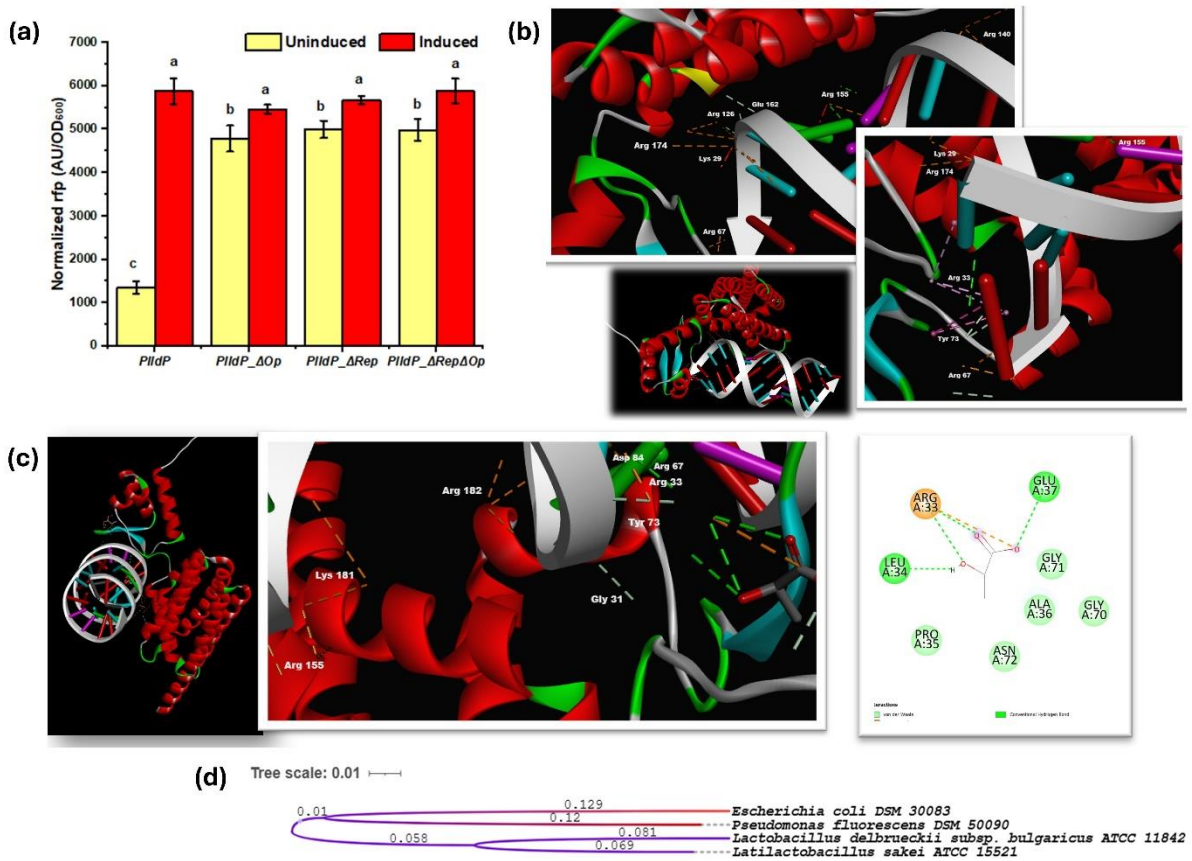


Figure 4.7 Analysis of the operator and repressor regions in the P_{GSP} promoter and molecular docking studies; (a)Functional evaluation of the operator and repressor regions in the P_{GSP} promoter, highlighting their regulatory roles in *Lactobacillus delbrueckii* subsp. *bulgaricus*. The same letter on bars represent insignificant variations among the levels of the factors (p>0.05); (b) Molecular docking analysis between the operator DNA sequence and the D-*lldR* repressor protein reveals key interactions responsible for promoter regulation; (c) Molecular docking analysis between the operator DNA sequence and the D-*lldR* repressor protein (docked with the DLA ligand) reveals a lack of significant interactions, indicating a potential sequestration mechanism. A magnified view highlights the specific interactions between D-*lldR* and the DLA ligand; (d) Phylogenetic tree analysis shows the evolutionary relationships of species from which the promoter regulatory sequences were derived, providing insights into the conservation of regulatory elements across different organisms.

4.4.4 Optimization of DLA-inducible promoter-repressor systems in LAB for enhancing functional efficacy

The systematic evaluation of the DLA inducible promoter-repressor system in recombinant *L. bulgaricus* VI104 revealed significant insights into optimizing gene expression under controlled fermentation conditions. The P_{GSP} promoter consistently outperformed other constructs, exhibiting high fluorescence intensity across most conditions tested (*Fig 4.8*). Peak

activity for P_{GSP} was observed at induction times between 6 to 8 hours, 200 rpm aeration conditions, temperatures between 37°C and 40°C, and when induced with pure DLA at acidic pH levels around 4.5. The P_{Hyb} promoter showed moderate activity overall, with increased fluorescence under similar conditions to P_{GSP}, while P_{Mut} demonstrated variable performance depending on specific environmental factors. Notably, P_{Mut} showed maximum fluorescence intensity at extended induction times of 12 hours and beyond, higher aeration rates, and better effectiveness in the presence of 3-hydroxypropionate compared to pure DLA. The P_{GSP} promoter's robust performance across conditions suggests its suitability for stable gene expression applications, owing to the native promoter sequence and codon-optimized repressor. The P_{Hyb} promoter's increased activity under specific conditions makes it ideal for precise control in response to environmental cues, while P_{Mut} sensitivity to short induction periods and extreme pH suggests its potential for short-term gene expression or applications involving rapid pH changes.

The distinct regulatory responses of each promoter to environmental changes provide a versatile toolkit for fine-tuning gene expression in *L. bulgaricus*. The promoters' differential responses to oxygen and pH highlight the organism's facultative anaerobic and acidophilic nature [246][247]. Based on OFAT experimental results, the P_{GSP} promoter-repressor system could be emerged as a promising candidate for further dynamic metabolic engineering applications using the optimized parameters identified in this chapter.

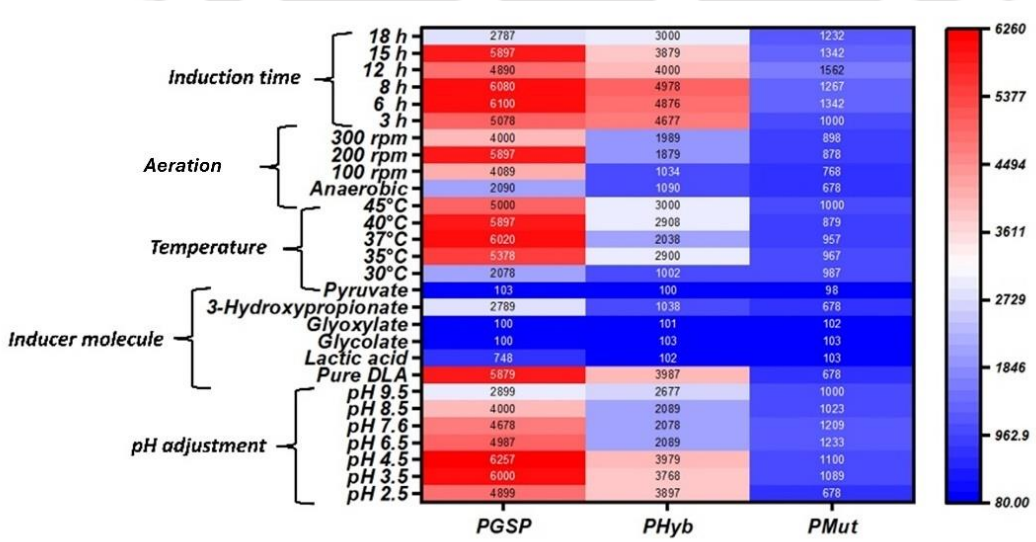


Figure 4.8 Heat map illustrating the optimization of promoter activities for P_{GSP}, P_{Hyb}, and P_{Mut} across diverse culture conditions.

4.4.5 Application of engineered promoter repressor system for enhanced DLA biosynthesis

The proposed dynamic control network for the DLA biosynthetic pathway is designed to autonomously regulate carbon flux between growth and production phases in response to DLA levels. The key features of this control logic are: (i) DLA, the metabolite of interest acts as the inducing molecule that activates the sensor-regulator system; (ii) During the lag phase, the *dldh* gene overexpression remains inactive, allowing the host cells to adapt to the fermentation environment without interference from DLA overproduction and cellular overburden; (iii) As cells enter exponential growth, the sensor-regulator system gradually activates the DLA production module by overexpressing the *dldh* gene; (iv) With increasing DLA accumulation, the carbon flux is redirected towards *dldh* overexpression, shifting cellular metabolism from growth to DLA production state (*Fig 4.8a*). This dynamic control network is expected to autonomously allocate carbon sources for growth and DLA production based on metabolic state, as sensed by DLA levels similar to literature reported by [193].

The dynamic $P_{GSP\text{-}dldh}$ system and the static P_{ldhL} control exhibited distinct patterns of DLA and biomass production in both induced and uninduced conditions in recombinant *Lactobacillus bulgaricus* VI104. However, for uninduced conditions the final DLA titres achieved by both systems were nearly similar by the end of the fermentation process. Maximum biomass accumulation was 0.96 g L^{-1} in the static control and 0.74 g L^{-1} in the dynamic system in shake-flask level (*Fig 4.9c*). The dynamic system peaked DLA production at 38th hour, stabilizing in the stationary phase and decreasing later in death phase (46th hour), while the DLA production for static control showed a gradual increase reaching a plateau in the death phase. Maximum DLA titres achieved by static and dynamic systems were 1.87 g L^{-1} and 1.82 g L^{-1} respectively. The main achievement of this study conducted in this chapter is the development of a refined dynamic regulatory network for DLA fermentation, resulting in significantly enhanced performance metrics and distinguished growth and production phases using the engineered P_{GSP} promoter repressor system. The dynamic control system also achieved a peak DLA titre with comparatively less biomass concentration than static system (*Fig. 4.9c*). In comparison, the static control reached a maximum DLA concentration (1.87 g L^{-1}) within the same timeframe, but the increase was gradual and occurred without external DLA induction. The static control entered the death phase early (28th hour), limiting overall DLA yield, while the dynamic system sustained cellular proliferation. This novel ADR system, engineered and tested for the first time, achieved a 2.27-fold increase in DLA titre compared to the wild-type, while

maintaining cellular viability and metabolic stability. The specific glucose utilization rate (*qs*) was comparable across recombinant strains, ranging from 0.98 to 1.1 g g^{-1} h^{-1}. Productivity (q_p) ranged from 0.076 to 0.078 g L^{-1} h^{-1}, and the optical purity of DLA remained consistently high across all conditions, exceeding 99% in recombinant VI104 strains (Table 4.3). Overall, productivity was enhanced by 2.23-fold in shake flask experiments for both recombinant VI104 strains, demonstrating its potential to optimize fermentation further and boost metabolic output. Similarly, the DLA yield from glucose ($Y_{DLA/Glucose}$) varied across the strains, with the highest yield of 0.198 g g^{-1} observed in the control strain, which had constitutive expression of *dldh*, followed by 0.194 g g^{-1} in the wild-type strain. The ADR exhibited a slightly lower yield of 0.175 g g^{-1}, which could be due to delayed and controlled overexpression of *dldh* gene which shifts carbon flux from glucose towards more DLA biosynthesis. Overall, the dynamic system produced more DLA from less biomass concentration, where the yield ($Y_{DLA/Biomass}$) was 1.36-folds enhanced than static system is a significant achievement (Table 4.3). The dynamic regulatory system's ability to modulate cellular proliferation and extend fermentation timeframe aligns with previous studies on two-stage metabolic control, decoupling biomass accumulation and metabolite overproduction. This approach preserves cellular viability, delays the death phase, and optimizes process efficiency and product yields [248].

In experiments with external 60 mM DLA induction (after 3 hours), DLA production remained consistent, but cellular biomass was reduced than uninduced controls (*Fig 4.9d*). The dynamic control system reached a peak DLA concentration of 1.78 g L^{-1}, while the static control showed a 1.56-fold reduction to 1.14 g L^{-1}. SDS-PAGE analysis of whole cell protein lysate from dynamic system (induced conditions) showed *dldh* expression post-induction (*Fig 4.9b*). The reduction in DLA titres could be due to inhibition from high DLA concentrations, which triggered the promoter-repressor system but negatively impacted the static control. Cellular biomass also declined, with cells entering the death phase within 30 hours reducing overall DLA titre. Overall, the ADR (1.82 g L^{-1}) outperformed the induced dynamic control (1.67 g L^{-1}), optimizing DLA production and sustaining cell growth. This highlights the efficiency of the engineered P_{GSP} promoter-repressor system in driving transcription without external induction, being regulated by the native DLA produced by the wild-type VI104.

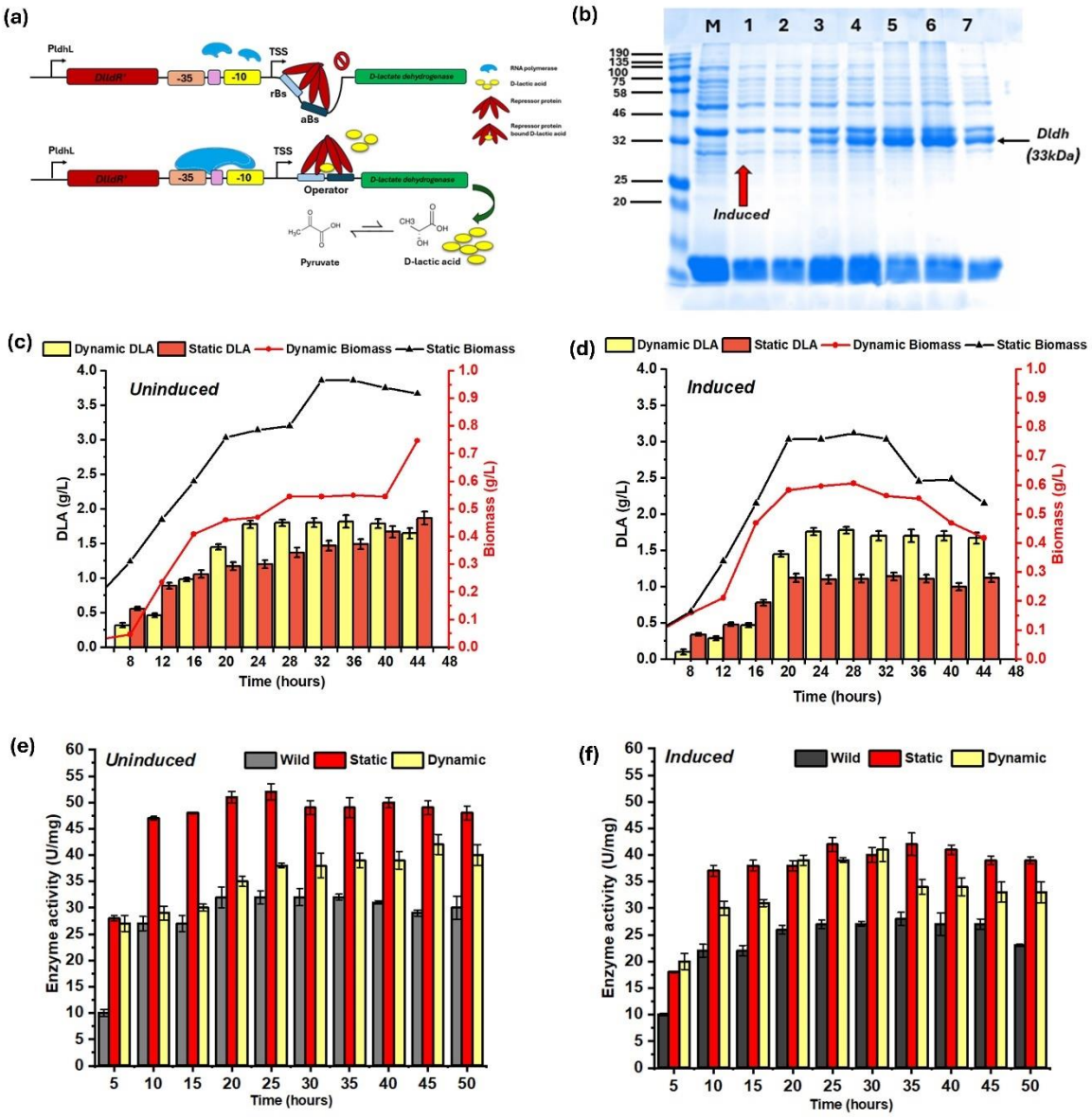


Figure 4.9 Dynamic metabolic engineering application of $P_{GSP\text{-}dldh}$ for enhanced DLA biosynthesis in *Lactobacillus bulgaricus* VI104; (a) Schematic of the mechanism of action for $P_{GSP\text{-}dldh}$ under both induced and uninduced conditions; (b) Protein expression analysis of whole cell lysates showing *dldh* protein levels after induction with DLA at various time points: Lane M (marker), Lane 1 (6 hours), Lane 2 (9 hours), Lane 3 (12 hours), Lane 4 (15 hours), Lane 5 (18 hours), Lane 6 (21 hours), and Lane 7 (24 hours); (c) The $P_{GSP\text{-}dldh}$ construct is referred to as 'dynamic,' while the P_{ldhL} promoter is considered 'static' under uninduced conditions, reflecting their distinct regulatory behaviors in DLA biosynthesis. The dynamic profiles of DLA production and biomass accumulation for both $P_{GSP\text{-}dldh}$ and P_{ldhL} constructs have been plotted; (d) Both constructs were externally induced with DLA to examine their distinct regulatory behaviors in DLA biosynthesis. The dynamic profiles of DLA production and biomass accumulation for $P_{GSP\text{-}dldh}$ and P_{ldhL} constructs were plotted, highlighting their differential responses to induction; (e) the *dldh* enzymatic activity is compared between wild, $P_{GSP\text{-}dldh}$ (dynamic) and P_{ldhL} (static) under uninduced

conditions; (f) the *dldh* enzymatic activity is compared between wild, $P_{GSP\text{-}dldh}$ (dynamic) and P_{ldhL} (static) under externally induced conditions. Error bars represent standard deviations from triplicate measurements of a single sample.

To further validate the promoter-repressor system, *dldh* enzymatic activity was quantified under induced and uninduced conditions (*Fig 4.9e&f*). Results showed higher *dldh* activity (53 U mg^{-1}) in the static control, likely due to the strong P_{ldhL} promoter. Under induced conditions, *dldh* activity decreased (48 U mg^{-1}), likely due to feedback inhibition from accumulated DLA in the fermentation media and less cellular biomass due to cell death, corroborating with the decrease in DLA titres. In contrast, the $P_{GSP\text{-}dldh}$ construct showed similar activity in both conditions (42 U mg^{-1}), but with differing trajectories: a gradual increase under uninduced conditions and a surge followed by a decline under induction, likely due to DLA accumulation and feedback inhibition. The dynamic control system sustained a DLA concentration of 1.78 g L^{-1}, outperforming the static control, which exhibited a marked decline in DLA production. Overall, the ADR system, using the $P_{GSP\text{-}dldh}$ promoter-repressor, demonstrates strong potential for controlled DLA biosynthesis in *L. bulgaricus* VI104, with 99.09% optical purity, and offers a platform for further optimization through targeted promoter engineering to enhance gene expression, metabolic flux, and overall production efficiency. Overall, although the DLA titres achieved with the ADR system were comparable to those of the static system, the ADR system was further evaluated in a large-scale fermenter to assess its potential as a dynamic metabolic control tool.

4.4.6 Scaling up fermentation and evaluating $P_{GSP\text{-}dldh}$ as a dynamic tool for metabolic burden alleviation

The production kinetics of DLA were comprehensively characterized using the VI104-$P_{GSP\text{-}dldh}$ recombinant strain in a controlled bioreactor batch fermentation scale-up over a 48-hour period under optimized parameters obtained from OFAT studies and previous reports [150]. Compared to flask-level studies, the $P_{GSP\text{-}dldh}$ strain exhibited significantly enhanced performance in the bioreactor set-up, achieving higher DLA titres and superior specific growth rate relative to the control strain (*Fig.4.10a*). The pH of the fermentation medium showed a sharp decline for all recombinant strains, including the wild-type VI104, due to the biosynthesis of organic acids, and was maintained at 4.5 to support optimized production. Lactic acid bacteria, including *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, and *L. rhamnosus*, are known to shift pyruvate metabolism towards acetyl-CoA and fatty acid production under medium acidification, altering membrane fluidity despite protective mechanisms against pH drop [249][250]. This likely

explains the significant accumulation of acetic acid as a major by-product by the recombinant strain in this study (data not shown). The $P_{ldhL\text{-}dldh}$ strain (control) showed enhanced biomass accumulation compared to both wild-type VI104 and dynamic system ($P_{GSP\text{-}dldh}$) (*Fig 4.10b*). The control strain with constitutive promoter attained a maximum biomass of 1.77 g L^{-1} and a DLA titre of 8.16 g L^{-1} by 36th hours. In comparison, the dynamic system ($P_{GSP\text{-}dldh}$ strain) exhibited a slightly lower maximum biomass of 1.03 g L^{-1} but achieved a higher DLA concentration of 9.02 g L^{-1} at the same time point. The extended exponential phase in the $P_{GSP\text{-}dldh}$ strain, lasting until the 29th hour, highlights its enhanced DLA biosynthesis and metabolic capacity, providing a significant advantage for industrial DLA production [250]. The dynamic $P_{GSP\text{-}dldh}$ promoter system enabled a sustained increase in DLA biosynthesis (99.09% optical purity), shifting metabolic flux towards DLA production after 10th hour, similar to flask studies signifying a segregation between growth phase and production phase. Beyond 38th hour, the DLA yield began to decline, likely due to the accumulation of excess product or the reconversion of DLA to pyruvate, as *dldh* catalyses a reversible reaction [251]. These results underscore the potential of the system for further exploration in larger-scale fermenters integrated with continuous product removal mechanisms to improve efficiency and scalability.

Constitutive expression of genes often increases metabolic burden, reducing growth rates and elevating biomass accumulation [252]. This trend was also evident in this study, where P_{ldhL} strain showed a 1.78-fold decrease in specific growth rate due to metabolic burden accumulation, while $P_{GSP\text{-}dldh}$ had a 1.52-fold reduction compared to wild VI104. Overall, $P_{GSP\text{-}dldh}$ exhibited improved DLA production efficiency, achieving higher DLA yield per unit biomass compared to the constitutive system, as evidenced by a 1.28-fold increase in yield ($Y_{DLA/Biomass}$). Both recombinant strains demonstrated substantial improvements in DLA specific productivity, with the constitutive expression system yielding a 1.93-fold increase, while the dynamic control system achieved an impressive 2.41-fold enhancement compared to the wild-type strain. Similarly, the volumetric productivity of the dynamic system was 1.06-folds enhanced than control with constitutive expression of key *dldh* gene. These findings highlight the metabolic advantages of implementing inducible promoter over constitutive promoters for optimized metabolite (DLA) biosynthesis. (*Fig 4.10c*).

Growth curve analysis showed that the $P_{GSP\text{-}dldh}$ strain exhibited gradual biomass production, reaching a robust stationary phase, indicative of a finely tuned metabolic network. This dynamic regulation promoted prolonged DLA biosynthesis, a growth-associated product. Differences in DLA titres and production rates between strains underscore the impact of genetic

modifications and promoter systems on metabolic flux [253]. Overall, the specific lactose utilization rate (*qs*) was highest in the control system (1.42 g g^{-1} h^{-1}), followed by the wild strain (1.3 g g^{-1} h^{-1}) and the autonomous DLA- inducible dynamic system (1.29 g g^{-1} h^{-1}). Although the DLA yield from lactose was 0.188 g g^{-1} for control, 1.13-folds higher than dynamic system, the overall volumetric and specific productivity for DLA was efficient in the ADR, maintaining excellent optical purity, exceeding 99%. Gradual activation of the *dldh* gene effectively alleviated cellular stress, leading to a significant enhancement in DLA production. These characteristics highlight the dynamic system's ability to optimize resource use, maintain cellular viability, and boost overall productivity. The superior DLA production of *L. bulgaricus* strains in bioreactor conditions, compared to flask studies, also significantly highlights the importance of optimized fermentation strategies, including pH control, temperature regulation, and media composition [254].

To further assess the efficiency of dynamic control system, the NAD^+ /NADH ratio was determined during fermentation because this redox couple serves as a critical indicator of the cell's metabolic state and *dldh* activity profile [255]. A higher NAD^+/NADH ratio indicates an oxidative environment favouring catabolism, while a lower ratio suggests a reductive environment linked to anabolism or stress. A skewed ratio, often due to impaired glycolysis or oxidative phosphorylation, reflects increased metabolic burden. The *dldh* enzyme in *L. bulgaricus* also regulates this ratio by catalysing the reversible conversion of D-lactate to pyruvate, influencing NAD^+/NADH balance [256]. The NAD^+/NADH ratios in the wild-type VI104, constitutive control, and inducible $P_{GSP\text{-}dldh}$ systems highlight the impact of genetic expression on metabolic regulation *(Fig. 4.10b*).

The wild type showed an initial peak in NAD^+/NADH in exponential phase, followed by a decline as cells adapted to byproducts and nutrient scarcity in death phase. The constitutive control maintained a lower ratio, peaking at 3.0 and declining to 1.5, indicating a higher metabolic burden due to persistent NADH production. The inducible $P_{GSP\text{-}dldh}$ system exhibited a low NAD^+/NADH ratio initially, which increased steadily over time, reaching a peak of approximately 4.0 at 35 hours. Subsequently, the ratio gradually declined, stabilizing around 3.0 by 45 hours. This trend can also be correlated with DLA titres, as *dldh* catalyses the conversion of pyruvate to DLA in the presence of NADH. In the ADR system, an increase in NAD^+ levels during the later stages likely led to the reverse conversion of DLA back to pyruvate, resulting in a decline in DLA titres at later stages. This trend indicates a dynamic modulation of the redox balance during the fermentation process, likely reflecting the

metabolic shifts associated with the system's activity. Total protein concentrations were higher in the control (79 ± 8.98 µg mL^{-1}) than in the dynamic system (48 ± 5.58 µg mL^{-1}), confirming the dynamic system's efficiency in balancing metabolite production (including DLA) and minimizing cellular burden.

Apart from *dldh* enzyme, NADH oxidase enzyme activity is crucial for maintaining this balance by converting NADH to NAD^+, thereby reducing oxidative stress through the production of hydrogen peroxide, which can act as both a signalling molecule and a stressor [257][258]. Excessive hydrogen peroxide can increase metabolic burden and trigger early stationary phase onset. In *L. bulgaricus* modulating the NAD^+/NADH ratio is crucial for survival and efficient yogurt fermentation, where oxidative and reductive stresses shape fermentation dynamics. These findings underscore the potential of dynamic promoter regulation in *L. bulgaricus* for optimizing DLA biosynthesis, setting a foundation for future promoter engineering efforts to enhance promoter activity, improve metabolic flux, and maximize product yield in scalable bioprocesses [245]. Most importantly, this chapter makes a significant contribution toward expanding the molecular cloning toolbox of *L. bulgaricus.*

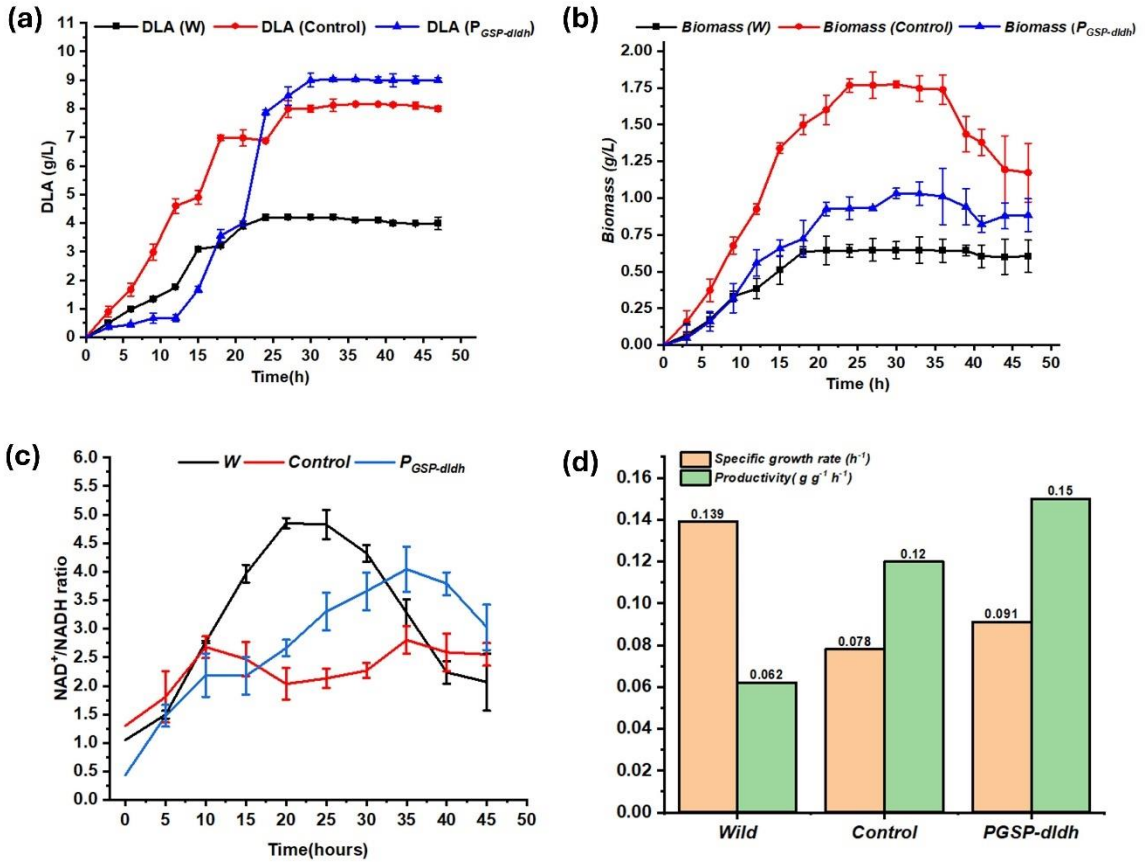


Figure 4.10 Scaling up dynamic metabolic control for DLA biosynthesis and cellular metabolic assessment; (a) Dynamic profiles of DLA production and (b) Dynamic profiles of biomass accumulation in wild-type, $P_{ldhL\text{-}dldh}$ (constitutively expressed), and $P_{GSP\text{-}dldh}$ (inducible) strains during batch fermentation, (c) Comparative analysis of specific growth rates and specific productivity among wild-type, $P_{ldhL\text{-}dldh}$, and $P_{GSP\text{-}dldh}$ strains in batch fermentation. Error bars represent standard deviations from triplicate measurements of a single sample

Table 4.3: Comparison of the kinetic parameters for shake-flask and bioreactor experiments

Strain-Shake flask experiments	**Specific glucose utilization rate *qs* (g g^{-1} h^{-1})**	**DLA titre (g L^{-1})**	**DLA yield (DLA/S) (g g^{-1})**	**DLA yield (DLA/X) (g g^{-1})**	**Maximum biomass (g L^{-1})**	**Productivity q_p (g L^{-1} h^{-1})**	**Optical purity (%)**
Wild	1.0	0.80	0.194	2.78	0.80	0.034	99.09
Control	1.1	1.87	0.198	4.47	0.966	0.078	99.05
Autonomous Dynamic system	0.98	1.82	0.175	6.1	0.744	0.076	99.07
Strain-Bioreactor experiments	**Specific lactose utilization rate *qs* (g g^{-1} h^{-1})**	**DLA titre (g L^{-1})**	**DLA yield (DLA/S) (g g^{-1})**	**DLA yield (DLA/X) (g g^{-1})**	**Maximum biomass (g L^{-1})**	**Productivity q_p (g L^{-1} h^{-1})**	**Optical purity (%)**
Wild	1.3	4.2	0.158	3.78	1.7	0.090	99.09
Control	1.42	8.16	0.188	5.87	1.77	0.171	99.06
Autonomous Dynamic system	1.29	9.02	0.165	7.54	1.033	0.182	99.09

DLA Yield ($Y_{DLA/S}$, gg^{-1}) was calculated as a ratio of DLA produced (g) to substrate consumed (g)

DLA Yield ($Y_{DLA/X}$, gg^{-1}) was calculated as a ratio of DLA produced (g) from biomass (g)

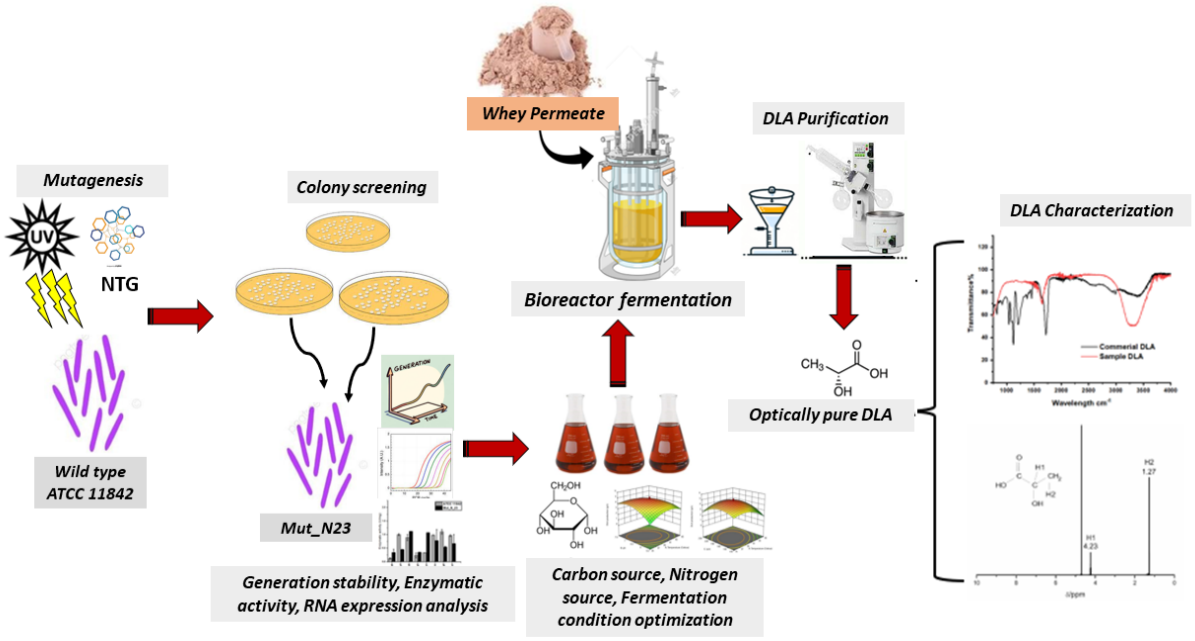
4.5 Conclusion

This chapter introduces a novel DLA-responsive promoter-repressor system from *Pseudomonas sp.* for controlled DLA biosynthesis in *Lactobacillus delbrueckii* subsp. *bulgaricus.* The engineered P_{GSP} promoter drove a 3.2-fold increase in DLA production, achieving 9.02 g L^{-1} in bioreactor cultivation compared to the wild-type strain. This

enhancement was accomplished by alleviating metabolic burden and prolonging fermentation duration. Through dynamic regulation of the key DLA-producing gene, *dldh*, this system optimized carbon flux while preserving cellular viability and sustaining extended fermentation times. These results advance dynamic metabolic engineering in LAB, offering a new tool for industrial applications and enhancing the potential for probiotic and biotherapeutic development.

CHAPTER 5:

Random mutagenesis and optimization studies for improved DLA biosynthesis from diverse carbon sources in mutant *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842



5.1 Summary

This chapter aims to augment the DLA biosynthetic capacity of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 through random mutagenesis. The mutant strain, Mut_N23, developed through synergistic application of ultraviolet (UV) irradiation and chemical mutagenesis using N-methyl-N′-nitro-N-nitrosoguanidine (NTG), exhibited 97% increase in DLA production and 37% enhancement in glucose uptake rate at flask level. Mut_N23 consistently produced optically pure DLA across seven generations, efficiently metabolizing lactose and sucrose to yield 4.47 g L^{-1} and 3.38 g L^{-1} of DLA, respectively. Optimal conditions identified through One-Factor-At-a-Time (OFAT), and Response Surface Methodology (RSM) facilitated maximum DLA concentration of 7.88 g L^{-1} (300% increase) from lactose-MRS (deMan Rogosa Sharpe) with specific productivity of 0.110 g g^{-1} h^{-1}. When lactose was replaced with whey permeate as an application, 4.89 g L^{-1} (140% increase) of DLA was obtained, with specific productivity of 0.066 g g^{-1} h^{-1} in lab-scale bioreactor setups, achieving 99.09% optical purity. Transcriptomics and enzymatic activity analyses substantiated enhanced performance of Mut_N23 signifying beneficial random mutations. Furthermore, characterization of purified DLA derived from whey permeate using Fourier Transform Infrared (FTIR) spectroscopy and proton Nuclear Magnetic Resonance (NMR) spectroscopy demonstrated parity with commercially available standards. This study highlights Mut_N23's potential for efficient DLA production exploiting a spectrum of carbon sources, providing a foundation for future metabolic engineering to enhance biosynthetic productivity.

5.2 Introduction

Lactobacillus delbrueckii subsp. *bulgaricus,* is an obligately homofermentative LAB renowned for its role in dairy industries as starter culture for yoghurt production. It is also majorly valued in the health industry as a probiotic, offering potential benefits for various health conditions. Despite its significant role in these sectors, its potential in the polymer industry remains scarcely explored. It could be used to produce lactic acid, a raw material for biodegradable and environmentally friendly polymers, offering a sustainable alternative to petrochemical-derived plastics [211] [80]. Bioconversion processes for lactic acid production present numerous benefits over traditional chemical synthesis for instance, they yield a high-quality optically pure isomer in substantial volumes and offers environmental sustainability and cost-effectiveness by harnessing waste by-product feedstocks as a carbon source [211]. DLA is a versatile organic acid with a broad range of applications across various industries, making it a product of significant industrial importance [211] [79]. DLA's significant role in producing

biodegradable polymer PLA lies in its use of optically pure lactic acid monomers, which enhance PLA's thermal stability and versatility for sustainable applications. There are many DLA producing microorganisms amongst which American Type cell culture ATCC 11842, one of the publicly available cultures for *L. bulgaricus*, produces optically pure DLA and also has capability to efficiently utilize lactose as carbon source as observed in Chapter 2 [211]. The metabolic engineering of *Lactobacillus delbrueckii* subsp. *bulgaricus* faces several challenges, which have been systematically addressed in our previous chapters by developing robust molecular cloning and protein expression tools, as well as optimizing electroporation protocols specifically for strain VI104 [117]. *L. bulgaricus* ATCC 11842, being an elite strain screened for the production of optically pure DLA in Chapter 2, suffers from extremely low transformation efficiency. To overcome this limitation, an alternative strain improvement strategy will be employed to enhance its genetic tractability and metabolic performance.

Random mutagenesis, a technique utilizing diverse mutagenic agents to induce genomic alterations, has been efficaciously employed in LAB to enhance strain performance and metabolic efficiency [259][260][261]. Chemical mutagens like NTG and Ethyl Methanesulfonate (EMS) induce point mutations by alkylating DNA bases while Ethidium Bromide (EtBr) intercalates into DNA, causing frameshift mutations that enhance the phenotype [262] [263]. Physical mutagens such as UV light induce pyrimidine dimers, particularly thymine dimers, and ionizing radiations like X-rays and Gamma rays causes a range of DNA damage including strand breaks and crosslinking [264] [265]. Biological mutagens include transposons, known as mobile genetic elements causing insertional mutagenesis, and site-specific mutases that introduce mutations at specific DNA sequences for various applications [266]. Each mutagen exhibits distinct mechanisms, eliciting diverse mutational spectra such as point mutations, insertions, deletions, or chromosomal rearrangements, tailored to specific experimental requisites. Random mutagenesis is advantageous over genetic engineering because it does not necessitate prior knowledge of the genome or specific targets, and it can generate a large diversity of mutants with unexpected beneficial traits. It has been systematically employed on a variety of organisms with the objective of augmenting the yield of diverse biochemical products. Mutants of *Rhizopus oryzae* with high LLA production were generated using combined UV and NTG mutagenesis[267] [268]. A high-efficiency heavy-ion mutagenesis technique was used on *Lactobacillus thermophilus* SRZ50 to enhance LLA production [269]. Microwave radiation was used to induce random mutagenesis in *Lactobacillus rhamnosus*, yielding high LLA in the mutant strain, W4-3-9 where mutations in lactate dehydrogenase gene and pyruvate kinase gene were

found to enhance lactic acid production [270]. Despite several studies focused on enhancing LLA production via random mutagenesis, no significant literature reports exist for improving DLA production through similar methods. *Lactobacillus delbreuckii* was employed to generate optically pure DLA from inedible, organosolv-treated biomass of hardwood and softwood, enabling the production of bio-based plastics, and from inulin, a plant-derived carbohydrate, using the simultaneous saccharification and fermentation (SSF) technique [271][188]. Feasibility of utilizing WP as a renewable feedstock for DLA biosynthesis by co-culture of *L. delbreuckii* and recombinant *L. lactis* to achieve complete lactose utilization was reported [79]. Since genetic manipulation ATCC 11842 is challenging, random mutation could be considered as promising alternative for developing a potential mutant strain with enhanced DLA biosynthesis capability. Despite *L. bulgaricus* being a significant probiotic strain, the application of detailed random mutagenesis studies in this strain is rarely documented in the current literature and this study aims to bridge this research gap. This chapter seeks to address this research gap by delivering in-depth insights into the impact of mutagenesis on *L. bulgaricus*. By exploring the mutagenic potential and optimizing fermentation conditions, this research not only enhances our understanding of *L. bulgaricus* but also paves the way for its industrial applications in bioprocessing and biotechnology. The synergy between chemical and physical mutagenesis has the potential to yield successful mutations was proclaimed [272].

In this present chapter, mutagenesis was implemented, integrating both physical and chemical mutagenesis treatments, to augment DLA production of the *L. bulgaricus* strain ATCC 11842. The chosen mutant, characterized by superior glucose and sucrose utilization kinetics, augmented biomass accumulation, and heightened DLA biosynthetic prowess, underwent extensive optimization of carbon and nitrogen sourcing as well as fermentation parameters to amplify DLA titre, rate, and yield (TRY). Furthermore, the stability of the selected mutant was rigorously assessed over successive generations, while its capacity to harness a spectrum of carbon sources was showcased as a sustainable bioproduction paradigm. The dairy industry's quest for sustainability necessitates innovative approaches to by-product utilization. WP, traditionally viewed as waste, harbors potential as a fermentation substrate. Herein, we also report the application of DLA production using WP by *L. bulgaricus* mutant, leveraging its innate metabolic pathways and mutagenesis-induced capabilities.

5.3 Materials and methods

5.3.1 Strain, media and culture conditions

All chemicals including enzymes employed in this present study were acquired from M/s Sigma-Aldrich Chemicals, St. Louis, USA. NTG was acquired from M/s Sigma Aldrich, dissolved in acetone, and then diluted with sterile sodium acetate buffer (50 mM, pH 6.0). Powdered WP was procured from M/s Nutrimed Healthcare Limited, New Delhi, India.

L. bulgaricus ATCC 11842 was procured from the American Type Culture Collection located in Manassas, Virginia. It was maintained in a frozen state with a glycerol solution comprising 50% (v/v) at a temperature of -80°C. For preculture preparation, the strain was propagated in 10 mL MRS broth at 37°C shaking conditions prior to the culture entered its phase of exponential growth (~10^6 CFU ml^{-1}). MRS medium composition: glucose 20 g L^{-1}, beef extract 10 g L^{-1}, peptone 10 g L^{-1}, yeast extract 5 g L^{1}, $CH_3COONa.3H_2O$ 5 g L^{-1}, K_2HPO_4 2 g L^{-1}, tri-ammonium citrate 2 g L^{-1}, $MgSO_4.7H_2O$ 0.2 g L^{-1}, $MnSO_4.4H_2O$ 0.5 g L^{-1} and Tween 80 1 g L^{-1}. For carbon source optimization experiments, glucose in MRS medium was substituted with lactose, sucrose, mannose, or fructose. For shake-flask experiments, starting pH of the fermentation medium was set to 6.5 by adding either 1 M HCl or 1 M NaOH, followed by sterilization by heating at 121°C for 20 minutes.

5.3.2 Random mutagenesis and screening

5.3.2.1 UV Mutagenesis

To conduct an UV sensitivity assay, *L. bulgaricus* ATCC11842 were cultured at a 37°C in 20 ml of MRS medium until they reached an optical density (OD_{600}) of 0.4 at 600 nm. The cells underwent separation via centrifugation at 5,000 x g for 15 minutes, followed by double rinsing with 20 ml of chilled phosphate buffer solution (PBS). Subsequently, 2 ml aliquots of cell suspensions were meticulously transferred to sterile Petri dishes with a diameter of 5 cm and subjected to UV irradiation at wavelengths of 254 nm and 365 nm for varied durations spanning 10, 20, 30, 40, and 50 minutes. Post-exposure, serial dilutions were performed, and 0.1 ml aliquots of cell suspensions from each treatment were aseptically spread onto MRS agar plates, shielded with aluminium foil to prevent unwanted photoreactivation effects. Following incubation to facilitate cellular proliferation, viable cell counts were meticulously enumerated and further assessed through shake flask studies. To prevent photo-reactivation, cultures in flasks were always shielded with aluminium foil, and the plates were stored in complete darkness.

5.3.2.2 NTG Mutagenesis

L. bulgaricus ATCC 11842 was propagated in 10 mL of MRS broth until the OD_{600} reached 1.2 at 600 nm. Following that, the fermentation broth was centrifuged for 10 minutes at 4°C at 10,000 rpm. The obtained cell pellet was harvested and washed twice with sterile sodium acetate buffer [273]. The cell suspension was reconstituted in 5 mL of the same buffer solution, supplemented with NTG to reach final concentrations ranging from 100 to 500 µg mL^{-1}. The resulting suspension was subjected to incubation at 37°C, wherein 0.5 mL aliquots were periodically withdrawn at designated intervals and subsequently centrifuged at 8,000 rpm for 5 minutes. Post-centrifugation, supernatants were carefully decanted, while cells were resuspended in a sterile 0.9% (w/v) saline solution. Following this, the cell suspension was uniformly spread onto MRS agar plates and incubated at 37°C overnight to facilitate growth. After allowing time for growth, the number of live cells was ascertained through enumeration of colony forming units and further evaluated for enhanced DLA synthesis.

5.3.2.3 Combined UV and NTG mutagenesis

L. bulgaricus ATCC 11842 underwent initial exposure to UV irradiation at 365 nm for 30 minutes, following the outlined protocol in section 5.3.2.1. Subsequently, UV-treated cells were harvested and resuspended in sodium acetate buffer supplemented with NTG at 500 µg mL^{-1}, undergoing incubation at 37°C for 60 minutes as detailed in section 5.3.2.2. The resultant NTG-treated cells underwent dilution and plating on MRS agar plates to quantify viable cells and assess phenotypic changes induced by mutagenesis. The combined UV and NTG mutagenic regimen aimed to augment genetic diversity, enhancing the likelihood of obtaining mutants with heightened DLA production. The cell viability count was conducted using the spread plate method and % cell mortality was calculated using Eq 2:

$$M = (Ni - Nd)/Ni \ * \ 100 \ldots\ldots\ldots\ldots\ldots \text{(Eq2)}$$

where M is the % cell mortality, N_i represents the initial viable cell count and N_d represents the viable cell count after mutation [274].

5.3.3 Screening of mutants in shake flask level

The prominent and fast-growing colonies obtained after mutagenesis were selected and cultured for further screening. An overnight preculture was inoculated to 100 ml of MRS medium in 250 ml screw-cap conical flasks, where it was subjected to agitation (150 rpm) at 37°C for 48 hours. Culture samples collected at intervals subjected to centrifugation at 1000×g

for 10 minutes and supernatant was subsequently analysed for sugar, DLA, and biomass. Individual colony was grown in separate shake flasks, and the mean values of the analysed metabolites were reported. The efficient mutant strains were screened based on their DLA production and efficient sugar consumption capabilities. Genetic stability test of the mutants was performed as reported by [269] [275].

5.3.4 Carbon and Nitrogen source optimization using One-Factor-At-a-Time (OFAT)

The wild-type strain ATCC 11842 and the selected mutant strain exhibiting enhanced DLA biosynthesis were propagated in MRS media. Subsequent shake-flask experiments for evaluating metabolite profiles were conducted using five distinct 250-ml baffled Erlenmeyer flasks, each containing 100 mL of MRS broth supplemented with either of the following sugars: glucose, lactose, sucrose, mannose, or fructose (at a concentration of 20 g L^{-1}). Each flask was inoculated with 5% (v/v) of the preculture and incubated at temperature 37°C with shaking at 150 rpm. Carbon source yielding superior results were further optimized by varying its initial concentrations (10, 20, 30, 40, 50 g L^{-1}) in subsequent experiments, which allowed the comparative analysis of DLA production across different carbon substrates and concentrations. At different time intervals, 1 mL samples were collected and centrifuged to separate the cells from the medium. The resultant optimized medium for carbon substrate was further investigated for DLA, sugar and biomass production Similarly, the effect of additional nitrogen source (casein hydrolysate, urea, ammonium chloride, ammonium acetate, and yeast nitrogen base) in the MRS medium was studied. All reported experimental data in this study represent the mean values derived from experiments conducted in duplicates.

5.3.5 Fermentation parameter optimization using Response Surface Methodology

The optimization of fermentation parameter (temperature, pH, rpm and fermentation time) on medium constituted with screened elite carbon and nitrogen source (discussed in section 5.3.4) was performed using Response Surface Methodology (RSM). The Central Composite Design (CCD) of RSM was employed to construct a statistical model that would elucidate the individual and interactive effects of variables on fermentation process using the obtained mutated strain. Considering that mutated strain may require altered growth conditions for its metabolism, the major fermentation parameters were optimized. Based on the findings of OFAT evaluates and analytical results, each factor was investigated at five levels. The CCD was configured implementing Design-Expert software (version 8.0.6, Stat-Ease, Inc., Minneapolis,

MN, USA). The specific levels for each factor are given in Table 5.1. The primary objective of optimizing the fermentation conditions was to maximize DLA production. CCD design facilitated the efficient estimation of first-order, second-order, and interaction terms of the individual factors.

Table 5.1: Independent variables and their corresponding levels for DLA production

Independent variables	Symbol	Coded levels				
		-α	**-1**	**0**	**+1**	**+ α**
Temperature	X1	30	35	40	45	50
pH	X2	4.5	5.5	6.5	7.5	8.5
Rpm	X3	25	100	175	250	325
Fermentation time	X4	4.5	28	51.5	75	98.5

5.3.6 Batch fermentation in shake flask

Batch fermentation involving the selected mutant strain was conducted in a 250 mL flask, which comprised 200 mL of the optimized fermentation medium. The fermentation was performed under optimal media components and fermentation parameters, determined from both OFAT and RSM studies previously, with supplementation of 5% (v/v) inoculum as outlined in previous sections. Samples were periodically collected and centrifuged at a speed of 6000 rpm for 10 minutes. The supernatants were subsequently analysed for DLA, other by-products and biomass. All fermentation experiments were executed in duplicates, with the results represented as mean values.

5.3.7 Valorization of whey permeate in bioreactor

Bioreactor experiments were conducted using pure lactose (99.09% purity) and whey permeate (composition mentioned in literature reported by [276]) in MRS medium simultaneously. WP containing lactose were supplemented with yeast nitrogen base 5 g L^{1}, $CH_3COONa.3H_2O$ 5 g L^{-1}, K_2HPO_4 2 g L^{-1}, tri-ammonium citrate 2 g L^{-1} , $MgSO_4.7H_2O$ 0.2 g L^{-1} , $MnSO_4.4H_2O$ 0.05 g L^{-1} and Tween 80 1 g L^{-1} was used as production media[277]. Inoculum was cultured for 6 hour in 100 mL MRS media supplemented with 10 g L^{-1} lactose. DLA production was performed in a 1.5 L bench-scale bioreactor (Minifors 2, Infors AG) containing 30% lactose

(WP). The bioreactor operating specifications (pH, temperature, stirring speed) were adopted from the values optimized using RSM-CCD. Dissolved oxygen and air flow rate was maintained at 20% and 0.2 vvm [150] . pH was maintained by automatic feeding of 4 N KOH. All experiments were conducted in duplicates, and the mean outcomes of the experimental data were provided.

5.3.8 Transcript level analysis and enzymatic activity

The extraction of RNA from *L. bulgaricus* cells was conducted using the procedure outlined by [156]. cDNA was acquired by reverse transcription of 4 to 5 µg of DNA-free RNA with iScript^{TM} cDNA Synthesis kit (Bio-RAD). q-RT PCR was conducted with iTaq^{TM} Universal SYBR Green Supermix (Bio-RAD). Primers corresponding to specific genes, as indicated in Table 5.2, were formulated in accordance with the publicly available genome sequences of *L. bulgaricus* ATCC 11842 (GenBank accession number: NC_008054.1). The quantification of these genes was executed using 16s ribosomal RNA (16S rRNA) as the internal standard. The real-time PCR was conducted in a LightCycler instrument (ABI PRISM® 7500 Real-Time PCR System, Applied Biosystems, USA) following the manufacturer's guidelines. The samples underwent a 30-second denaturation at 95 °C, followed by 40 cycles of 5 seconds at 95 °C and 34 seconds at 60 °C. The data were analysed using the ABI PRISM 7500 System Sequence Detection software. Three independent experiments were performed to compute relative gene expression, which was calculated using the $2^{-\Delta\Delta CT}$ method [157]. Enzymatic activity for each of the key enzymes were measured at 340 nm by monitoring the decline or rise of NAD (phosphate) [NAD(P)H or NAD(P)$^{+}$] using methods as described in published literature [81].

Table 5.2: Oligonucleotide pairs used for determining gene expression of Mut_N23

Primers	Protein/gene	Amplicon size (bp)	Tm (°C)
Sense: ATCGGAAACTGTCATTCTTG Antisense: CTAATCCTGTTCGCTACCC	16s rRNA	157 bp	52
Sense:CATCCTGCGTCAAGACAAG Antisense:TAAGCGATAACCTTAGCGC	*Ldb0101*	174 bp	53
Sense: TTCCGCTACCGTATGGAC Antisense: CACGTCATAGGCGATGAC	*Ldb1010*	165 bp	50

Sense: TAACTTCATCTTTGCTTC Antisense: CACCACGGGCAACCATCA	*Ldb0839*	179 bp	55
Sense: TGTTGTCGTTATTGGTG Antisense:CGCGTCCATTGCCGTCATG	*Ldb0838*	160 bp	52
Sense:GAAGCTCAACCTGGACG Antisense:ATCCAGGTATCTGGTGGCA	*Ldb1772*	184 bp	48
Sense:GCTGACTGGCCTGGAC Antisense:CGGAAGCCCCAAAGGC	*Ldb0119*	179 bp	50
Sense:TTACACTTCAGTTTTG Antisense:TCAGCAGTTTCAGC	*Ldb0230*	149 bp	45
Sense:GATTCACACTTCCCTTTTG Antisense:CTGGACACGTCCTTGTCC	*Ldb1734*	150 bp	50
Sense: ACTTCATTCCGTGACGG Antisense:CACGTTCAATAGCTTCG	*Ldb1294*	170 bp	51
Sense:ACGGCCATGAAGGCTG Antisense: GTGATAACGCCGATC	*Ldb0636*	152 bp	55
Sense:AGTCGTTTTGGCCGGGACTGT Antisense:TCAAAGGCCGGAATCC	*Ldb1593*	180 bp	62

5.3.9 Purification and characterization of DLA

Subsequent to bioreactor fermentation, the residual media containing DLA was centrifuged at 14,000 rpm for 30 minutes to remove bacterial biomass and particulates. Acidification was performed in a 2-litre glass vessel with a working volume of 1 litre using H_2SO_4, with continuous pH monitoring until pH 1.0 was reached. Subsequently, 100 g L^{-1} activated carbon was added for decolourization over 5 hours. The activated carbon was separated by centrifugation at 14,000 rpm for 30 minutes, and the supernatant was filtered through a Whatman® grade 1 filter to remove residual carbon particles [278] .

The purification of the obtained DLA was further achieved through phase partitioning technique as reported by [279]. Ammonium sulfate (3 g) was dissolved in the flask, followed by the addition of 300 mL n-butanol. The mixture was vortexed and shaken for 2 hours at 30 °C. Subsequently, the mixture was moved to a separation funnel, left undisturbed until aqueous and organic phases were clearly distinguishable. The upper organic phase was then separated post phase separation. The isolated organic phase was evaporated at a temperature of 50 °C using a rotavapor. The resulting DLA in the dried organic phase was solvated in 5 mL of Milli Q water [279]. The pure DLA obtained was characterized using Fourier Transform Infrared (FTIR) analysis in the attenuated total reflectance (ATR) mode, (IR Affinity-1, M/s Shimadzu, Kyoto, Japan). The analysis involved an average of five scans at a resolution of 2 cm^{-1}, spanning a wavelength range of 400 to 4000 cm^{-1}[278] . The obtained pure DLA was then compared with commercially available pure DLA for reference. In a similar manner, the purified DLA was analysed using a 600 MHz proton-NMR analysis (^1H NMR) conducted on Bruker Ascend 600 MHz NMR spectrometer (Bruker, MA, USA) with TOPSPIN data acquisition software (Bruker) where purified DLA was dissolved in 0.6 mL of D_2O, analysed in 5 mm standard NMR tubes. ^1H chemical shifts were expressed in δ and data compared with previous reports.

5.3.10 Analytical methods

Glucose, lactate, and other metabolites including all types of reducing sugars in the collected supernatant were assessed using Phemomenex Aminex HPX-87H column and Phenomenex guard column attached to HPLC-RID detector system (M/s. Shimadzu, Corp., Kyoto, Japan). The isocratic elution procedure was adopted with a flow rate of 0.6 mL $minute^{-1}$ and a mobile phase of 5 mM H_2SO_4. Glucose, sucrose, mannose, fructose, and lactose concentration was determined by using the Refractive Index Detection (RID) detector system. Lactate and other metabolites concentrations were evaluated using UV detector at 210nm. Cell growth was measured using a UV-Vis spectrophotometer (Spectronics 200, ThermoFisher, UK. The presence of DLA was analyzed by a D-lactate dehydrogenase enzyme kit (K-DATE, M/s Megazyme, Ireland) and LLA was analysed using an L-lactate oxidase kit (K-LATE, M/s Megazyme, Ireland) based on manufacturers protocol in a multimode plate reader (Varioskan™ Lux, M/s ThermoFisher Scientific). Optical purity was measured using the formula given in Eq 1.

All the analysis were performed in duplicates, and the metabolite concentrations were represented as mean of duplicates. Experiments on enzymatic activity, nitrogen source

optimization, and generation stability were conducted in triplicates, and the results are reported as mean ± standard deviation.

5.3.11 Statistical analysis

The experiments in this study were carried out in duplicates or in triplicates. Graphs were plotted using Origin2021 (OriginLab Corporation, USA) software for statistical analysis and evaluated using analysis of variance (ANOVA) and Tukey Post-hoc test. Student's t-test was performed using Microsoft Excel (Microsoft, Redmond, WA, USA). Statistical significances were determined at a 95% confidence level ($p < 0.05$).

5.4 Results and discussion

5.4.1 Effect of UV and NTG on survivability rate

The number of colony forming units (cfu mL^{-1}) obtained were plotted for wild ATCC11842 based on their exposure to UV radiation at wavelengths of 245 nm and 365 nm. It was observed that UV radiation at 365 nm exhibited a greater effectiveness on strain ATCC 11842 compared to radiation at 254 nm, leading to a substantial reduction in the number of cfu mL^{-1} after 30 minutes of exposure (*Fig. 5.2a*). The untreated wild strain, which served as the control group, exhibited no reduction in cfu mL^{-1}, highlighting the effectiveness of mutagenesis treatments (data not shown). These findings highlight the inherent resistance of LAB to UV irradiation in contrast to *E. coli* where UV radiations is highly impactful when exposed for few seconds [280][281]. The mechanism behind the observed sensitivity can be attributed to UV light-induced DNA damage, primarily the formation of covalent bonds (dimers) between neighbouring pyrimidines, with a particular emphasis on thymine, where more than 85% of these bonds are formed. In this investigation, two specific wavelengths were meticulously selected based on their categorical classifications: the 365 nm wavelength is situated within the UV-C spectrum, representing a longer wavelength, whereas the 245 nm wavelength is categorized under the UV-A spectrum, indicative of a shorter wavelength. These radiations can be equally effective on Gram positive bacteria depending on the varied degree of effectiveness. As per previous published reports [282], UV-C radiation showed more effectiveness in this present study.

Additionally, we report primarily that ATCC 11842 possess a significantly higher intrinsic resistance to chemical mutagen, NTG. To determine the ideal conditions for NTG mutagenesis, two factors were evaluated: NTG concentration (ranged from 100 to 500 µg ml^{-1}), and exposure

time (varied between 20 and 120 minutes). The survivability rate exhibited a substantial decrease when the concentration of NTG exceeded 200 µg ml^{-1} and exposure time extended beyond 60 minutes (*Fig. 5.2b*). Moreover, as the survivability rate declined, a corresponding increase in the rate of mutagenesis was observed during mutagen screening probably due to occurrence of random point mutations [283]. The degree of potency in NTG mutagenesis can vary from species to species, encompassing diverse organisms inclusive of bacteria, fungi, and algae [76] [284] [285]. The cell mortality rate for ATCC 11842 ranged from 5.45% to 14.9% under a UV wavelength of 265 nm, however, a significant increase in cell mortality rate was noted at a UV wavelength of 365 nm, with rates escalating substantially from 28% to 87.09%. The highest cell death was recorded at NTG concentrations of 400 and 500 µg ml^{-1} and this observation was consistent across different time frames of exposure, namely 60, 90, and 120 minutes. The surviving cells exhibit a substantial accumulation of both genotypic and phenotypic mutations. This random mutagenesis study on ATCC 11842 reveals insights for future research shedding light on strain's unique adaptability, possibly due to distinct DNA repair mechanisms, highlights its resilience.

Lactobacillus bulgaricus is an important probiotic organism and have exhibited various responses to mutagenesis treatments, demonstrating both phenotypic and genotypic changes. Mutant strains have shown enhanced acid production capabilities and improved fructose utilization, which could be beneficial for yogurt production [286] [287]. Notably, some mutants obtained through ultraviolet light and chemical mutagenesis methods have displayed an increased capacity to decompose uremic toxins, with significant improvements in the breakdown of substances like creatinine, urea nitrogen, and uric acid [288]. These mutant strains often exhibit genetic stability, maintaining their newly acquired traits over multiple generations [288]. While these mutations can lead to beneficial changes, it's crucial to note that careful screening and extensive testing are necessary to ensure that the enhanced traits do not compromise other essential probiotic features or safety aspects of the bacteria. The findings of this present study provide valuable insights into the mutagenic responses of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 to both UV radiation and chemical mutagen NTG. The differential response to UV wavelengths is particularly noteworthy, with 365 nm proving more effective in reducing cell viability compared to 254 nm. This heightened sensitivity to 365 nm UV light could be attributed to several factors. While DNA typically absorbs more strongly at 254 nm, the greater effectiveness of 365 nm suggests that other cellular components or photosensitizers may be involved in the mutagenic process at this wavelength [289]. This could

lead to indirect DNA damage through the generation of reactive oxygen species or other intermediates. The longer wavelength of 365 nm UV light may penetrate cellular structures more effectively, potentially causing more widespread damage throughout the bacterial cell. The DNA repair systems in *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 may be more adept at addressing damage caused by 254 nm UV, which is closer to the wavelengths encountered in natural sunlight. The observed resistance to NTG at lower concentrations and shorter exposure times is intriguing. This resilience could be attributed to efficient DNA repair mechanisms system present in ATCC 11842. However, the sharp decline in survivability at higher NTG concentrations and longer exposure times indicates a threshold beyond which these protective mechanisms are overwhelmed. The increase in mutagenesis rate with prolonged NTG exposure suggests a cumulative effect of DNA damage [290]. This could be due to the saturation of repair enzymes or the induction of error-prone repair mechanisms under severe stress conditions. Such a response is consistent with the SOS response observed in many bacteria when faced with extensive DNA damage [281]. Future research could focus on elucidating the specific molecular mechanisms underlying the observed responses, such as identifying the DNA repair pathways activated by different mutagenic treatments. Additionally, investigating the phenotypic changes in the surviving mutants, particularly in terms of technological properties relevant to dairy fermentations, could provide valuable insights for strain improvement strategies. In conclusion, this study enhances our understanding of the mutagenic responses of *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 and provides a foundation for developing more targeted and effective mutagenesis strategies for this industrially important LAB.

5.4.2 Screening of mutants with enhanced DLA biosynthesis and genetic stability

115 mutant strains were screened from combined UV and NTG mutagenesis treatments and best three strains, Mut_N5, Mut_N23, and Mut_ N28, that exhibited notable enhancements in biomass, DLA production and yield, were selected for further experiments (*Fig. 5.2c*). Selected from combined UV and NTG treatment, these strains demonstrated superior effectiveness compared to individual treatments. The selected mutants exhibited maximal DLA production ranging from 3.1 to 3.5 g L^{-1} within 35 hours of fermentation, representing a 1.94-fold increase over wild-type strain in flask level. Moreover, these selected strains exhibited improved growth performance and DLA yield contrast to wild strain which means more amount of glucose was utilized for DLA and energy production through the Embden Meyerhof pathway [271].

Although both the wild-type and mutant strains produced acetic acid as the major by-product in comparable amounts (by-product profile data not shown), no discernible correlation was observed in this study between biomass concentration and DLA production. This observation is intriguing as it contradicts the conventional understanding that lactic acid production is inherently linked to microbial growth [150]. When lactose concentrations exceed 20 g L^{-1}, non-growth-associated lactic acid production occurs in *L. bulgaricus* has been prevalent was reported previously [291]. This also signifies that in mutant strains, DLA production is not solely dependent on the existence of rapidly dividing cells but also primarily relies on increased enzyme activity in mutated strain, which subsequently amplifies DLA production [292]. Growth variability in *Lactobacillus delbreuckii* subsp. *bulgaricus* ATCC 11842 is influenced by environmental and nutritional factors. In this study, mutant strains Mut_N23 and Mut_N5 showed higher net specific growth rates than the wild-type ATCC 11842, indicating enhanced metabolic efficiency or altered regulatory mechanisms, which likely facilitated faster growth through improved nutrient utilization. Table 5.3 provides a comparative analysis of all major kinetic parameters between the wild ATCC 11842, and the efficient mutants selected after screening. Among the three shortlisted mutants, Mut_N23 was selected as the most efficient strain, showing improved performance with a maximal DLA biosynthesis of 3.5 g L^{-1} and a yield of 0.175 g g^{-1} of DLA from glucose.

Genetic stability test serves as a critical factor to verify the persistence of mutations over time. Previous reports indicate that *L. thermophilus* mutants, after random mutagenesis, showed good stability and enhanced LLA production [269]. In concordance with the experimental findings, the mutant strains Mut_N5, Mut_N23, and Mut_N28 consistently manifested elevated DLA titres relative to the wild-type counterpart (*Fig. 5.2c*). Mut_N23 consistently produced DLA over seven generations, ranging from 3.50±0.20g L^{-1} to 3.59±0.23g L^{-1}, demonstrating its robustness and suitability for further research (*Fig 5.1*). This research highlights the potential of combined UV and NTG mutagenesis to generate unique mutants with enhanced metabolite production in gram-positive lactic acid bacteria.

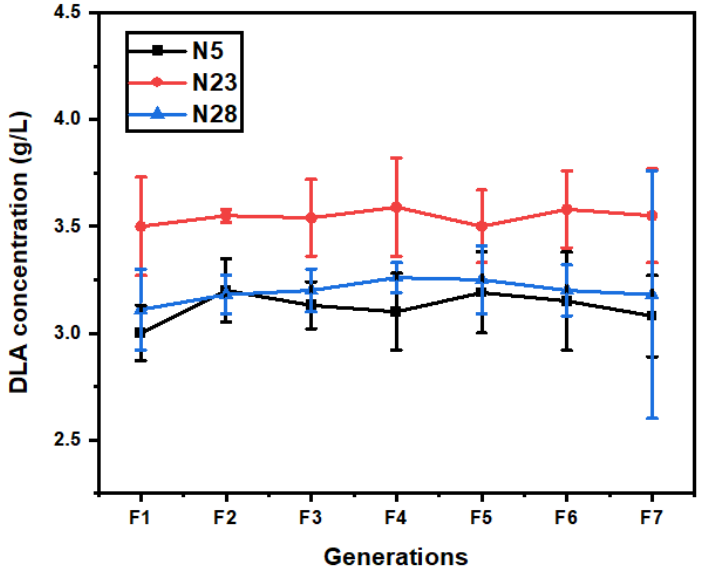


Figure. 5.1 DLA production across seven generations for the selected mutated strains. The error bars indicate standard deviations of three parallel replicates.

Table 5.3: Comparative analysis of kinetic parameters in MRS-glucose medium for wild-type and three selected mutants.

Strain	Fermentation time (hours)	Max. biomass concentration ($g\ L^{-1}$)	Max. DLA concentration ($g\ L^{-1}$)	$Y_{DLA/S}$ [a] ($g\ g^{-1}$)	$Y_{X/S}$ [b] ($g\ g^{-1}$)	DLA Optical purity (%)	r_P [c] ($g\ L^{-1}\ hr^{-1}$)	Specific growth rate (μ)
Wild	48	1.89	1.8	0.048	0.069	99.06	0.033	0.038
Mut_N5	38	1.99	3.13	0.169	0.092	99.08	0.052	0.060
Mut_N23	36	2.01	3.55	0.175	0.100	99.12	0.076	0.056
Mut_N28	35	2.03	3.11	0.116	0.093	99.07	0.046	0.037

a DLA Yield ($Y_{DLA/S}$, gg^{-1}) was calculated as a ratio of DLA produced (g) to glucose consumed (g)

b DLA Yield ($Y_{X/S}$, gg^{-1}) was calculated as a ratio of biomass produced (g) to glucose consumed (g)

c Volumetric productivity (rp, $g\ L^{-1}\ h^{-1}$) was calculated as a ratio of concentration of DLA produced ($g\ L^{-1}$) to fermentation time (h).

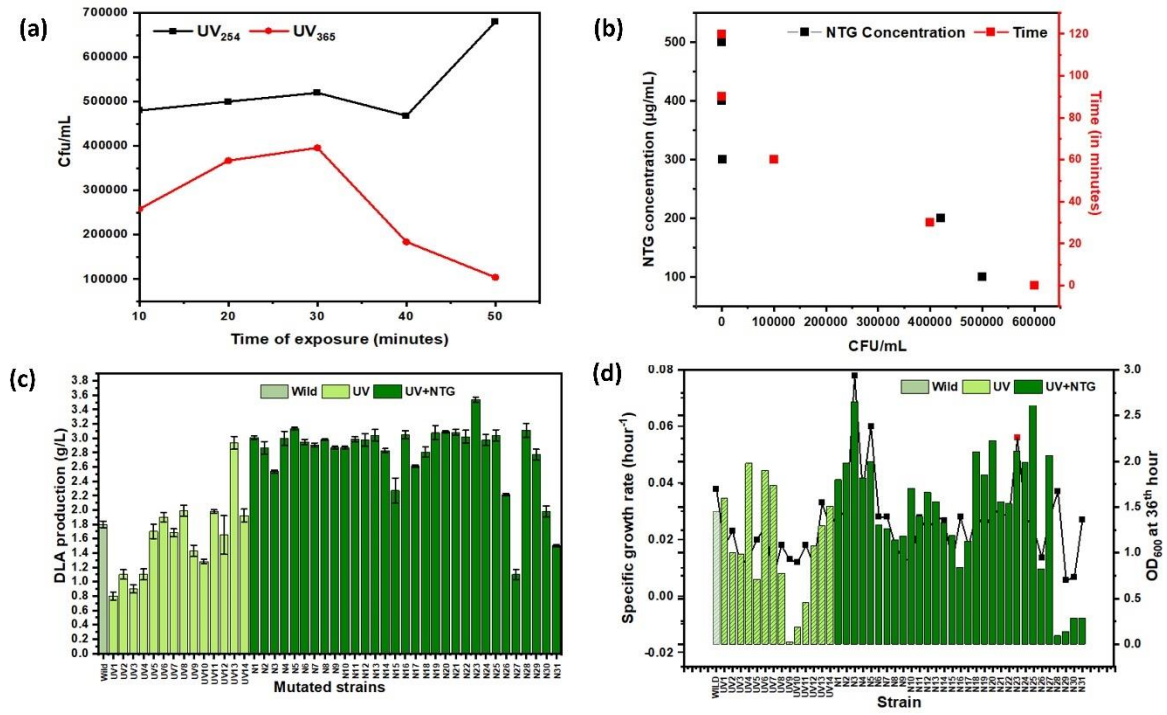


Figure.5.2 Survivability of ATCC 11842 following mutagenic treatments and screening of enhanced DLA biosynthesis in mutant strains (a) The number of colony-forming units (cfu mL^{-1}) as a function of exposure time at UV wavelengths of 254 nm and 365 nm, illustrating the differential impact of UV light on microbial viability. (b) Colony-forming units (cfu mL^{-1}) plotted against various concentrations of NTG over time, providing insight into the dose-dependent effects of NTG on cell survival, (c) DLA production comparison among wild-type, UV-treated, NTG-treated, and combined UV-NTG-treated mutant strains, (d) Assessment of specific growth rates and optical density (OD_{600}) at the 36^{th} hour across wild-type, UV-treated, NTG-treated, and combined UV-NTG-treated mutants. This comprehensive analysis highlights the differential impacts of mutagenic treatments on DLA biosynthesis and growth characteristics where the bars represent specific growth rate, and the connected dots represent OD_{600}.

5.4.3 One-Factor-at-a-time optimization of carbon and nitrogen source for Mut_N23

The sugar catabolism efficiency of the mutated strain Mut_N23 was investigated, comparing its growth and DLA production with the wild-type ATCC 11842 strain using different reducing sugars. Wild *Lactobacillus bulgaricus* strains can ferment lactose, glucose, fructose, and mannose, but not galactose, also exhibiting a preference for lactose over glucose which contradicts the common bacterial preference for glucose [293] [294]. It is reported that the accumulation of galactose in the medium led to feedback inhibition during the conversion of lactose into cells and lactic acid [291]. It has also been reported that *L. bulgaricus* doubles faster in presence of lactose than in glucose, with similar final biomass after 24 to 48 hours

[295]. In this study, both wild and mutant strains efficiently utilized glucose, lactose, mannose, and fructose but not galactose due to the absence of galactose pathways. Mut_N23 consistently produced more DLA in shorter times across all carbon sources than the wild strain. For example, Mut_N23 produced 3.558 g L^{-1} DLA from glucose in 36 hours, while the wild strain produced 1.806 g L^{-1} in 48 hours with similar final biomass. Similarly, Mut_N23 produced 3.819 g L^{-1} of DLA from lactose in 38 hours, whereas the wild strain produced 3.06 g L^{-1} in 63 hours. Mut_N23 exhibited faster growth on lactose (0.092 h^{-1}) than on glucose (0.056 h^{-1}), unlike the wild strain, which had similar growth rates on both sugars. Residual galactose in the medium during lactose fermentation did not affect cell growth using lactose's glucose moiety but reduced overall growth by 25% at high concentrations for both wild and mutant (30 g L^{-1} and above). *Lactobacillus bulgaricus* demonstrates concentration-dependent growth on lactose, with no glucose-induced repression, indicating simultaneous glucose and lactose consumption without diauxic growth, highlighting a unique aspect of lactose metabolism in both strains [295]. The Mut_N23 strain demonstrated a superior DLA titre of 3.819 g L^{-1} and exhibited favorable kinetic parameters when utilizing lactose, highlighting lactose as a more efficient carbon source for DLA biosynthesis compared to other substrates (Table 5.4). Notably, the residual glucose in the Mut_N23 strain was lower (2.32 g L^{-1}) than in the wild-type strain (7.09 g L^{-1}), indicating a higher efficiency of glucose utilization in the mutant strain despite the presence of galactose moieties. The Mut_N23 strain exhibited a higher glucose utilization rate (0.845 $gg^{-1}h^{-1}$) inspite of leftover galactose moiety compared to the wild-type strain (0.616 $gg^{-1}h^{-1}$), and the DLA production from lactose and fructose was higher compared to that from glucose alone (Table 5.4). This differential response in the mutant strain could be attributed to variations in the regulation of the lactose operon or the glucose transport system caused due to beneficial mutations.

A notable observation from results obtained from this chapter was that the Mut_N23 strain's ability to grow and produce DLA using sucrose, reaching a maximal DLA production of 3.31 g L^{-1}, a capability not observed in the ATCC 11842 wild strain. This novel discovery challenges current knowledge, as sucrose utilization by *Lactobacillus delbrueckii* subsp. *bulgaricus* has not been previously documented. The inability of ATCC 11842 to utilize sucrose is likely due to genetic factors that result in either a deficiency or inadequate expression of sucrase, as well as insufficient transport mechanisms for sucrose and its breakdown products. Despite the presence of key enzymes such as sucrose phosphorylase, sucrose-6-phosphate hydrolase, and the PTS system in the genomic DNA of ATCC 11842, sucrose remains unutilized. In contrast,

the ability of the Mut_N23 strain to utilize sucrose potentially involves novel or upregulated functional enzymes capable of sucrose hydrolysis and metabolism similar reports recently published by [265] where a halophilic bacterium also showed enhanced sucrose utilization rate after random mutagenesis. This adaptation could also confer an advantage under certain environmental circumstances, such as varied sugar availability or stress responses when using different waste feedstocks.

Fermentation experiments were executed out at different initial lactose and sucrose concentrations (10 to 50 g L^{-1}) to investigate on growth and DLA production kinetics (Table 5.5 and 5.6). The choice of the initial sugar concentration range in this study was informed by existing literature, while exceeding this range leads to substrate inhibition. It was observed that 30 g L^{-1} of lactose is the ideal concentration using wild *L. delbreuckii* strain for achieving highest DLA production and maximum DLA yield, which corroborates with values reported in the literature [79]. It was also noted that while the glucose moiety of lactose was completely metabolized at lower lactose concentrations, its accumulation was evident at higher concentrations (30 g L^{-1} and above). In contrast to the wild-type strain, which lacks sucrose utilization capability, the mutant strain Mut_N23 demonstrates the ability to produce a maximum of 3.36 g L^{-1} DLA from an initial sucrose concentration of 30 g L^{-1}. Moreover, Mutant_N23 achieves a maximal productivity rate of 0.100 g L^{-1} h^{-1} when utilizing both 30 g L^{-1} lactose and sucrose as substrates.

Nitrogen substrate serves as a vital component for DLA synthesis and yeast extract (10 g L^{-1}) was stated to be the optimal nitrogen source for lactic acid production in wild *Lactobacillus delbreuckii* [296][297]. This study found that adding yeast nitrogen base (YNB) with yeast extract boosted DLA production in Mut_N23 more than other nitrogen sources (*Fig. 5.3*). YNB's composition, including ammonium sulfate, vitamins, and trace elements, likely contributed to this improvement. Overall, Mut_N23's ability to use a wider range of carbon sources, including sucrose, and its high DLA production efficiency (99.06% optical purity) have significant industrial bioprocessing benefits. Utilizing Mut_N23 as a potential strain can increase DLA yields and productivity from various substrates through fed-batch and continuous operations, enhancing cost-effectiveness and flexibility in biotechnological applications in future research. The genetic stability of the obtained mutants over seven generations, particularly Mut_N23, underscores the potential for future industrial applications in this study. The consistent DLA production across generations indicates that the beneficial

mutations are stable and do not revert easily, which is crucial for maintaining production efficiency in large-scale fermentations [298].

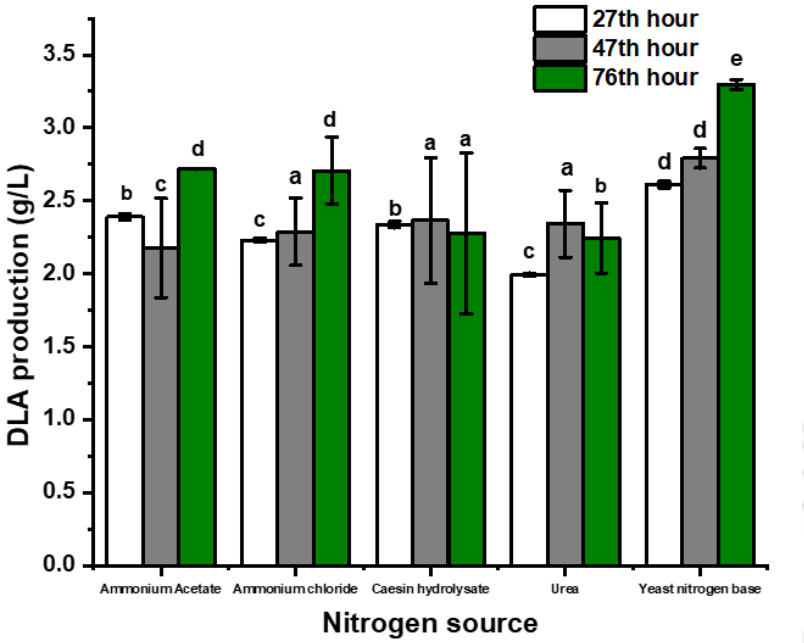


Figure. 5.3 Variations in DLA production using additional nitrogen sources. The same letter indicates a non-significant difference in data ($p > 0.05$) and different letters indicate a significant difference ($p < 0.05$)

Table 5.4: Kinetic parameter comparison for wild-type ATCC 11842 and mutant Mut_N23 across various carbon sources

Strain	Carbon source	Fermentation time (h)	Max. DLA production ($g\ L^{-1}$)	q_s [a] ($gg^{-1}\ h^{-1}$)	$Y_{DLA/S}$ [b] ($g\ g^{-1}$)	$Y_{X/S}$ [c] ($g\ g^{-1}$)	$Y_{DLA/X}$ [d] ($g\ g^{-1}$)	r_p [e] ($g\ L^{-1}\ h^{-1}$)	Max. biomass concentration($g\ L^{-1}$)
WILD	Glucose	48	1.80	0.61	0.03	0.09	1.02	0.03	1.89
	Lactose	63	3.06	0.39	0.05	0.10	1.69	0.04	2.01
	Sucrose	41	0.54	0.02	0.01	0.02	0.01	0.01	0.56
	Mannose	53	0.57	0.06	0.01	0.04	0.53	0.01	0.89
	Fructose	63	3.08	1.76	0.04	0.09	1.33	0.05	1.89
Mut_N23	Glucose	36	3.55	0.84	0.09	0.10	1.75	0.07	2.01
	Lactose	38	3.81	0.57	0.10	0.11	1.50	0.10	2.32
	Sucrose	41	3.31	0.17	0.07	0.09	0.67	0.08	1.86
	Mannose	53	0.60	0.08	0.01	0.03	0.68	0.02	0.95
	Fructose	63	3.24	1.84	0.05	0.10	1.70	0.05	1.85

a Specific substrate utilization rate is the ratio of substrate consumed to fermentation time

b DLA Yield ($Y_{DLA/S}$, gg^{-1}) was calculated as a ratio of DLA produced (g) to substrate consumed (g)

c DLA Yield ($Y_{X/S}$, gg^{-1}) was calculated as a ratio of biomass produced (g) to substrate consumed (g)

d DLA Yield ($Y_{DLA/X}$, gg^{-1}) was calculated as a ratio of biomass produced (g) to glucose consumed (g)

Table 5.5: Kinetic parameters of Mut_N23 grown on varied initial lactose concentrations

Initial Lactose conc. ($g\ L^{-1}$)	Maximum DLA production ($g\ L^{-1}$)	Maximum biomass produced ($g\ L^{-1}$)	$Y_{DLA/S}$ a ($g\ g^{-1}$)	$Y_{X/S}$ b ($g\ g^{-1}$)	$Y_{DLA/X}$ c ($g\ g^{-1}$)	r_P d ($g\ L^{-1}\ h^{-1}$)	Optical purity (%)
10	3.71	2.01	0.37	0.19	1.75	0.07	99.01
20	3.82	2.34	0.18	0.11	1.60	0.07	99.08
30	4.16	2.65	0.13	0.09	1.54	0.10	99.11
40	4.08	2.89	0.10	0.07	1.42	0.09	99.01
50	4.05	2.88	0.08	0.05	1.40	0.07	99.00

a DLA Yield (YDLA⁄S, gg^{-1}) was calculated as a ratio of DLA produced (g) to lactose consumed (g)

b DLA Yield (YX⁄S, gg^{-1}) was calculated as a ratio of biomass produced (g) to lactose consumed (g)

c DLA Yield (YDLA⁄X, gg^{-1}) was calculated as a ratio of DLA produced (g) to biomass produced (g)

d Volumetric productivity (r_P, $g\ L^{-1}\ h^{-1}$) was calculated as a ratio of concentration of DLA produced ($g\ L^{-1}$) to fermentation time (h).

Table 5.6: Kinetic parameters of Mut_N23 grown on varied initial sucrose concentrations

Initial Sucrose conc ($g\ L^{-1}$)	Maximum DLA production ($g\ L^{-1}$)	Maximum biomass produced ($g\ L^{-1}$)	$Y_{DLA/S}$ a (g/g)	$Y_{X/S}$ b (g/g)	$Y_{DLA/X}$ c ($g\ g^{-1}$)	rP d ($g\ l^{-1}\ h^{-1}$)	Optical purity (%)
10	3.06	1.01	0.03	0.01	0.45	0.05	99.00
20	3.31	1.84	0.07	0.09	0.67	0.08	99.17
30	3.36	1.98	0.08	0.09	0.78	0.10	99.09
40	3.08	1.39	0.03	0.04	0.48	0.07	99.17
50	3.00	1.88	0.02	0.02	0.67	0.06	99.00

a DLA Yield ($Y_{DLA/S}$, gg^{-1}) was calculated as a ratio of DLA produced (g) to sucrose consumed (g)

b DLA Yield ($Y_{X/S}$, gg^{-1}) was calculated as a ratio of biomass produced (g) to sucrose consumed (g)

c DLA Yield ($Y_{DLA/X}$, gg^{-1}) was calculated as a ratio of DLA produced (g) to biomass produced (g)

d Volumetric productivity (rp, g L^{-1} h^{-1}) was calculated as a ratio of concentration of DLA produced (g L^{-1}) to fermentation time (h).

5.4.4 Fermentation parameter optimization using Response Surface Methodology

The effects of varying fermentation conditions on DLA production were investigated, using a Central Composite Design in Response Surface Methodology (RSM CCD) approach. The factors considered were temperature, pH, rpm, and fermentation time, which are crucial for enhancing DLA yield and efficiency in industrial processes [299]. Based on published literature [300], the ranges for these factors were set as follows: temperature 35 to 45°C, pH 5.5 to 7.5, rpm 100 to 250, and fermentation time 28 to 75 hours, with central values of 45°C, 7.5, 250, and 75, respectively. The mutated strain of *L. bulgaricus*, being a facultative anaerobe, also performs well under aerated conditions. This enhanced adaptability allows it to optimize its metabolic processes in both oxygen-rich and oxygen-poor environments, making it highly efficient for various applications.

A range of experiments were performed (Table 5.8), and a quadratic model equation for DLA production was obtained (Eq3). To verify the reliability of these models, validation experiments were conducted within the specified ranges. The outcomes of these 30 runs are presented in the Table 5.6 where the predicted values aligned well with the experimental results. The quality of fit using statistical parameters such as the coefficient of determination (R^2), lack of fit, and p-values were evaluated. The assigned codes to the variables were: temperature (A), pH (B), rpm (C), and fermentation time (D). Terms such as AB, AC, and BD were significant, indicating important interactions between the variables. The coefficients in the coded quadratic model equation reflected the individual and interactive effects of these factors on DLA production. The p-value for the quadratic regression equation was less than 0.0001, indicating its high significance. The lack of fit's p-value was 0.0512, implying its insignificance. The R-squared (R^2) and adjusted R-squared (Adjusted R^2) values were 0.9788 and 0.9591, respectively, demonstrating the equation's ability to accurately capture the relationship between each factor and the response value. ANOVA tables generated (Table 5.7) examined the significance of the model terms and their interactions. The experiments resulted in DLA production varying from 9.8 mg L^{-1} to 4.89 g L^{-1} under different experimental conditions. The contour plots were analysed to determine the significance of interactions between variables (*Fig.5.4*). Elliptical or circular contours obtained suggested significant interactions, while rounder contours implied

less significance[272]. Our findings in a coded equation for DLA production were summarized as given in Eq3:

$$DLA\ production = 4.79 - 0.27 * A + 0.21 * B + 0.12 * C + 0.30 * D - 0.63 * AB + 0.48 * AC - 0.20 * AD - 0.39 * BC + 0.47 * BD + 0.11 * CD - 0.94 * A^2 - 1.07 * B^2 - 1.05 * C^2 - 0.57 * D^2 \quad \text{..... (Eq 3)}$$

Using Design-Expert software, these models were applied to optimize fermentation parameters and predicted that DLA production can reach a maximum of 4.928 g L^{-1} under the following conditions: temperature at 38.4°C, pH adjusted to 6.79, rpm set to 171, and a fermentation time of 61.6 hours. This was validated by performing a shake flask experiment at all the optimized conditions and DLA titre of 4.698 g L^{-1} with optical purity of 99.09% could be obtained after 65 hours of fermentation.

Table 5.7: ANOVA Table for RSM-CCD analysis

	Sum of Squares	df	Mean Square	F-value	p-value	
Model	91.46	14	6.53	49.55	<0.0001	significant
A-Temperature	1.78	1	1.78	13.48	0.0023	
B-pH	1.09	1	1.09	8.27	0.0116	
C-rpm	0.3602	1	0.3602	2.73	0.1191	
D-fermentation time	2.17	1	2.17	16.45	0.0010	
AB	6.50	1	6.50	49.29	<0.0001	
AC	3.81	1	3.81	28.92	<0.0001	
AD	0.6496	1	0.6496	4.93	0.0423	
BC	2.46	1	2.46	18.66	0.0006	
BD	3.63	1	3.63	27.53	<0.0001	
CD	0.2242	1	0.2242	1.70	0.2119	
A^2	24.25	1	24.25	183.95	<0.0001	
B^2	31.53	1	31.53	239.21	<0.0001	
C^2	30.55	1	30.55	231.71	<0.0001	
D^2	8.96	1	8.96	67.96	<0.0001	
Residual	1.98	15	0.1318			
Lack of Fit	1.79	10	0.1787	4.68	0.0512	not significant
Pure Error	0.1909	5	0.0382			
Cor Total	93.44	29				

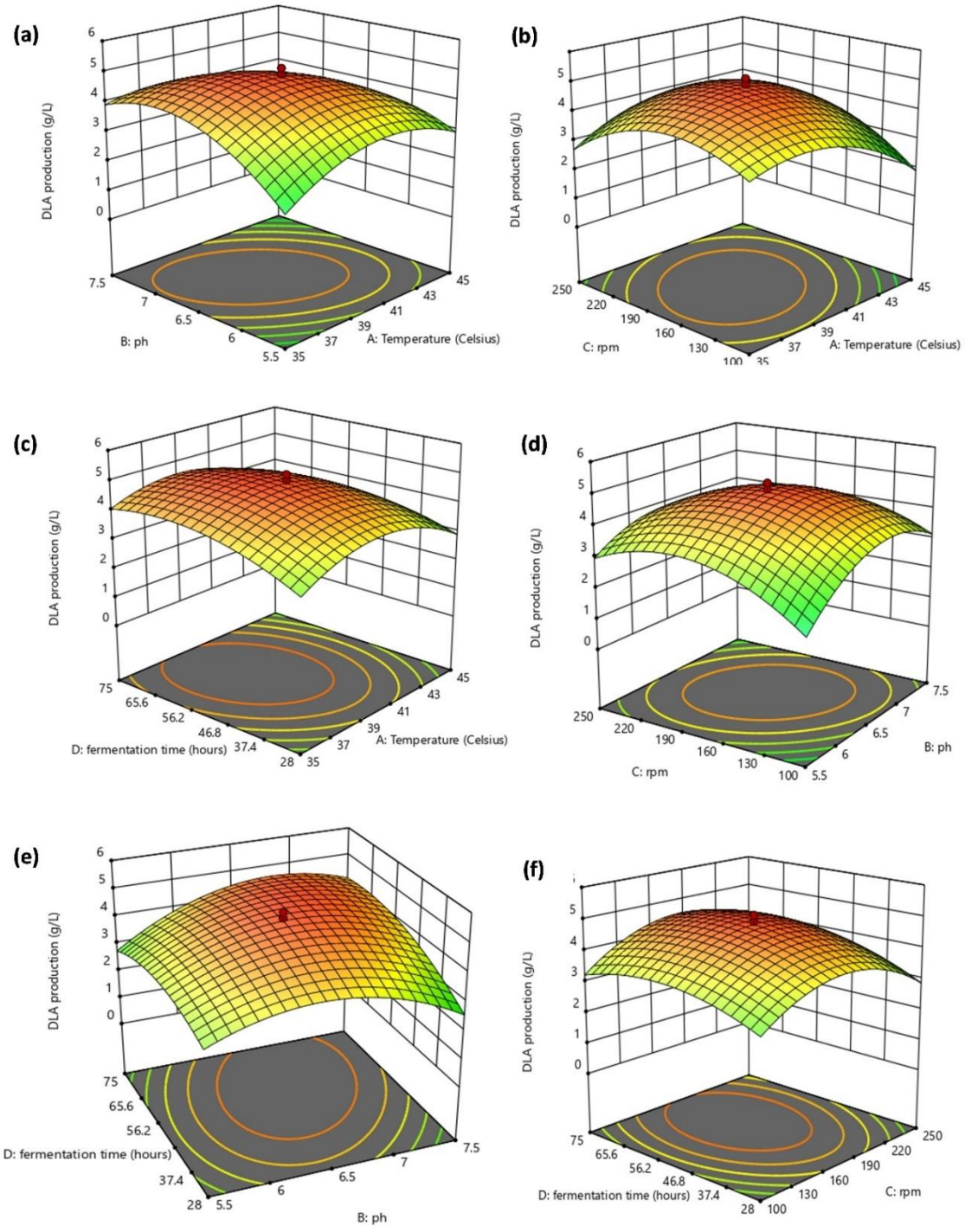


Fig.5.4 Surface plot for the RSM showing interactions between (a) pH and temperature, (b) rpm and temperature, (c) fermentation time and temperature, (d) rpm and pH, (e) fermentation time and pH, (f) fermentation time and rpm. The contour lines on each plot represent the response levels, with the highest point indicating the optimal conditions for each factor combination. These plots provide insights into the complex interactions

between fermentation parameters and highlight the critical factors influencing the production of D-lactic acid. (*A, Temperature; B, pH; C, rpm; D, Fermentation time)*

Table 5.8: Experimental design with predicted and actual values for DLA production

Run	Independent variables			Response value			
	Temperature	pH	rpm	Fermentation time	DLA production		
					Experimental	Predicted	Residual
1	40	7.5	250	51.5	1.98	1.94	0.0408
2	35	6.5	175	28	2.35	2.27	0.0763
3	35	6.5	25	75	3.28	3.16	0.1246
4	45	6.5	175	28	1.24	1.18	0.0596
5	40	6.5	175	51.5	3.26	3.26	-0.0017
6	40	7.5	100	51.5	3.45	3.50	-0.0542
7	40	6.5	175	98.5	2.55	2.49	0.0592
8	40	5.5	250	51.5	3.10	3.26	-0.1617
9	45	5.5	250	75	3.35	3.29	0.0646
10	40	8.5	175	4.5	0.89	0.89	-0.0042
11	45	6.5	175	28	0.98	1.13	-0.1471
12	35	7.5	100	75	2.19	2.30	-0.1054
13	40	6.5	175	51.5	2.09	2.08	0.0142
14	40	6.5	325	51.5	3.23	3.26	-0.0317
15	45	7.5	250	28	2.01	1.93	0.0829
16	40	5.5	100	51.5	3.21	3.26	-0.0517
17	40	7.5	250	51.5	2.09	1.98	0.1092
18	45	5.5	100	75	1.88	1.98	-0.0988
19	45	7.5	100	28	1.89	1.76	0.1312
20	35	6.5	175	75	2.10	2.26	-0.1637

21	45	6.5	175	75	3.89	3.99	-0.1021
22	35	6.5	175	28	3.00	3.23	-0.2321
23	40	5.5	100	51.5	3.32	3.26	0.0583
24	30	5.5	250	51.5	3.96	3.78	0.1775
25	40	5.5	250	51.5	3.45	3.26	0.1883
26	35	6.5	175	75	2.98	3.07	-0.0921
27	35	7.5	250	28	1.96	2.06	-0.0971
28	45	7.5	250	75	3.00	2.80	0.1996
29	50	6.5	175	51.5	2.86	2.98	-0.1225
30	35	6.5	250	28	2.98	2.90	0.0796

5.4.5 Optimized batch fermentation of Mut_N23 for enhanced DLA biosynthesis

Verification experiments on strains ATCC 11842 and Mut_N23 focus on DLA production from lactose and sucrose under optimized OFAT and RSM conditions. Lactose uptake in *Lactobacilli* involves $PTS^{Lactose}$ and lactose permease, while a lactose-galactose antiport mechanism exports galactose in lactic acid bacteria [79]. It was observed that Mut_N23 exhibited complete lactose utilization within 55 hours, with residual glucose at 1.22 g L^{-1} and galactose at 11.40 g L^{-1} (*Fig 5.5b*). In contrast, ATCC 11842 showed slower lactose utilization, with residual glucose and galactose at 5.0 g L^{-1} and 9.23 g L^{-1}, respectively. Mut_N23 continued to consume glucose even after DLA concentration reached 3.08 g L^{-1}[301] [150] (*Fig 5.5a*). The glucose concentration remained constant and was not consumed between the 45th and 65th hours of fermentation for wild strain. However, for the Mut_N23 strain, the glucose remained constant for a shorter duration and was subsequently consumed, resulting in increased DLA and other by-product synthesis (including acetic acid). Overall, comparative analysis revealed the superior performance of the mutant strain, with enhanced glucose utilization capability, a 45% increase in DLA yield from lactose ($Y_{DLA/S}$), a 115% increase in DLA yield from biomass ($Y_{DLA/X}$), and a 13% increase in productivity in presence of MRS-Lactose media.

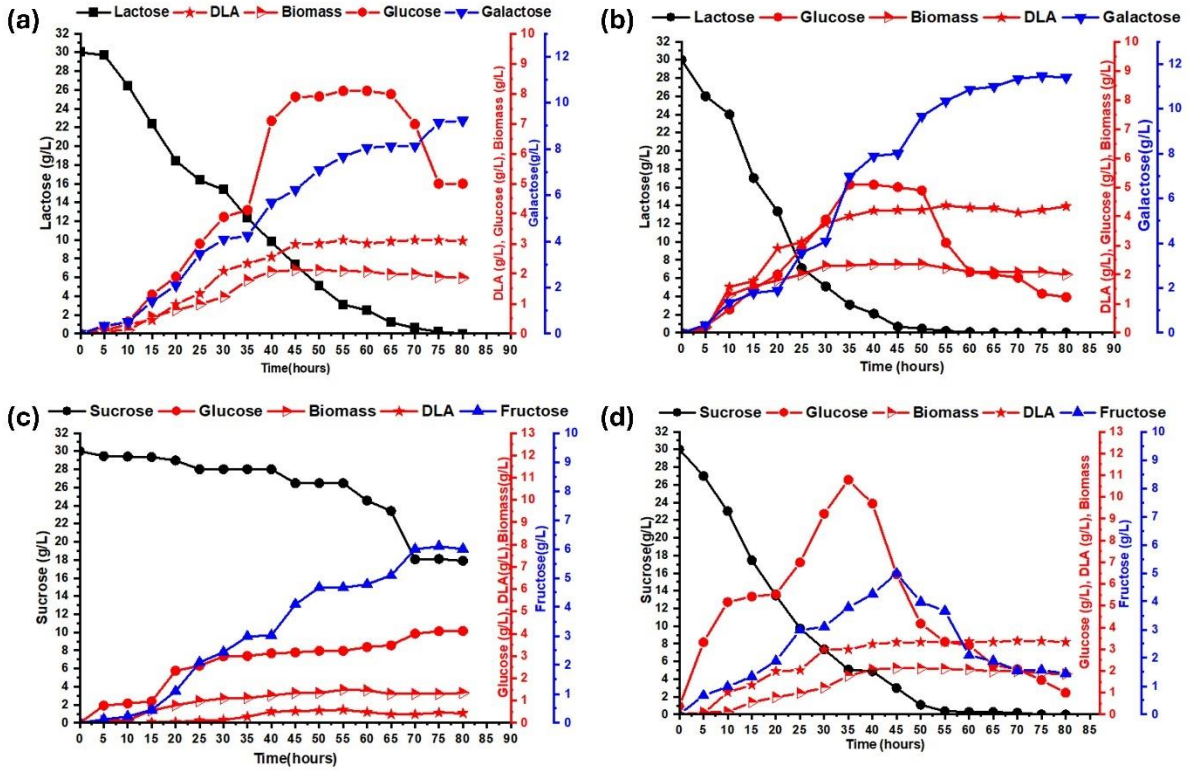


Figure 5.5 Batch fermentation dynamic profile of wild ATCC 11842 and Mut_N23. (a) Wild ATCC 11842 in presence of MRS-lactose in shake flask, (b) Mut_N23 in presence of MRS-lactose in shake flask, (c) Wild ATCC 11842 in presence of MRS-sucrose in shake flask and (d) Mut_N23 in presence of MRS-sucrose in shake flask.

It was observed that wild ATCC 11842 had a minimal sucrose utilization while the Mut_N23 demonstrated a notable enhancement in the dissociation rate of sucrose into its constituent monosaccharides, fructose, and glucose (*Fig.5.5c*). This observation contradicts to what was reported, [302] that *L. bulgaricus* lacks the property for sucrose utilization, which also indicates that the mechanism underlying sucrose utilization by *L. delbreuckii* subsp. *bulgaricus* warrants further investigation. Mut_N23 strain utilized released glucose for DLA biosynthesis, with a notable reduction in glucose concentration after 35 hours, stabilizing at 0.99 g L^{-1} (*Fig. 5.5d*). As DLA accumulated, the glucose utilization rate remained same, but the acidity of the environment increased, leading to a decline in microbial growth and a subsequent drop in productivity due to lack of pH maintenance in shake flask experiments [303]. The Mut_N23 strain produced a maximum of 4.47 g L^{-1} and 3.38 g L^{-1} DLA titre using lactose and sucrose as carbon sources, respectively, under optimized conditions, maintaining 99.09% optical purity. The proficient utilization of these carbon substrates by mutant strain Mut_N23 for DLA

biosynthesis underscores its capacity to serve as a platform for enhancing metabolic pathway in *Lactobacillus delbreuckii* subsp. *bulgaricus.* This contradicts previous suppositions about sucrose metabolism in *L. bulgaricus* and opens new research avenues. Future studies can aim to elucidate the molecular mechanisms behind these metabolic capabilities through transcriptomic analysis, optimizing *L. bulgaricus* strains for sustainable and efficient industrial use.

5.4.6 Valorization of whey permeate in lab-scale bioreactor

Bioreactor experiments were performed by cultivating Mut_N23 strain in both lactose (30 g L^{-1}) and WP (30 g L^{-1} lactose) – based MRS medium under regulated pH, temperature, agitation and dissolved oxygen (*Fig. 5.6a* and *b*). An initial increase in pH to 7.1 was observed during initial phase (0 to 4 hours) and subsequently, there was a marked decrease in pH, indicative of metabolic activity and organic acid production. This was further substantiated by an extended lag phase observed when WP was utilized, implying an adjustment period for Mut_N23 to acclimatize to the more complex medium. Lactose metabolism in the MRS-Lactose was significantly expedited, where 98% of the lactose was entirely depleted within 44 hours culminating in a DLA titre of 7.9 g L^{-1}. It is also important to note, despite the exhaustion of lactose, only the glucose moiety was metabolized, leaving the galactose component unutilized. In contrast, fermentation with WP yielded a maximum DLA titre of 4.89 g L^{-1} with 92% lactose consumption in 80 hours where a partial assimilation of galactose was observed. The lactose consumption rate in bioreactor was accelerated in regulated parameters compared to shake flask studies devoid of pH control [303]. Despite DLA titre differences, biomass concentrations were consistent in both media types, indicating cell growth wasn't restricted by lactose source. This confirms enhanced DLA production in Mut_N23 isn't solely dependent on cell growth but also on efficient enzymatic activity. Specific productivity for lactose-MRS and WP-MRS media were 0.110 and 0.066 g g^{-1} h^{-1} respectively.

The WP constituted the bulk of the fermentation media volume, overshadowing other nutrients. This could potentially elucidate the diminished DLA production and lactose consumption, as suggested by literature reports [304]. Lactose dissociation in MRS-pure lactose milieu demonstrated superior efficacy. Residual monosaccharides in lactose-MRS medium were 3.89 g L^{-1} glucose and 14.75 g L^{-1} galactose, whereas WP-MRS media exhibited residuals of 1 g L^{-1} glucose and 2.77 g L^{-1} galactose, signifying decelerated lactose dissociation. Contrasting [79] findings of *Lactobacillus delbreuckii*'s high galactose consumption, our study observed negligible uptake at elevated lactose concentration (30 g L^{-1}) in MRS medium. Galactose

uptake was observed in WP-MRS medium, likely due to WP's intricate nature affecting Mut_N23's fermentation and metabolic pathways. Along with DLA, huge amounts of acetic acid production were noted for both Lactose-MRS and WP-MRS based fermentation. The maximum productivity of batch bioreactor using lactose was 93% higher (0.208 g $L^{-1}h^{-1}$) than flask level experiments, which corroborates with published literature [305]. Both bioreactor experiments exhibited DLA optical purity of 99.09%.

Various *Lactobacillus* strains have demonstrated significant potential for DLA production using different substrates. *L. bulgaricus* CGMCC 1.6970 achieved the highest reported titre of 123.6 g L^{-1} with a yield of 97.9% and productivity of 1.72 g L^{-1} h^{-1} using chicory-derived inulin[188]. An evolutionarily engineered *L. bulgaricus* strain reached a titre of approximately 108 g L^{-1} using pretreated rice straw, highlighting the benefits of strain improvement through evolutionary engineering[80]. Additionally, *L. bulgaricus* CGMCC 1.6970 has been reported to utilize dairy waste as a low-cost substrate for DLA production, although specific metrics were not provided [152]. These results underscore the versatility of *Lactobacillus* strains in producing DLA from various feedstocks, including inulin, lignocellulosic biomass, and dairy waste. *L. delbrueckii* subsp. *bulgaricus* ATCC 11842 produced 62 g L^{-1} of DLA from organosolv pretreated beechwood, showcasing the viability of lignocellulosic biomass as a substrate [271]. This is the first kind of research where we report a mutated ATCC11842 strain with enhanced sugar utilization capability to produce optically pure DLA using WP. In another reported literature, the investigation focused on the inhibitory effects of DLA and LLA on *Lactobacillus delbrueckii* subsp. *bulgaricus*, revealing that DLA notably extended the lag phase and reduced both growth rate and cell yield more than LLA. This differential inhibition underscores the complexities inherent in lactic acid fermentation processes, where specific lactic acid isomers can significantly influence microbial performance [306]. The mutant strain Mut_N23 of *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842 exhibits significant improvements over traditional strains in several key areas. Mut_N23 can metabolize a wider variety of sugars, including sucrose and galactose from WP, making it highly suitable for converting dairy by-products into valuable products. The strain's genetic stability across multiple generations ensures the retention of these advantageous traits, which is crucial for reliable industrial applications. Moreover, its metabolic versatility allows for the simultaneous production of acetic acid and DLA, offering potential for multi-product bioprocesses that enhance overall economic feasibility.

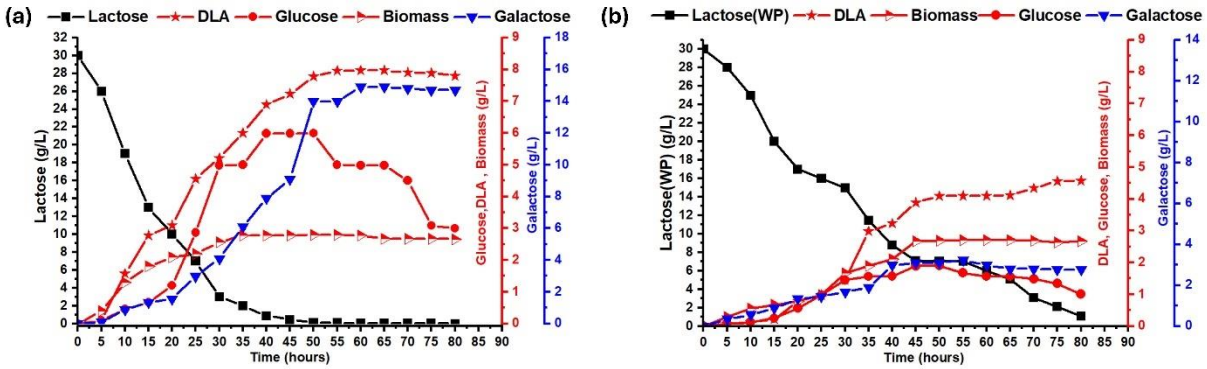


Figure 5.6 Batch fermentation dynamic profile of Mut_N23 (a) Fermentation dynamic profiles of mutant strain Mut_N23 in MRS-Lactose media scaled up in lab-scale bioreactor and (b) Fermentation dynamic profiles of mutant strain Mut_N23 in WP based MRS media in lab-scale bioreactor. These profiles illustrate the strain's performance and metabolic activity in optimized and regulated nutritional environments, providing insights into its scale-up properties and efficiency in utilizing waste feedstocks.

The comparative analysis of DLA production using pure lactose and WP in bioreactor highlights the metabolic efficiency of Mut_N23 in different substrate environments at higher scale. The findings underscore the potential of employing pure lactose as a more favourable and readily available substrate for DLA production in terms of fermentation kinetics and end-product concentration by *L. bulgaricus* [307]. The decrease in DLA titre and specific productivity when using WP as a carbon source, compared to lactose, may be attributed to high levels of dissolved minerals that inhibit microbial growth and disrupt metabolic processes, along with the presence of inhibitory compounds that negatively impact cell metabolism. Moreover, the capability of *L. bulgaricus* Mut_N23 to transform dairy waste into DLA was addressed, particularly through the utilization of lactose and galactose from WP. While the initial yields of DLA were modest and accompanied by altered metabolic behaviour, these challenges serve as a framework for further investigation and optimization. Through appropriate pretreatment of WP, such as ultrafiltration and deproteinization, the Mut_N23 strain demonstrates significant potential for DLA production. Further refining the fermentation media composition and adding industrial enzymes could enhance the efficiency of lactose hydrolysis has potential to significantly improve the utilization rates of complex substrates like WP, as evidenced by previous studies [308] [309, 310] [310]. This chapter underscores the value of re-purposing dairy waste by-product, WP and positions *L. bulgaricus* Mut_N23 as a key contributor to sustainable bio-based commodities manufacturing, including optically pure DLA and other crucial metabolites like acetic acid, fostering more efficient and cost-effective bioprocessing methods.

5.4.7 Transcript level and enzymatic activity analysis of Mut_N23

Glycolytic pathway is the major pathway for DLA biosynthesis in lactic acid bacteria and the major genes involved for DLA biosynthesis have been reported (Mukherjee et al., 2023).Enzymatic activity and gene expression in major glycolytic genes for DLA biosynthesis were compared between mutant strain Mut_N23 and wild type ATCC 11842 (*Fig.5.7a* and *5.7 b*). RT-qPCR analysis showed Mut_N23's glycolytic genes upregulated by 20.23% to 84.89% at 12^{th}, 24^{th}, and 48^{th} hours. In Mut_N23, a comparative enzymatic assay delineated an augmented enzymatic activity for *d-ldh*, *pgm*, *glk*, and *pfk*. Conversely, a diminished enzyme function was noted for *pyk*, *eno*, *pgk*, and *CcpA*, in relation to the wild-type counterpart. Contrast to reduced enzymatic activity, a pronounced transcriptional upsurge was observed for pivotal glycolytic genes, including *CcpA*. Reports have elucidated the pivotal role of the *CcpA* gene in channelling metabolic flux towards the production of DLA [311]. The pronounced transcriptional presence of the *CcpA* gene underscores its pivotal role in metabolic rerouting, while the *dldh* homologs *Ldb1010* and *Ldb0101* exhibit increased activity and transcription by 89.87% and 68.87%, respectively, likely due to enhanced energy production and pyruvate conversion to DLA. [294]. Research indicates that mutations can affect enzyme behavior, gene control, and metabolic processes in bacteria in both beneficial and ineffectual way [312] [313]. In Mut_N23, elevated *pyk* activity and transcripts suggest increased Phosphoenolpyruvate (PEP) to pyruvate conversion, potentially from advantageous mutations at the catalytic site [314]. *Pyk* also generates ATP, which is required for *d-ldh* activation by fructose-1,6-bisphosphate (F1,6BP) and has been demonstrated to have acid tolerance property in *L. bulgaricus* [315]. Thus, *pyk* plays a dual role in enhancing DLA production by providing both substrate and cofactor. Additionally, reports state that more than 90% of the pyruvate is converted to DLA in wild *L. bulgaricus* [294]. *Pfk* increase the pool of F1,6BP, which is also a key regulator of glycolysis and DLA production, further activates *d-ldh* and *pfk* genes favoring metabolic flux towards DLA biosynthesis [316]. Literature also indicates that PEP, the substrate for *pyk*, functions as an allosteric inhibitor of *pfk*. Concurrently, fructose-6-bisphosphate (F6BP) acts as an allosteric activator of *pyk*, thereby facilitating the biosynthetic pathway towards DLA production [317]. Most glycolytic genes in Mut_N23 exhibited a gradual increase in transcriptional abundance from 12^{th} to 48^{th} hours signifying the beneficial mutations, with asterisks indicating statistical significance (*Fig. 5.7b*). These findings suggest that Mut_N23 leverages these transcriptional shifts to effectively streamline carbon allocation, improving metabolic efficiency and stability under production conditions. The observed

temporal transcriptional trends further support that the mutations may have conferred adaptive advantages, enhancing both metabolic flexibility and overall strain performance in continuous DLA production systems. An increases transcriptional abundance coupled with decreased enzymatic activity can be associated with glucose conversion yield falling below 50%. This suggests a complex interplay between gene expression and enzyme function that influences the efficiency of glucose utilization in mutant strain. Further exploration of Mut_N23's enhanced DLA production could utilize omics methods (genomics and transcriptomics) to identify key genes and mutations responsible for increased DLA biosynthesis. These insights could be leveraged for future metabolic engineering efforts.

Mut_N23, the mutant strain, exhibited upregulated expression of glycolytic genes (major genes including *CcpA* and *d-ldh* homologs) which suggests that the mutagenesis process triggered metabolic rerouting, favouring DLA and other organic acid production including acetic acid. Sucrose utilization, absent in the wild-type strain, emerged in Mut_N23. This adaptation could involve upregulated activity of transporters or enzymes that allow sucrose uptake and conversion. Exact mutations can be revealed after whole genome sequencing or omics analysis, which we will conduct in our future work. In summary, the combination of mutagenesis-induced genetic alterations and metabolic adaptations led to the relatively enhanced performance of Mut_N23 for production of optically pure DLA.

5.4.8 Characterization of purified DLA from WP

DLA purification from WP-based MRS media was done using a phase partitioning method [279] [318]. This involved shifting DLA into an organic phase regulated by ammonium sulphate [$(NH_4)_2SO_4$] and n-butanol. A modified procedure was used to enhance DLA purity and efficiency [278]. After the bioreactor run, 1.2 litres of fermentation media underwent purification, yielding 78% lactic acid with 90% chemical purity. The remaining 10% impurities primarily consisted of organic acids, including acetic acid and residual butanol. HPLC analysis confirmed lactic acid presence.

FTIR analysis of purified DLA revealed peaks indicative of its molecular structure (*Fig 5.7c*). The peak at 3400 cm^{-1} corresponds to O-H stretching, while those around 2900 and 1500 cm^{-1} are attributed to sp3 C-H stretching and CH2 bending of residual n-butanol, respectively. The range of 1725-1705 cm^{-1} signifies C=O stretching of DLA's carboxylic acid group. Peaks around 1450 cm^{-1} and 1127 cm^{-1} are attributed to O-H bond deformation and C-O-C stretching, respectively, in DLA. The 3300-2500 cm^{-1} range may also indicate O-H stretching of DLA's

carboxylic acid group. The presence of carboxylic acid, O-H, and C-O-C functional groups in both commercial and sample DLAs is confirmed by these peaks, aligning with published spectral data [319]. The prominent peak at 4.70 ppm in the NMR spectra is attributed to the solvent (D_2O) (*Fig.5.6d*). The methyl (CH_3) and methine (CH) protons appeared as a doublet at approximately 1.26 ppm and a quartet around 4.24 ppm, respectively. The NMR spectra of DLA purified from WP closely resembled those of commercial lactic acid, indicating a high purity of the DLA obtained from fermentation. These findings are consistent with previous literature [320] [319] and [279] further validating our results.

The result of this chapter also underscores the significant potential of process optimization in enhancing DLA production by *Lactobacillus delbrueckii* subsp. *bulgaricus* mutant strain [321] [322]. The ability of Mut_N23 to utilize galactose from whey permeate, demonstrates its potential for valorizing dairy industry by-products, aligning with sustainable bioprocessing principles. The achievement of 99.09% optical purity and high sugar uptake potential, despite relatively low titres, is particularly noteworthy as it meets stringent requirements for high-value pharmaceutical and chemical applications. The scale-up to bioreactors, resulting in 93% increase in productivity, highlights the importance of controlled fermentation conditions and the strain's potential for industrial application apart from probiotic use. The co-production of acetic acid alongside DLA presents both a challenge and an opportunity for developing multi-product bioprocesses [323]. Future research directions should focus on elucidating the genetic basis of the observed phenotypes, optimizing fermentation conditions for complex substrates, and exploring the simultaneous production of multiple high-value compounds to enhance the overall economics of the bioprocess. Overall, this study enhanced DLA production in *L. bulgaricus* ATCC 11842 by developing mutant strain Mut_N23, which showed superior biosynthesis and carbon assimilation efficiency. Under optimized conditions, Mut_N23 produced 7.8 g L^{-1} DLA, a 300% increase over ATCC 11842 with 99.09% optical purity. Mut_N23 also exhibited superior wide spectrum sugar metabolism, suggesting its potential for valorizing various waste feedstocks. Optically pure DLA, with a purity of 99.09%, was successfully purified from whey permeate, achieving a yield of 78% and a chemical purity of 90%, thus meeting commercial standards.

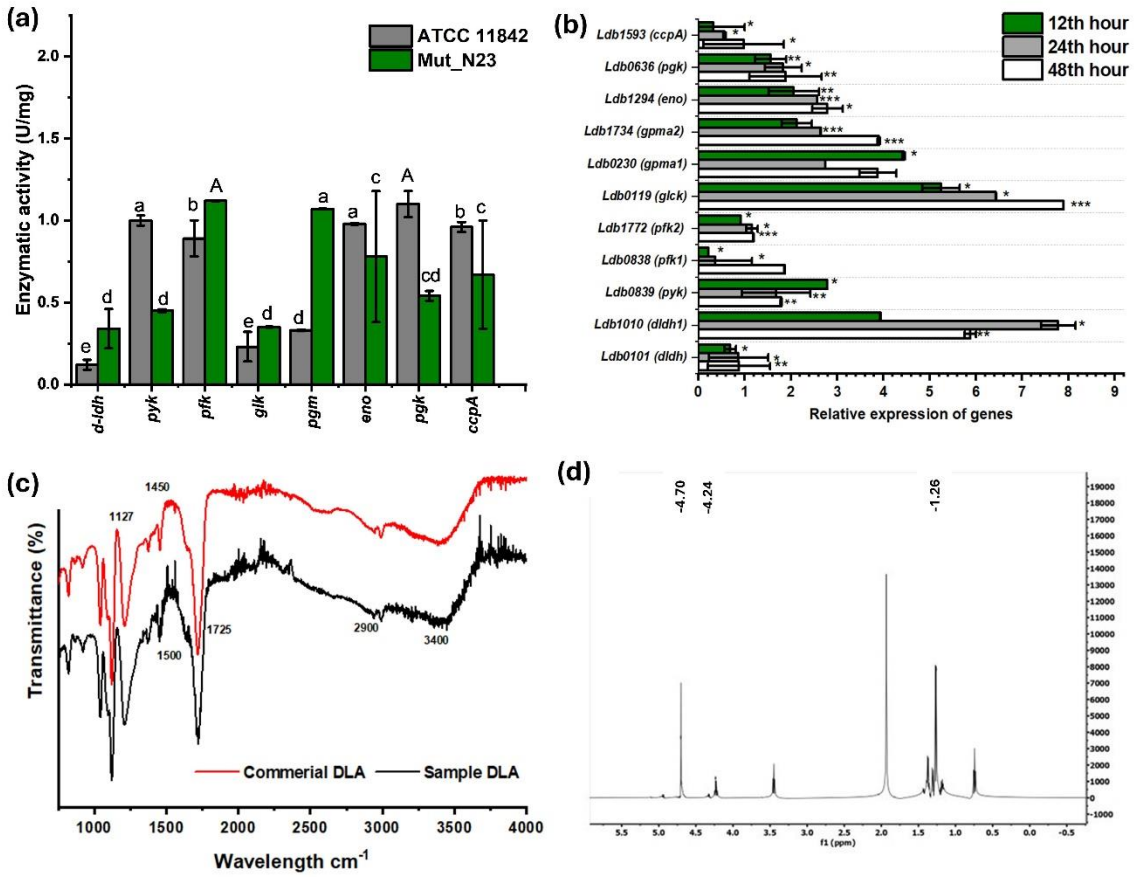


Figure 5.7. Comparative enzymatic activity and transcriptional abundance between ATCC 11842 and Mut_N23 (a) Enzymatic activity of the key glycolytic genes responsible for DLA production. The same letter on the bars denotes insignificant variations among the levels of the factors ($p > 0.05$); (b) Transcript level analysis indicating relative expression of key enzymes responsible for DLA biosynthesis. Results are mean ± standard deviation: *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate significant difference between ATCC11842 and Mut_N23, analysed by Student's t-test. Each experiment included three biological replicates. *(Ldb0101: dldh, Ldb1010: dldh1, Ldb 0838: pfk1, Ldb0839: pyk, Ldb1772: pfk2, Ldb0119: glck, Ldb0230: gpma1, Ldb1734: gpma2, Ldb1294: eno, Ldb0636: pgk, Ldb1593: ccpA)* (c) Characterization of DLA purified from whey permeate by mutant strain Mut_N23 using Fourier-transform infrared spectroscopy (FTIR) profiles, with commercially available DLA depicted in black and DLA purified in this study from whey permeate shown in red, demonstrating spectral similarity and purity, (d) Proton nuclear magnetic resonance (^{1H} NMR) analysis, confirming that the spectral peaks align with those of commercial lactic acid profiles reported previously (data not shown in this study), underscoring the high fidelity of the purification process.

5.5 Conclusion

In conclusion, the mutagenesis experiments with the mutant strain Mut_N23 have elucidated significant insights into the metabolic dynamics of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

The strain demonstrated a 97% augmentation in DLA biosynthesis, concomitant with enhanced glucose assimilation. Optimization protocols delineated ideal fermentation parameters, culminating in a peak DLA concentration of 7.88 g L^{-1} with 99.09% enantiomeric purity. Mut_N23's proficiency in metabolizing a spectrum of carbon substrates, including sucrose and whey permeate, underscores its potential for sustainable technological applications. These findings not only advance our comprehension of the strain's metabolic capabilities but also pave the way for its deployment in therapeutic and probiotic formulations. The genetic stability across successive generations further corroborates its industrial viability. Future research should focus on comprehensive genomic analyses to pinpoint specific mutations and engineer pivotal enzymes, thereby optimizing the strain for enhanced DLA production and broader biotechnological applications.

CHAPTER 6:

Conclusions and Future perspectives

6.1 Conclusion

This thesis work marks a pivotal advancement in the exploration and utilization of *Lactobacillus delbrueckii* subsp. *bulgaricus* as a biotechnological platform for the biosynthesis of industrially important biochemicals. The comprehensive approach employed, which integrates mutagenesis, metabolic engineering, and fermentation optimization, has led to significant enhancements in DLA production compared to wild strain. The results obtained from this research represent the first reported studies exploring *Lactobacillus bulgaricus* as a microbial cellular host and engineering this organism for the synthesis of value-added products. This work highlights the development of genetic engineering approaches and the establishment of compatible cloning tools, paving the way for its utilization in advanced biotechnological applications.

The mutant strain Mut_N23 emerged as a standout performer, exhibiting a remarkable 97% increase (7.88 g L^{-1}) in DLA synthesis compared to its parental strain. This enhancement is attributed to its superior glucose utilization capabilities and its ability to metabolize a diverse array of carbon substrates. Such versatility is particularly advantageous for industrial applications where the availability of various feedstocks can vary significantly. The capacity of Mut_N23 to thrive on different carbon sources positions it as a promising candidate for large-scale production processes that require flexibility and efficiency. In parallel, the engineered strain *L. bulgaricus* VI104 demonstrated an exceptional 240% improvement in DLA production through targeted pathway reconstruction. This involved meticulous adjustments to enhance ATP flux balance and the co-expression of glycolytic and DLA-specific genes. As a result, this engineered strain LdbVI104_07 achieved an impressive DLA titre of up to 9.39 g L^{-1}, with an optical purity of 99.09% in bioreactor levels. Although previously reported studies demonstrate DLA titres significantly higher than those achieved here, this thesis stands out as the first documented work on metabolic engineering of *Lactobacillus bulgaricus*, exploring its potential as a versatile microbial host for value-added product synthesis and biotechnological applications. This thesis work establishes a foundational framework for future research aimed at developing advanced engineering tools and strategies for *L. bulgaricus*, paving the way for its further optimization and industrial application apart from dairy industries.

The development of a novel DLA-responsive promoter system was pivotal in achieving enhanced dynamic metabolic regulation in *Lactobacillus bulgaricus*. This thesis work also marks the first successful establishment of an inducible promoter system in this strain,

providing a significant advancement in genetic engineering tools for its metabolic optimization. This innovative system facilitated prolonged fermentation durations and improved carbon flux efficiency, which are essential for maximizing product yields. The ability to fine-tune metabolic pathways dynamically not only enhances productivity but also underscores the versatility and industrial viability of *L. bulgaricus* as a chassis organism for sustainable chemical production and probiotic innovations. It is also concluded that interspecies promoter compatibility is achievable and influenced by various factors, with the specific sequences of the -10 and -35 regions playing a pivotal role. The findings suggest that *Lactobacillus bulgaricus* and *Pseudomonas* may share certain similarities in their promoter regulatory sequences, which could facilitate cross-species compatibility for gene expression.

Overall, the establishment of molecular cloning tools, transformation protocols, and promoter systems specifically tailored for *L. bulgaricus* addresses long-standing challenges associated with its genetic engineering. Historically, the genetic manipulation of this organism has been fraught with difficulties due to its genetic intractability. However, the advancements made in this study provide a robust framework for future research endeavours aimed at optimizing *L. bulgaricus* strains for various biotechnological applications. The implications of these findings extend beyond mere academic interest; they present real opportunities for industrial applications that can contribute to sustainable practices in chemical production. The ability to produce high-purity DLA positions *L. bulgaricus* favourably within the context of global efforts aimed at reducing reliance on fossil fuels and minimizing environmental impact through biotechnological innovations.

6.2 Future Perspectives

Looking ahead, there are several promising avenues for further research that could significantly enhance the application of *Lactobacillus delbrueckii* subsp. *bulgaricus* in biotechnology. Comprehensive genomic studies aimed at identifying key mutations and regulatory elements will be instrumental in providing deeper insights into strain performance and metabolic pathways. Understanding these genetic underpinnings will enable researchers to make informed decisions regarding future modifications and optimizations. Advanced metabolic engineering strategies hold great promise for further optimizing DLA biosynthesis in *L. bulgaricus*. Techniques such as CRISPR-based gene editing offer precise control over genetic modifications, allowing researchers to target specific genes or regulatory elements with high accuracy. This precision can lead to more effective strain improvements that enhance DLA

production while maintaining or improving other desirable traits such as growth rate and substrate utilization efficiency. In addition to CRISPR technology, adaptive laboratory evolution (ALE) presents another powerful strategy for optimizing microbial strains like *L. bulgaricus.* By subjecting populations of *L. bulgaricus* to selective pressures that favor higher DLA production or improved substrate utilization, researchers can drive evolutionary changes that result in enhanced performance over time. This approach not only complements traditional metabolic engineering techniques but also provides an avenue for discovering novel mutations that may confer advantageous traits.

Lactobacillus bulgaricus has long been utilized in various therapeutic and probiotic applications due to its unique metabolic properties and health benefits. However, the research outcomes from this thesis establish a foundational framework for advancing the potential of *L. bulgaricus* through metabolic engineering. By building on these findings, future efforts can focus on further optimizing this organism for enhanced therapeutic and probiotic functionalities. Additionally, this thesis work opens avenues to explore innovative applications, such as engineering *L. bulgaricus* for the efficient production of bioplastics, biofuels, or valuable organic acids. The adaptability of this strain to diverse substrates positions it as a promising candidate for developing sustainable biotechnological solutions in both healthcare and industrial contexts.

In future studies, further exploration of interspecies promoter compatibility can be conducted to identify additional regulatory elements and factors influencing cross-species gene expression. Detailed comparative analyses of promoter architectures across diverse bacterial species could provide insights into conserved sequences and mechanisms that enable compatibility. Additionally, engineering synthetic promoters tailored to optimize expression in *L. bulgaricus* could enhance its potential as a microbial host. Future research could also focus on expanding the repertoire of compatible genetic tools and regulatory systems, enabling more precise control of gene expression. These advancements would not only improve *L. bulgaricus* as a chassis for biotechnological applications but also facilitate its use in co-culture systems or synthetic microbial consortia for complex metabolic engineering projects.

Consumer acceptance of genetically modified products derived from mutant strains depends on regulatory compliance, transparency, and public perception. Since random mutagenesis mimics natural evolution and does not involve foreign gene insertion, it is generally more acceptable than recombinant GMOs. To address concerns, regulatory approvals must confirm

the genetic stability and safety of these strains, ensuring compliance with agencies like the FDA or EFSA. Additionally, obtaining non-GMO certifications and using naturally evolved high-yield strains through adaptive laboratory evolution can further mitigate concerns and improve acceptance in the market. In conclusion, this work not only advances the current state-of-the-art in metabolic engineering of *Lactobacillus delbrueckii* subsp. *bulgaricus* but also lays a transformative foundation for its deployment in sustainable and innovative biotechnological applications across various industries. The integration of advanced genetic tools with innovative fermentation strategies paves the way for future research that could harness this organism's full potential in addressing some of today's pressing environmental challenges while simultaneously contributing to economic growth through sustainable practices in chemical production and biotechnology.

Bibliography

1. Mukherjee P, Raj N, Sivaprakasam S (2023) Harnessing valorization potential of whey permeate for D-lactic acid production using lactic acid bacteria. Biomass Conversion and Biorefinery 2023 13:17 13:15639–15658. https://doi.org/10.1007/S13399-023-05038-3

2. Mukherjee P, Pal S, Sivaprakasam S (2024) Optimization of D-lactic acid biosynthesis from diverse carbon sources in mutant Lactobacillus delbrueckii subsp. bulgaricus via random mutagenesis. Systems Microbiology and Biomanufacturing 2024 1–20. https://doi.org/10.1007/S43393-024-00316-1

3. Mehta R, Kumar V, Bhunia H, Upadhyay SN (2005) Synthesis of poly (lactic acid): a review. Journal of Macromolecular Science, Part C: Polymer Reviews 45:325–349

4. Raef M, Sarasua JR, Etxeberria A, Ugartemendia JM (2023) Stereocomplexation: From molecular structure to functionality of advanced polylactide systems. Polymer (Guildf) 280:126066. https://doi.org/10.1016/J.POLYMER.2023.126066

5. Gali KK, Mukherjee P, Katiyar V, Sivaprakasam S (2022) Process Efficacy in Cassava-Based Biorefinery: Scalable Process Technology for the Development of Green Monomer d-Lactic Acid. 107–134. https://doi.org/10.1007/978-981-19-4316-4_5

6. Jia S, Yu D, Zhu Y, et al (2017) Morphology, crystallization and thermal behaviors of PLA-based composites: wonderful effects of hybrid GO/PEG via dynamic impregnating. Polymers (Basel) 9:528

7. Klotz S, Kaufmann N, Kuenz A, Prüße U (2016) Biotechnological production of enantiomerically pure d-lactic acid. Appl Microbiol Biotechnol 100:9423–9437

8. Lebarbe T (2013) Synthesis of novel “green” polyesters from plant oils: application to the rubber-toughening of poly (L-lactide)

9. Gali KK, Mukherjee P, Katiyar V, Sivaprakasam S (2022) Process Efficacy in Cassava-Based Biorefinery: Scalable Process Technology for the Development of Green Monomer d-Lactic Acid. 107–134. https://doi.org/10.1007/978-981-19-4316-4_5

10. Fernandes MC, Alves-Ferreira J, Duarte LC, et al (2023) D-lactic acid production from hydrothermally pretreated, alkali delignified and enzymatically saccharified rockrose with the metabolic engineered Escherichia coli strain JU15. Biomass Convers Biorefin 13:12849–12858. https://doi.org/10.1007/S13399-021-02207-0/TABLES/5

11. Arshadi M, Attard TM, Lukasik RM, et al (2016) Pre-treatment and extraction techniques for recovery of added value compounds from wastes throughout the agri-food chain. Green Chemistry 18:6160–6204

12. Yamada R, Yokota M, Matsumoto T, et al (2023) Promoting cell growth and characterizing partial symbiotic relationships in the co-cultivation of green alga

Chlamydomonas reinhardtii and Escherichia coli. Biotechnol J 18:2200099. https://doi.org/10.1002/BIOT.202200099

13. Yankov D (2022) Fermentative Lactic Acid Production From Lignocellulosic Feedstocks: From Source to Purified Product. Front Chem 10:823005. https://doi.org/10.3389/FCHEM.2022.823005

14. Kacaribu AA, Darwin (2024) Biotechnological lactic acid production from low-cost renewable sources via anaerobic microbial processes. BioTechnologia 105:179–194. https://doi.org/10.5114/BTA.2024.139757

15. Nagarajan D, Nandini A, Dong C Di, et al (2020) Lactic acid production from renewable feedstocks using poly(vinyl alcohol)-Immobilized lactobacillus plantarum 23. Ind Eng Chem Res 59:17156–17164. https://doi.org/10.1021/ACS.IECR.0C01422/ASSET/IMAGES/LARGE/IE0C01422_0004.JPEG

16. Suganya T, Varman M, Masjuki HH, Renganathan S (2016) Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach. Renewable and Sustainable Energy Reviews 55:909–941. https://doi.org/10.1016/J.RSER.2015.11.026

17. Nagarajan D, Chen CY, Ariyadasa TU, et al (2022) Macroalgal biomass as a potential resource for lactic acid fermentation. Chemosphere 309:136694. https://doi.org/10.1016/J.CHEMOSPHERE.2022.136694

18. Mazumdar S, Clomburg JM, Gonzalez R (2010) Escherichia coli strains engineered for homofermentative production of D-lactic acid from glycerol. Appl Environ Microbiol 76:4327–4336. https://doi.org/10.1128/AEM.00664-10/ASSET/A6A3DF1C-78A0-4EAF-B49B-0F604BAE6DEE/ASSETS/GRAPHIC/ZAM9991011160006.JPEG

19. Qiao J, Fang Y, Li Z, et al (2024) Experimental evolution reveals an effective avenue for d-lactic acid production from glucose-xylose mixtures via enhanced Glk activity and a cAMP-independent CRP mutation. Biotechnol Bioeng 121:3514–3526. https://doi.org/10.1002/BIT.28819

20. Alexandri M, Schneider R, Mehlmann K, Venus J (2019) Recent advances in d-lactic acid production from renewable resources: case studies on agro-industrial waste streams. Food Technol Biotechnol 57:293–304

21. Yin FW, Sun XL, Zheng WL, et al (2023) Development of a Strategy for L-Lactic Acid Production by Rhizopus oryzae Using Zizania latifolia Waste and Cane Molasses as Carbon Sources. Molecules 2023, Vol 28, Page 6234 28:6234. https://doi.org/10.3390/MOLECULES28176234

22. Cho SW, Yim J, Seo SW (2020) Engineering tools for the development of recombinant lactic acid bacteria. Biotechnol J 15:1900344

23. Heiss S, Hörmann A, Tauer C, et al (2016) Evaluation of novel inducible promoter/repressor systems for recombinant protein expression in Lactobacillus plantarum. Microb Cell Fact 15:1–17

24. Van Pijkeren J-P, Britton RA (2012) High efficiency recombineering in lactic acid bacteria. Nucleic Acids Res 40:e76–e76

25. Awasthi D, Wang L, Rhee MS, et al (2018) Metabolic engineering of Bacillus subtilis for production of D-lactic acid. Biotechnol Bioeng 115:453–463. https://doi.org/10.1002/BIT.26472

26. Ishida N, Suzuki T, Tokuhiro K, et al (2006) d-Lactic acid production by metabolically engineered Saccharomyces cerevisiae. J Biosci Bioeng 101:172–177. https://doi.org/10.1263/JBB.101.172

27. Grabar TB, Zhou S, Shanmugam KT, et al (2006) Methylglyoxal bypass identified as source of chiral contamination in L(+) and D(-)-lactate fermentations by recombinant Escherichia coli. Biotechnol Lett 28:1527–1535. https://doi.org/10.1007/S10529-006-9122-7/METRICS

28. Wang Q, Ingram LO, Shanmugam KT (2011) Evolution of D-lactate dehydrogenase activity from glycerol dehydrogenase and its utility for D-lactate production from lignocellulose. Proc Natl Acad Sci U S A 108:18920–18925. https://doi.org/10.1073/PNAS.1111085108/SUPPL_FILE/PNAS.1111085108_SI.PDF

29. Baek SH, Kwon EY, Kim YH, Hahn JS (2016) Metabolic engineering and adaptive evolution for efficient production of D-lactic acid in Saccharomyces cerevisiae. Appl Microbiol Biotechnol 100:2737–2748. https://doi.org/10.1007/S00253-015-7174-0/TABLES/2

30. Sangproo M, Polyiam P, Jantama SS, et al (2012) Metabolic engineering of Klebsiella oxytoca M5a1 to produce optically pure d-lactate in mineral salts medium. Bioresour Technol 119:191–198. https://doi.org/10.1016/J.BIORTECH.2012.05.114

31. In S, Khunnonkwao P, Wong N, et al (2020) Combining metabolic engineering and evolutionary adaptation in Klebsiella oxytoca KMS004 to significantly improve optically pure D-(−)-lactic acid yield and specific productivity in low nutrient medium. Appl Microbiol Biotechnol 104:9565–9579. https://doi.org/10.1007/S00253-020-10933-0/TABLES/3

32. Zhang Y, Vadlani P V., Kumar A, et al (2016) Enhanced D-lactic acid production from renewable resources using engineered Lactobacillus plantarum. Appl Microbiol Biotechnol 100:279–288. https://doi.org/10.1007/S00253-015-7016-0/FIGURES/3

33. Tsuge Y, Kato N, Yamamoto S, et al (2019) Metabolic engineering of Corynebacterium glutamicum for hyperproduction of polymer-grade l- and d-lactic acid. Appl Microbiol Biotechnol 103:3381–3391. https://doi.org/10.1007/S00253-019-09737-8/TABLES/3

34. Yamada R, Wakita K, Mitsui R, Ogino H (2017) Enhanced d-lactic acid production by recombinant Saccharomyces cerevisiae following optimization of the global metabolic pathway. Biotechnol Bioeng 114:2075–2084. https://doi.org/10.1002/BIT.26330

35. Zhong W, Yang M, Hao X, et al (2021) Improvement of D-lactic acid production at low pH through expressing acid-resistant gene IoGAS1 in engineered Saccharomyces cerevisiae. Journal of Chemical Technology & Biotechnology 96:732–742. https://doi.org/10.1002/JCTB.6587

36. Varman AM, Yu Y, You L, Tang YJ (2013) Photoautotrophic production of D-lactic acid in an engineered cyanobacterium. Microb Cell Fact 12:1–8. https://doi.org/10.1186/1475-2859-12-117/TABLES/2

37. Watcharawipas A, Sae-Tang K, Sansatchanon K, et al (2021) Systematic engineering of Saccharomyces cerevisiae for D-lactic acid production with near theoretical yield. FEMS Yeast Res 21:. https://doi.org/10.1093/FEMSYR/FOAB024

38. Ahmad A, Banat F, Taher H (2020) A review on the lactic acid fermentation from low-cost renewable materials: Recent developments and challenges. Environ Technol Innov 101138

39. Mimitsuka T, Sawai K, Kobayashi K, et al (2015) Production of d-lactic acid in a continuous membrane integrated fermentation reactor by genetically modified Saccharomyces cerevisiae: Enhancement in d-lactic acid carbon yield. J Biosci Bioeng 119:65–71

40. Beitel SM, Coelho LF, Contiero J (2020) Efficient conversion of agroindustrial waste into D (-) lactic acid by Lactobacillus delbrueckii using fed-batch fermentation. Biomed Res Int 2020:

41. Bai Z, Gao Z, Sun J, et al (2016) D-Lactic acid production by Sporolactobacillus inulinus YBS1-5 with simultaneous utilization of cottonseed meal and corncob residue. Bioresour Technol 207:346–352

42. Ahring BK, Traverso JJ, Murali N, Srinivas K (2016) Continuous fermentation of clarified corn stover hydrolysate for the production of lactic acid at high yield and productivity. Biochem Eng J 109:162–169

43. Abdel-Rahman MA, Tashiro Y, Zendo T, et al (2016) Highly efficient L-lactic acid production from xylose in cell recycle continuous fermentation using Enterococcus mundtii QU 25. RSC Adv 6:17659–17668

44. Lee HD, Lee MY, Hwang YS, et al (2017) Separation and purification of lactic acid from fermentation broth using membrane-integrated separation processes. Ind Eng Chem Res 56:8301–8310

45. Komesu A, de Oliveira JAR, da Silva Martins LH, et al (2017) Lactic acid production to purification: a review. Bioresources 12:4364–4383

46. Su C-Y, Yu C-C, Chien I-L, Ward JD (2015) Control of highly interconnected reactive distillation processes: purification of raw lactic acid by esterification and hydrolysis. Ind Eng Chem Res 54:6932–6940

47. Komesu A, Wolf Maciel MR, Rocha de Oliveira JA, et al (2017) Purification of lactic acid produced by fermentation: focus on non-traditional distillation processes. Separation & Purification Reviews 46:241–254

48. Wang L, Cai Y, Zhu L, et al (2014) Major role of NAD-dependent lactate dehydrogenases in the production of l-lactic acid with high optical purity by the thermophile Bacillus coagulans. Appl Environ Microbiol 80:7134–7141

49. Mazzoli R, Bosco F, Mizrahi I, et al (2014) Towards lactic acid bacteria-based biorefineries. Biotechnol Adv 32:1216–1236

50. van de Guchte M, Penaud S, Grimaldi C, et al (2006) The complete genome sequence of Lactobacillus bulgaricus reveals extensive and ongoing reductive evolution. Proceedings of the National Academy of Sciences 103:9274–9279

51. Yadav M, Shukla P (2020) Efficient engineered probiotics using synthetic biology approaches: a review. Biotechnol Appl Biochem 67:22–29

52. Raja BR, Arunachalam KD (2011) Market potential for probiotic nutritional supplements in India. African Journal of Business Management 5:5418–5423. https://doi.org/10.5897/AJBM11.494

53. Haris NIN, Salleh SF, Nolasco-Hipolito C, et al (2025) A review on lactic acid production via microbial fermentation from biowaste: insights on scaling up to industrial process. Biofuels, Bioproducts and Biorefining 19:437–452. https://doi.org/10.1002/BBB.2704

54. Manome A, Okada S, Uchimura T, Komagata K (1998) The ratio of L-form to D-form of lactic acid as a criteria for the identification of lactic acid bacteria. J Gen Appl Microbiol 44:371–374

55. Tanaka T, Hoshina M, Tanabe S, et al (2006) Production of D-lactic acid from defatted rice bran by simultaneous saccharification and fermentation. Bioresour Technol 97:211–217

56. Calabia BP, Tokiwa Y (2007) Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugar beet juice by Lactobacillus delbrueckii. Biotechnol Lett 29:1329–1332

57. Yáñez R, Moldes AB, Alonso JL, Parajó JC (2003) Production of D (−)-lactic acid from cellulose by simultaneous saccharification and fermentation using Lactobacillus coryniformis subsp. torquens. Biotechnol Lett 25:1161–1164

58. Benthin S, Villadsen J (1995) Production of optically pure D-lactate by Lactobacillus bulgaricus and purification by crystallisation and liquid/liquid extraction. Appl Microbiol Biotechnol 42:826–829

59. Okano K, Zhang Q, Shinkawa S, et al (2009) Efficient production of optically pure D-lactic acid from raw corn starch by using a genetically modified L-lactate dehydrogenase gene-deficient and α-amylase-secreting Lactobacillus plantarum strain. Appl Environ Microbiol 75:462–467

60. Okano K, Zhang Q, Yoshida S, et al (2010) D-lactic acid production from cellooligosaccharides and β-glucan using L-LDH gene-deficient and endoglucanase-secreting Lactobacillus plantarum. Appl Microbiol Biotechnol 85:643–650

61. Kleerebezem M, Hugenholtz J (2003) Metabolic pathway engineering in lactic acid bacteria. Curr Opin Biotechnol 14:232–237

62. Son J, Jeong KJ (2020) Recent advances in synthetic biology for the engineering of lactic acid bacteria. Biotechnology and Bioprocess Engineering 1–12

63. de Vos WM (1999) Gene expression systems for lactic acid bacteria. Curr Opin Microbiol 2:289–295

64. Rud I, Jensen PR, Naterstad K, Axelsson L (2006) A synthetic promoter library for constitutive gene expression in Lactobacillus plantarum. Microbiology (N Y) 152:1011–1019

65. Thompson J (1987) Regulation of sugar transport and metabolism in lactic acid bacteria. FEMS Microbiol Rev 3:221–231

66. Kandler O (1983) Carbohydrate metabolism in lactic acid bacteria. Antonie Van Leeuwenhoek 49:209–224

67. Bhowmik T, Steele JL (1994) Cloning, characterization and insertional inactivation of the Lactobacillus helveticus D (−) lactate dehydrogenase gene. Appl Microbiol Biotechnol 41:432–439

68. Okano K, Tanaka T, Ogino C, et al (2010) Biotechnological production of enantiomeric pure lactic acid from renewable resources: recent achievements, perspectives, and limits. Appl Microbiol Biotechnol 85:413–423

69. Saitoh S, Ishida N, Onishi T, et al (2005) Genetically engineered wine yeast produces a high concentration of L-lactic acid of extremely high optical purity. Appl Environ Microbiol 71:2789–2792

70. Okano K, Uematsu G, Hama S, et al (2018) Metabolic engineering of Lactobacillus plantarum for direct l-lactic acid production from raw corn starch. Biotechnol J 13:1700517

71. Andersen HW, Solem C, Hammer K, Jensen PR (2001) Twofold reduction of phosphofructokinase activity in Lactococcus lactis results in strong decreases in growth rate and in glycolytic flux. J Bacteriol 183:3458–3467

72. de Felipe FL, Hugenholtz J (2001) Purification and characterisation of the water forming NADH-oxidase from Lactococcus lactis. Int Dairy J 11:37–44

73. Zhai Z, An H, Wang G, et al (2015) Functional role of pyruvate kinase from Lactobacillus bulgaricus in acid tolerance and identification of its transcription factor by bacterial one-hybrid. Sci Rep 5:17024. https://doi.org/10.1038/srep17024

74. Wee Y, Kim J, Ryu H (2006) Biotechnological production of lactic acid and its recent applications. Food Technol Biotechnol 44:163–172

75. Abdel-Rahman MA, Tashiro Y, Sonomoto K (2013) Recent advances in lactic acid production by microbial fermentation processes. Biotechnol Adv 31:877–902

76. Xie M, Li Q, Hu XP, et al (2017) Effects of a NTG-based chemical mutagenesis on the propamocarb-tolerance of the nematophagous fungus Lecanicillium attenuatum. Pestic Biochem Physiol 141:71–75. https://doi.org/10.1016/J.PESTBP.2016.11.011

77. Chen CC, Lan CC, Pan CL, et al (2019) Repeated-batch lactic acid fermentation using a novel bacterial immobilization technique based on a microtube array membrane. Process Biochemistry 87:25–32. https://doi.org/10.1016/J.PROCBIO.2019.09.016

78. Zhong W, Yang M, Mu T, et al (2019) Systematically redesigning and optimizing the expression of D-lactate dehydrogenase efficiently produces high-optical-purity D-lactic acid in Saccharomyces cerevisiae. Biochem Eng J 144:217–226. https://doi.org/10.1016/J.BEJ.2018.09.013

79. Sahoo TK, Jayaraman G (2019) Co-culture of Lactobacillus delbrueckii and engineered Lactococcus lactis enhances stoichiometric yield of d-lactic acid from whey permeate. Appl Microbiol Biotechnol 103:5653–5662

80. Vishnu Prasad J, Sahoo TK, Naveen S, Jayaraman G (2020) Evolutionary engineering of Lactobacillus bulgaricus reduces enzyme usage and enhances conversion of lignocellulosics to D-lactic acid by simultaneous saccharification and fermentation. Biotechnol Biofuels 13:1–11. https://doi.org/10.1186/S13068-020-01812-X/FIGURES/5

81. Yamamoto S, Gunji W, Suzuki H, et al (2012) Overexpression of Genes Encoding Glycolytic Enzymes in Corynebacterium glutamicum Enhances Glucose Metabolism and Alanine Production under Oxygen Deprivation Conditions. Appl Environ Microbiol 78:4447. https://doi.org/10.1128/AEM.07998-11

82. Kyla-Nikkila K, Hujanen M, Leisola M, Palva A (2000) Metabolic engineering of Lactobacillus helveticus CNRZ32 for production of pure L-(+)-lactic acid. Appl Environ Microbiol 66:3835–3841. https://doi.org/10.1128/AEM.66.9.3835-

3841.2000/ASSET/5A982259-B2B3-47DF-8A89-CF97FBB81910/ASSETS/GRAPHIC/AM0900449006.JPEG

83. Yi X, Zhang P, Sun J, et al (2016) Engineering wild-type robust Pediococcus acidilactici strain for high titer l- and d-lactic acid production from corn stover feedstock. J Biotechnol 217:112–121. https://doi.org/10.1016/J.JBIOTEC.2015.11.014

84. Okano K, Zhang Q, Yoshida S, et al (2010) D-lactic acid production from cellooligosaccharides and β-glucan using l-LDH gene-deficient and endoglucanase-secreting Lactobacillus plantarum. Appl Microbiol Biotechnol 85:643–650. https://doi.org/10.1007/S00253-009-2111-8/FIGURES/3

85. Aarnikunnas J, Von Weymarn N, Rönnholm K, et al (2003) Metabolic engineering of Lactobacillus fermentum for production of mannitol and pure L-lactic acid or pyruvate. Biotechnol Bioeng 82:653–663. https://doi.org/10.1002/BIT.10615

86. Okano K, Zhang Q, Shinkawa S, et al (2009) Efficient production of optically pure D-lactic acid from raw corn starch by using a genetically modified L-lactate dehydrogenase gene-deficient and α-amylase-secreting Lactobacillus plantarum strain. Appl Environ Microbiol 75:462–467. https://doi.org/10.1128/AEM.01514-08/ASSET/EE431AE8-52C5-49A5-94B4-8198D42FEBD2/ASSETS/GRAPHIC/ZAM0020995540004.JPEG

87. Tian X, Liu X, Zhang Y, et al (2021) Metabolic engineering coupled with adaptive evolution strategies for the efficient production of high-quality L-lactic acid by Lactobacillus paracasei. Bioresour Technol 323:124549. https://doi.org/10.1016/J.BIORTECH.2020.124549

88. Aso Y, Hashimoto A, Ohara H (2019) Engineering Lactococcus lactis for D-Lactic Acid Production from Starch. Curr Microbiol 76:1186–1192. https://doi.org/10.1007/S00284-019-01742-4/FIGURES/4

89. Sansatchanon K, Sudying P, Promdonkoy P, et al (2023) Development of a Novel D-Lactic Acid Production Platform Based on Lactobacillus saerimneri TBRC 5746. Journal of Microbiology 61:853–863. https://doi.org/10.1007/S12275-023-00077-X/FIGURES/7

90. Zhang Y, Vadlani P V., Kumar A, et al (2016) Enhanced D-lactic acid production from renewable resources using engineered Lactobacillus plantarum. Appl Microbiol Biotechnol 100:279–288. https://doi.org/10.1007/S00253-015-7016-0/FIGURES/3

91. Okano K, Uematsu G, Hama S, et al (2018) Metabolic Engineering of Lactobacillus plantarum for Direct l-Lactic Acid Production From Raw Corn Starch. Biotechnol J 13:1700517. https://doi.org/10.1002/BIOT.201700517

92. Tian X, Liu X, Zhang Y, et al (2021) Metabolic engineering coupled with adaptive evolution strategies for the efficient production of high-quality L-lactic acid by Lactobacillus paracasei. Bioresour Technol 323:124549. https://doi.org/10.1016/J.BIORTECH.2020.124549

93. Zhang Y, Vadlani P V., Kumar A, et al (2016) Enhanced D-lactic acid production from renewable resources using engineered Lactobacillus plantarum. Appl Microbiol Biotechnol 100:279–288. https://doi.org/10.1007/S00253-015-7016-0/FIGURES/3

94. Sansatchanon K, Sudying P, Promdonkoy P, et al (2023) Development of a Novel D-Lactic Acid Production Platform Based on Lactobacillus saerimneri TBRC 5746. Journal of Microbiology 61:853–863. https://doi.org/10.1007/S12275-023-00077-X/FIGURES/7

95. Duarte SOD, Monteiro GA (2021) Plasmid Replicons for the Production of Pharmaceutical-Grade pDNA, Proteins and Antigens by Lactococcus lactis Cell Factories. International Journal of Molecular Sciences 2021, Vol 22, Page 1379 22:1379. https://doi.org/10.3390/IJMS22031379

96. Llull D, Poquet I (2004) New expression system tightly controlled by zinc availability in Lactococcus lactis. Appl Environ Microbiol 70:5398–5406. https://doi.org/10.1128/AEM.70.9.5398-5406.2004/ASSET/AAD877C6-D02A-4461-9AF5-45A368171C52/ASSETS/GRAPHIC/ZAM0090447410005.JPEG

97. Fristot E, Cambray G, Bonnet J (2024) LactoSpanks: A Collection of IPTG Inducible Promoters for the Commensal Lactic Acid Bacteria Lactobacillus gasseri. ACS Synth Biol 13:951–957. https://doi.org/10.1021/ACSSYNBIO.3C00438

98. Peirotén Á, Landete JM (2020) Natural and engineered promoters for gene expression in Lactobacillus species. Appl Microbiol Biotechnol 104:3797–3805. https://doi.org/10.1007/S00253-020-10426-0/FIGURES/1

99. Rud I, Jensen PR, Naterstad K, Axelsson L (2006) A synthetic promoter library for constitutive gene expression in Lactobacillus plantarum. Microbiology (N Y) 152:1011–1019. https://doi.org/10.1099/MIC.0.28599-0/CITE/REFWORKS

100. Wu J, Xin Y, Kong J, Guo T (2021) Genetic tools for the development of recombinant lactic acid bacteria. Microb Cell Fact 20:1–10. https://doi.org/10.1186/S12934-021-01607-1/TABLES/2

101. Rottinghaus AG, Ferreiro A, Fishbein SRS, et al (2022) Genetically stable CRISPR-based kill switches for engineered microbes. Nature Communications 2022 13:1 13:1–17. https://doi.org/10.1038/s41467-022-28163-5

102. Teusink B, Wiersma A, Molenaar D, et al (2006) Analysis of growth of Lactobacillus plantarum WCFS1 on a complex medium using a genome-scale metabolic model. Journal of Biological Chemistry 281:40041–40048. https://doi.org/10.1074/JBC.M606263200/ATTACHMENT/84011731-63E9-4F0C-82EA-5A11A6E7A1DC/MMC1.ZIP

103. Kim B, Kim WJ, Kim DI, Lee SY (2015) Applications of genome-scale metabolic network model in metabolic engineering. J Ind Microbiol Biotechnol 42:339–348. https://doi.org/10.1007/S10295-014-1554-9

104. Kuhl GC, Mazzon RR, Simas Porto BL, et al (2021) Oleate Hydratase in Lactobacillus delbrueckii subsp. bulgaricus LBP UFSC 2230 Catalyzes the Reversible Conversion between Linoleic Acid and Ricinoleic Acid . Microbiol Spectr 9:. https://doi.org/10.1128/SPECTRUM.01179-21/SUPPL_FILE/REVIEWER-COMMENTS.PDF

105. Song K, Ling H, Wang L, et al (2024) Lactobacillus delbrueckii subsp. bulgaricus Alleviates Acute Injury in Hypoxic Mice. Nutrients 16:1465. https://doi.org/10.3390/NU16101465/S1

106. Wu T, Guo S, Kwok LY, et al (2025) Temperature-dependent metabolic interactions between Streptococcus thermophilus and Lactobacillus delbrueckii ssp. bulgaricus in milk fermentation: Insights from gas chromatography–ion mobility spectrometry metabolomics. J Dairy Sci 108:242–256. https://doi.org/10.3168/JDS.2024-25153

107. Bao S, Zhu L, Zhuang Q, et al (2013) Distribution Dynamics of Recombinant Lactobacillus in the Gastrointestinal Tract of Neonatal Rats. PLoS One 8:e60007. https://doi.org/10.1371/JOURNAL.PONE.0060007

108. Yamamoto E, Tooyama E, Honme Y (2024) Role of fumarate reductase on the fermentation properties of Lactobacillus delbrueckii ssp. bulgaricus. J Dairy Sci 107:3443–3450. https://doi.org/10.3168/JDS.2023-24091

109. Usui Y, Kimura Y, Satoh T, et al (2018) Effects of long-term intake of a yogurt fermented with Lactobacillus delbrueckii subsp. bulgaricus 2038 and Streptococcus thermophilus 1131 on mice. Int Immunol 30:319–331. https://doi.org/10.1093/INTIMM/DXY035

110. Mende S, Krzyzanowski L, Weber J, et al (2012) Growth and exopolysaccharide yield of Lactobacillus delbrueckii ssp. bulgaricus DSM 20081 in batch and continuous bioreactor experiments at constant pH. J Biosci Bioeng 113:185–191. https://doi.org/10.1016/J.JBIOSC.2011.10.012

111. Lee M, Bang WY, Lee H Bin, et al (2024) Safety Assessment and Evaluation of Probiotic Potential of Lactobacillus bulgaricus IDCC 3601 for Human Use. Microorganisms 12:2063. https://doi.org/10.3390/MICROORGANISMS12102063/S1

112. Qiao F, Wang S, He J, et al (2024) Investigating the role of membrane lipid composition differences on spray drying survival in Lactobacillus bulgaricus using non-targeted Lipidomics. Food Chem 459:140336. https://doi.org/10.1016/J.FOODCHEM.2024.140336

113. Gong P, Lin K, Zhang J, et al (2020) Enhancing spray drying tolerance of Lactobacillus bulgaricus by intracellular trehalose delivery via electroporation. Food Research International 127:108725. https://doi.org/10.1016/J.FOODRES.2019.108725

114. Pang XY, Cui WM, Liu L, et al (2014) Gene Knockout and Overexpression Analysis Revealed the Role of N-Acetylmuramidase in Autolysis of Lactobacillus delbrueckii

subsp. bulgaricus Ljj-6. PLoS One 9:e104829. https://doi.org/10.1371/JOURNAL.PONE.0104829

115. Kudo H, Nakano M, Kikuchi A, et al (2024) Lactobacillus delbrueckii subsp. bulgaricus OLL1073R-1 eps gene knockout mutants reduced exopolysaccharide synthesis and immunomodulatory activities. Int Dairy J 150:105827. https://doi.org/10.1016/J.IDAIRYJ.2023.105827

116. Zhang G, Liu L, Li C (2020) Effects of ccpA gene deficiency in Lactobacillus delbrueckii subsp. bulgaricus under aerobic conditions as assessed by proteomic analysis. Microb Cell Fact 19:1–12. https://doi.org/10.1186/S12934-020-1278-7/FIGURES/5

117. Serror P, Sasaki T, Ehrlich SD, Maguin E (2002) Electrotransformation of Lactobacillus delbrueckii subsp. bulgaricus and L. delbrueckii subsp. lactis with Various Plasmids. Appl Environ Microbiol 68:46. https://doi.org/10.1128/AEM.68.1.46-52.2002

118. Wang C, Cui Y, Qu X (2020) Optimization of electrotransformation (ETF) conditions in lactic acid bacteria (LAB). J Microbiol Methods 174:105944. https://doi.org/10.1016/J.MIMET.2020.105944

119. Brockman IM, Prather KLJ (2015) Dynamic metabolic engineering: New strategies for developing responsive cell factories. Biotechnol J 10:1360–1369. https://doi.org/10.1002/BIOT.201400422

120. Burg JM, Cooper CB, Ye Z, et al (2016) Large-scale bioprocess competitiveness: the potential of dynamic metabolic control in two-stage fermentations. Curr Opin Chem Eng 14:121–136. https://doi.org/10.1016/J.COCHE.2016.09.008

121. Hartline CJ, Schmitz AC, Han Y, Zhang F (2021) Dynamic control in metabolic engineering: Theories, tools, and applications. Metab Eng 63:126–140. https://doi.org/10.1016/J.YMBEN.2020.08.015

122. Stirling F, Bitzan L, Wk Oliver J, et al Rational Design of Evolutionarily Stable Microbial Kill Switches. https://doi.org/10.1101/129445

123. Garde R, Dea A, Herwig MF, et al (2024) Feedback control of the heat shock response by spatiotemporal regulation of Hsp70. Journal of Cell Biology 223:. https://doi.org/10.1083/JCB.202401082

124. Yin X, Shin HD, Li J, et al (2017) Pgas, a low-pH-induced promoter, as a tool for dynamic control of gene expression for metabolic engineering of Aspergillus niger. Appl Environ Microbiol 83:. https://doi.org/10.1128/AEM.03222-16/SUPPL_FILE/ZAM999117722S1.PDF

125. Salinas F, Rojas V, Delgado V, et al (2017) Optogenetic switches for light-controlled gene expression in yeast. Appl Microbiol Biotechnol 101:2629–2640. https://doi.org/10.1007/S00253-017-8178-8/METRICS

126. Kessenikh AG, Novoyatlova US, Bazhenov S V., et al (2021) Constructing of bacillus subtilis-based lux-biosensors with the use of stress-inducible promoters. Int J Mol Sci 22:9571. https://doi.org/10.3390/IJMS22179571/S1

127. Gawin A, Valla S, Brautaset T (2017) The XylS/Pm regulator/promoter system and its use in fundamental studies of bacterial gene expression, recombinant protein production and metabolic engineering. Microb Biotechnol 10:702–718. https://doi.org/10.1111/1751-7915.12701

128. Hwang HJ, Kim JW, Ju SY, et al (2017) Application of an oxygen-inducible nar promoter system in metabolic engineering for production of biochemicals in Escherichia coli. Biotechnol Bioeng 114:468–473. https://doi.org/10.1002/BIT.26082

129. Van Dien S (2013) From the first drop to the first truckload: commercialization of microbial processes for renewable chemicals. Curr Opin Biotechnol 24:1061–1068

130. Yu J, Guo H, Sun M, et al (2023) Milk fermented by combined starter cultures comprising three Lactobacillus strains exerts an alleviating effect on loperamide-induced constipation in BALB/c mice. Food Funct 14:5264–5276. https://doi.org/10.1039/D3FO00222E

131. Elean M, Albarracin L, Villena J, et al (2023) In Silico Comparative Genomic Analysis Revealed a Highly Conserved Proteolytic System in Lactobacillus delbrueckii. Int J Mol Sci 24:11309. https://doi.org/10.3390/IJMS241411309/S1

132. Uzunsoy I, Budak SO, Sanli T, et al (2023) OBSERVATION OF THE SUITABILITY OF SINGLE STRAINS OF STREPTOCOCCUS THERMOPHILUS AND LACTOBACILLUS DELBRUECKII SUBSP. BULGARICUS ISOLATED FROM LOCAL DAIRY SOURCES IN TURKEY AS YOGURT STARTER COMBINATIONS. Journal of Microbiology, Biotechnology and Food Sciences 13:. https://doi.org/10.55251/JMBFS.9241

133. Petrut S, Rusu E, Tudorache IS, et al (2019) Influence of Various Carbon Sources on Growth and Biomass Accumulation of Some Lactic Acid Bacteria Strains. Revista de Chimie 70:2434–2438. https://doi.org/10.37358/RC.19.7.7356

134. Sasaki C, Okumura R, Asakawa A, et al (2012) Production of D-lactic acid from sugarcane bagasse using steam-explosion. J Phys Conf Ser 352:012054. https://doi.org/10.1088/1742-6596/352/1/012054

135. Serror P, Ilami G, Chouayekh H, et al (2003) Transposition in Lactobacillus delbrueckii subsp. bulgaricus: Identification of two thermosensitive replicons and two functional insertion sequences. Microbiology (N Y) 149:1503–1511. https://doi.org/10.1099/MIC.0.25827-0/CITE/REFWORKS

136. Chouayekh H, Serror P, Boudebbouze S, Maguin E (2009) Highly efficient production of the staphylococcal nuclease reporter in Lactobacillus bulgaricus governed by the

promoter of the hlbA gene. FEMS Microbiol Lett 293:232–239. https://doi.org/10.1111/J.1574-6968.2009.01522.X

137. Licandro-Seraut H, Brinster S, van de Guchte M, et al (2012) Development of an efficient In vivo system (Pjunc-TpaseIS1223) for random transposon mutagenesis of Lactobacillus casei. Appl Environ Microbiol 78:5417–5423. https://doi.org/10.1128/AEM.00531-12/ASSET/556A9D42-FE22-4BE8-A6CB-7CDAF1121BFB/ASSETS/GRAPHIC/ZAM9991034390002.JPEG

138. Serror P, Dervyn R, Ehrlich SD, Maguin E (2003) csp-like genes of Lactobacillus delbrueckii ssp. bulgaricus and their response to cold shock. FEMS Microbiol Lett 226:323–330. https://doi.org/10.1016/S0378-1097(03)00594-9

139. Laxmi NP, Mutamed MA, Nagendra P (2011) Effect of nitrogen sources on production of β-galactosidase from Bifidobacterium animalis Bb12 and Lactobacillus delbrueckii ssp. bulgaricus ATCC 11842 grown in whey under different culture conditions. Int Food Res J

140. Nwamaioha NO, Ibrahim SA (2018) A selective medium for the enumeration and differentiation of Lactobacillus delbrueckii ssp. bulgaricus. J Dairy Sci 101:4953–4961. https://doi.org/10.3168/JDS.2017-14155

141. Evivie SE, Abdelazez A, Li B, et al (2019) In vitro organic acid production and in vivo food pathogen suppression by Probiotic S. thermophilus and L. Bulgaricus. Front Microbiol 10:782. https://doi.org/10.3389/FMICB.2019.00782/FULL

142. Urshev Z, Ishlimova D (2015) Distribution of clustered regularly interspaced palindrome repeats CRISPR2 and CRISPR3 in Lactobacillus delbrueckii ssp. bulgaricus strains. Biotechnology & Biotechnological Equipment 29:541–546. https://doi.org/10.1080/13102818.2015.1013351

143. Horiuchi J, Naito S, Kumada Y, et al (2021) Effective D-lactic acid production from corncobs by simultaneous saccharification and fermentation using metabolically engineered Lactobacillus plantarum. https://doi.org/10.22541/AU.161774066.61150349/V1

144. Gali KK, Reddy Tadi SR, E.V.R. A, et al (2021) Cost-effective valorization of cassava fibrous waste into enantiomerically pure D-lactic acid: Process engineering and kinetic modelling approach. Environ Technol Innov 22:101519. https://doi.org/10.1016/J.ETI.2021.101519

145. Sasaki C, Okumura R, Asakawa A, et al (2012) Production of D-lactic acid from sugarcane bagasse using steam-explosion. J Phys Conf Ser 352:012054. https://doi.org/10.1088/1742-6596/352/1/012054

146. Bai M, Yang S, Zhao Q, et al (2024) Fermentation characteristics of Lactobacillus delbrueckii subsp. bulgaricus T50 and Streptococcus thermophilus S10 complex starter:

Enhancing fermentation performance, metabolic interaction, and storage stability. LWT 208:116716. https://doi.org/10.1016/J.LWT.2024.116716

147. Lee M, Bang WY, Lee H Bin, et al (2024) Safety Assessment and Evaluation of Probiotic Potential of Lactobacillus bulgaricus IDCC 3601 for Human Use. Microorganisms 12:2063. https://doi.org/10.3390/MICROORGANISMS12102063/S1

148. Castro A, Aleman RS, Tabora M, et al (2023) Probiotic Characteristics of Streptococcus thermophilus and Lactobacillus bulgaricus as Influenced by New Food Sources. Microorganisms 2023, Vol 11, Page 2291 11:2291. https://doi.org/10.3390/MICROORGANISMS11092291

149. Zu H, Wu Y, Liao Z, et al (2024) Research progress on the biosynthesis of d-lactic acid from low-value biomass materials. Biomass Bioenergy 182:107067. https://doi.org/10.1016/J.BIOMBIOE.2024.107067

150. Prasad S, Srikanth K, Limaye AM, Sivaprakasam S (2014) Homo-fermentative production of d-lactic acid by Lactobacillus sp. employing casein whey permeate as a raw feed-stock. Biotechnol Lett 36:1303–1307

151. Reddy Tadi SR, Arun EVR, Limaye AM, Sivaprakasam S (2017) Enhanced production of optically pure d (–) lactic acid from nutritionally rich Borassus flabellifer sugar and whey protein hydrolysate based–fermentation medium. Biotechnol Appl Biochem 64:279–289. https://doi.org/10.1002/BAB.1470

152. Liu P, Zheng Z, Xu Q, et al (2018) Valorization of dairy waste for enhanced D-lactic acid production at low cost. Process Biochemistry 71:18–22

153. Bustamante D, Tortajada M, Ramón D, Rojas A (2019) Production of D-Lactic Acid by the Fermentation of Orange Peel Waste Hydrolysate by Lactic Acid Bacteria. Fermentation 2020, Vol 6, Page 1 6:1. https://doi.org/10.3390/FERMENTATION6010001

154. Calabia BP, Tokiwa Y (2007) Production of D-lactic acid from sugarcane molasses, sugarcane juice and sugar beet juice by Lactobacillus delbrueckii. Biotechnol Lett 29:1329–1332. https://doi.org/10.1007/S10529-007-9408-4/TABLES/1

155. Joshi DS, Singhvi MS, Khire JM, Gokhale D V. (2010) Strain improvement of Lactobacillus lactis for d-lactic acid production. Biotechnol Lett 32:517–520. https://doi.org/10.1007/S10529-009-0187-Y/FIGURES/1

156. Penaud S, Fernandez A, Boudebbouze S, et al (2006) Induction of heavy-metal-transporting CPX-type ATPases during acid adaptation in Lactobacillus bulgaricus. Appl Environ Microbiol 72:7445–7454. https://doi.org/10.1128/AEM.01109-06/ASSET/CF77CC12-D42C-4A95-AFDB-FC53BD25D39F/ASSETS/GRAPHIC/ZAM0120673130006.JPEG

157. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. Nature Protocols 2008 3:6 3:1101–1108. https://doi.org/10.1038/nprot.2008.73

158. Sun Y, Gao G, Cai T (2021) Enzymatic characterization of D-lactate dehydrogenase and application in alanine aminotransferase activity assay kit. Bioengineered 12:6459. https://doi.org/10.1080/21655979.2021.1972781

159. Zhang J, Wu C, Du G, Chen J (2012) Enhanced acid tolerance in Lactobacillus casei by adaptive evolution and compared stress response during acid stress. Biotechnology and Bioprocess Engineering 17:283–289. https://doi.org/10.1007/S12257-011-0346-6/METRICS

160. Bettenbrock K, Alpert CA (1998) The gal Genes for the Leloir Pathway of Lactobacillus casei 64H. Appl Environ Microbiol 64:2013. https://doi.org/10.1128/AEM.64.6.2013-2019.1998

161. Chervaux C, Ehrlich SD, Maguin E (2000) Physiological Study of Lactobacillus delbrueckii subsp. bulgaricus Strains in a Novel Chemically Defined Medium. Appl Environ Microbiol 66:5306. https://doi.org/10.1128/AEM.66.12.5306-5311.2000

162. Sørensen KI, Curic-Bawden M, Junge MP, et al (2016) Enhancing the Sweetness of Yoghurt through Metabolic Remodeling of Carbohydrate Metabolism in Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus. Appl Environ Microbiol 82:3683. https://doi.org/10.1128/AEM.00462-16

163. Wu Q, Cheung CKW, Shah NP (2015) Towards galactose accumulation in dairy foods fermented by conventional starter cultures: Challenges and strategies. Trends Food Sci Technol 41:24–36. https://doi.org/10.1016/J.TIFS.2014.08.010

164. Zhang X, Jin J, Zhao J, et al (2023) Study on a galactose decomposition strain of Lactiplantibacillus plantarum MWLp-4 from human milk: Safety evaluating and key genes analysis of potential probiotic function. Food Biosci 54:102934. https://doi.org/10.1016/J.FBIO.2023.102934

165. Liu K, Zeng X, Qiao L, et al (2014) The sensitivity and significance analysis of parameters in the model of pH regulation on lactic acid production by Lactobacillus bulgaricus. BMC Bioinformatics 15:1–9. https://doi.org/10.1186/1471-2105-15-S13-S5/FIGURES/4

166. Grossiord BP, Luesink EJ, Vaughan EE, et al (2003) Characterization, expression, and mutation of the Lactococcus lactis galPMKTE genes, involved in galactose utilization via the Leloir pathway. J Bacteriol 185:870–878. https://doi.org/10.1128/JB.185.3.870-878.2003

167. Zhang SS, Xu ZS, Qin LH, Kong J (2020) Low-sugar yogurt making by the co-cultivation of Lactobacillus plantarum WCFS1 with yogurt starter cultures. J Dairy Sci 103:3045–3054. https://doi.org/10.3168/JDS.2019-17347

168. Zhai Z, An H, Wang G, et al (2015) Functional role of pyruvate kinase from Lactobacillus bulgaricus in acid tolerance and identification of its transcription factor by

bacterial one-hybrid. Scientific Reports 2015 5:1 5:1–10. https://doi.org/10.1038/srep17024

169. Viana R, Pérez-Martínez G, Deutscher J, Monedero V (2005) The glycolytic genes pfk and pyk from Lactobacillus casei are induced by sugars transported by the phosphoenolpyruvate:sugar phosphotransferase system and repressed by CcpA. Arch Microbiol 183:385–393. https://doi.org/10.1007/S00203-005-0003-6/FIGURES/4

170. Leong-Morgenthaler P, Zwahlen MC, Hottinger H (1991) Lactose metabolism in Lactobacillus bulgaricus: analysis of the primary structure and expression of the genes involved. J Bacteriol 173:1951. https://doi.org/10.1128/JB.173.6.1951-1957.1991

171. El Kafsi H, Binesse J, Loux V, et al (2014) Lactobacillus delbrueckii ssp. lactis and ssp. bulgaricus: A chronicle of evolution in action. BMC Genomics 15:1–12. https://doi.org/10.1186/1471-2164-15-407/FIGURES/4

172. Huang Y, You C, Liu Z (2017) Cloning of d-lactate dehydrogenase genes of Lactobacillus delbrueckii subsp. bulgaricus and their roles in d-lactic acid production. 3 Biotech 7:1–7. https://doi.org/10.1007/S13205-017-0822-6/FIGURES/3

173. Awasthi D, Wang L, Rhee MS, et al (2018) Metabolic engineering of Bacillus subtilis for production of D-lactic acid. Biotechnol Bioeng 115:453–463. https://doi.org/10.1002/BIT.26472

174. Tsuge Y, Yamamoto S, Kato N, et al (2015) Overexpression of the phosphofructokinase encoding gene is crucial for achieving high production of D-lactate in Corynebacterium glutamicum under oxygen deprivation. Appl Microbiol Biotechnol 99:4679–4689. https://doi.org/10.1007/S00253-015-6546-9/METRICS

175. Tsuge Y, Kato N, Yamamoto S, et al (2019) Enhanced production of d-lactate from mixed sugars in Corynebacterium glutamicum by overexpression of glycolytic genes encoding phosphofructokinase and triosephosphate isomerase. J Biosci Bioeng 127:288–293. https://doi.org/10.1016/J.JBIOSC.2018.08.002

176. Bourniquel AA, Mollet B (2002) Purification and characterization of the 3-phosphoglycerate kinase from the thermophile Lactobacillus delbrueckii subsp. lactis. Int Dairy J 12:723–728. https://doi.org/10.1016/S0958-6946(02)00069-9

177. Tsuge Y, Kato N, Yamamoto S, et al (2019) Metabolic engineering of Corynebacterium glutamicum for hyperproduction of polymer-grade L- and D-lactic acid. Appl Microbiol Biotechnol 103:3381–3391. https://doi.org/10.1007/S00253-019-09737-8

178. Peetermans A, Foulquié-Moreno MR, Thevelein JM (2021) Mechanisms underlying lactic acid tolerance and its influence on lactic acid production in Saccharomyces cerevisiae. Microb Cell 8:111–130. https://doi.org/10.15698/MIC2021.06.751

179. Chervaux C, Ehrlich SD, Maguin E (2000) Physiological Study of Lactobacillus delbrueckii subsp. bulgaricus Strains in a Novel Chemically Defined Medium. Appl Environ Microbiol 66:5306. https://doi.org/10.1128/AEM.66.12.5306-5311.2000

180. Kawai M, Harada R, Yoda N, et al (2020) Suppression of lactate production by using sucrose as a carbon source in lactic acid bacteria. J Biosci Bioeng 129:47–51. https://doi.org/10.1016/J.JBIOSC.2019.06.017

181. Srivastava A, Poonia A, Tripathi AD, et al (2014) Optimization of nutritional supplements for enhanced lactic acid production utilizing sugar refinery by-products. Ann Microbiol 64:1211–1221. https://doi.org/10.1007/S13213-013-0761-3/TABLES/6

182. Karnaouri A, Asimakopoulou G, Kalogiannis KG, et al (2020) Efficient d-lactic acid production by Lactobacillus delbrueckii subsp. bulgaricus through conversion of organosolv pretreated lignocellulosic biomass. Biomass Bioenergy 140:105672. https://doi.org/10.1016/J.BIOMBIOE.2020.105672

183. Yang PB, Tian Y, Wang Q, Cong W (2015) Effect of different types of calcium carbonate on the lactic acid fermentation performance of Lactobacillus lactis. Biochem Eng J 98:38–46. https://doi.org/10.1016/J.BEJ.2015.02.023

184. Burgos-Rubio CN, Okos MR, Wankat PC (2000) Kinetic Study of the Conversion of Different Substrates to Lactic Acid Using Lactobacillus bulgaricus. Biotechnol Prog 16:305–314. https://doi.org/10.1021/BP000022P

185. Adamberg K, Kask S, Laht TM, Paalme T (2003) The effect of temperature and pH on the growth of lactic acid bacteria: a pH-auxostat study. Int J Food Microbiol 85:171–183. https://doi.org/10.1016/S0168-1605(02)00537-8

186. Silva J, Carvalho AS, Pereira H, et al (2004) Induction of stress tolerance in Lactobacillus delbrueckii ssp. bulgaricus by the addition of sucrose to the growth medium. J Dairy Res 71:121–125. https://doi.org/10.1017/S0022029903006411

187. Gui-cheng H (2012) The correlation of growth performance and postacidification properties of Lactobacillus delbrueckii subsp. bulgaricus. Food Science and Technology International

188. Xu Q, Zang Y, Zhou J, et al (2016) Highly efficient production of d-lactic acid from chicory-derived inulin by Lactobacillus bulgaricus. Bioprocess Biosyst Eng 39:1749–1757. https://doi.org/10.1007/S00449-016-1650-5/TABLES/3

189. Guo Y, Wang G, Li K, et al (2023) [Immobilization of Lactobacillus bulgaricus with gellan gum and its application in continuous fermentation of D-lactic acid from corn straw]. Sheng Wu Gong Cheng Xue Bao 39:1083–1095. https://doi.org/10.13345/J.CJB.220542

190. Guo Y, Wang G, Chen H, et al (2022) Continuous Fermentation by Lactobacillus bulgaricus T15 Cells Immobilized in Cross-Linked F127 Hydrogels to Produce D-Lactic Acid. Fermentation 8:. https://doi.org/10.3390/FERMENTATION8080360

191. Guo Y, Zhao Y, Gao Y, et al (2023) Low acyl gellan gum immobilized Lactobacillus bulgaricus T15 produce d-lactic acid from non-detoxified corn stover hydrolysate.

Biotechnology for Biofuels and Bioproducts 16:43. https://doi.org/10.1186/S13068-023-02292-5

192. Giorno L, Chojnacka K, Donato L, Drioli E (2002) Study of a cell-recycle membrane fermentor for the production of lactic acid by Lactobacillus bulgaricus. Ind Eng Chem Res 41:433–440. https://doi.org/10.1021/IE010201R/ASSET/IMAGES/LARGE/IE010201RF00012.JPEG

193. Yang Y, Lin Y, Wang J, et al (2018) Sensor-regulator and RNAi based bifunctional dynamic control network for engineered microbial synthesis. Nature Communications 2018 9:1 9:1–10. https://doi.org/10.1038/s41467-018-05466-0

194. Boo A, Ellis T, Stan GB (2019) Host-aware synthetic biology. Curr Opin Syst Biol 14:66–72. https://doi.org/10.1016/J.COISB.2019.03.001

195. Snoeck S, Guidi C, De Mey M (2024) "Metabolic burden" explained: stress symptoms and its related responses induced by (over)expression of (heterologous) proteins in Escherichia coli. Microbial Cell Factories 2024 23:1 23:1–19. https://doi.org/10.1186/S12934-024-02370-9

196. Jones JA, Toparlak TD, Koffas MAG (2015) Metabolic pathway balancing and its role in the production of biofuels and chemicals. Curr Opin Biotechnol 33:52–59. https://doi.org/10.1016/J.COPBIO.2014.11.013

197. Ni C, Dinh C V., Prather KLJ (2021) Dynamic Control of Metabolism. Annu Rev Chem Biomol Eng 12:519–541. https://doi.org/10.1146/ANNUREV-CHEMBIOENG-091720-125738/1

198. Zu Y, Prather KL, Stephanopoulos G (2020) Metabolic engineering strategies to overcome precursor limitations in isoprenoid biosynthesis. Curr Opin Biotechnol 66:171–178. https://doi.org/10.1016/J.COPBIO.2020.07.005

199. Heiss S, Hörmann A, Tauer C, et al (2016) Evaluation of novel inducible promoter/repressor systems for recombinant protein expression in Lactobacillus plantarum. Microb Cell Fact 15:1–17. https://doi.org/10.1186/S12934-016-0448-0/FIGURES/8

200. Lalwani MA, Zhao EM, Avalos JL (2018) Current and future modalities of dynamic control in metabolic engineering. Curr Opin Biotechnol 52:56–65. https://doi.org/10.1016/J.COPBIO.2018.02.007

201. Hartline CJ, Schmitz AC, Han Y, Zhang F (2021) Dynamic control in metabolic engineering: Theories, tools, and applications. Metab Eng 63:126–140. https://doi.org/10.1016/J.YMBEN.2020.08.015

202. Olson EJ, Hartsough LA, Landry BP, et al (2014) Characterizing bacterial gene circuit dynamics with optically programmed gene expression signals. Nature Methods 2014 11:4 11:449–455. https://doi.org/10.1038/nmeth.2884

203. Rohlhill J, Sandoval NR, Papoutsakis ET (2017) Sort-Seq Approach to Engineering a Formaldehyde-Inducible Promoter for Dynamically Regulated Escherichia coli Growth on Methanol. ACS Synth Biol 6:1584–1595. https://doi.org/10.1021/ACSSYNBIO.7B00114/SUPPL_FILE/SB7B00114_LIVESLIDES.MP4

204. Wu S, Liu J, Liu C, et al (2020) Quorum sensing for population-level control of bacteria and potential therapeutic applications. Cellular and Molecular Life Sciences 77:1319–1343. https://doi.org/10.1007/S00018-019-03326-8/FIGURES/9

205. Maidin T, Shawal M (2013) Identification of a heat-shock protein promoter and construction of a novel stress inducible Lactobacillus-lactococcus shuttle vector

206. Carrillo Rincón AF, Farny NG (2023) Unlocking the strength of inducible promoters in Gram-negative bacteria. Microb Biotechnol 16:961. https://doi.org/10.1111/1751-7915.14219

207. Guan C, Yuan Y, Ma Y, et al (2022) Development of a novel expression system in lactic acid bacteria controlled by a broad-host-range promoter PsrfA. Microb Cell Fact 21:1–10. https://doi.org/10.1186/S12934-022-01754-Z/TABLES/2

208. Ream M, Prather KLJ (2023) Engineered autonomous dynamic regulation of metabolic flux. Nature Reviews Bioengineering 2023 2:3 2:233–243. https://doi.org/10.1038/s44222-023-00140-7

209. Augustiniene E, Malys N (2022) Identification and characterization of L- and D-lactate-inducible systems from Escherichia coli MG1655, Cupriavidus necator H16 and Pseudomonas species. Scientific Reports 2022 12:1 12:1–11. https://doi.org/10.1038/s41598-022-06028-7

210. Singh K, Ainala SK, Kim Y, Park S (2019) A novel D(−)-lactic acid-inducible promoter regulated by the GntR-family protein D-LldR of Pseudomonas fluorescens. Synth Syst Biotechnol 4:157–164. https://doi.org/10.1016/J.SYNBIO.2019.08.004

211. Mukherjee P, Raj N, Sivaprakasam S (2023) Harnessing valorization potential of whey permeate for D-lactic acid production using lactic acid bacteria. Biomass Conversion and Biorefinery 2023 13:17 13:15639–15658. https://doi.org/10.1007/S13399-023-05038-3

212. Hasan GMMA, Das AK, Satter MA (2024) CRISPR/Cas9-mediated gene editing of L. bulgaricus d-lactate dehydrogenase (ldh) gene to monitor yogurt characteristics. Food Chemistry Advances 5:100772. https://doi.org/10.1016/J.FOCHA.2024.100772

213. Van De Guchte M, Penaud S, Grimaldi C, et al (2006) The complete genome sequence of Lactobacillus bulgaricus reveals extensive and ongoing reductive evolution. Proc Natl Acad Sci U S A 103:9274–9279. https://doi.org/10.1073/PNAS.0603024103/SUPPL_FILE/03024FIG13.PDF

214. Gong P, Lin K, Zhang J, et al (2020) Enhancing spray drying tolerance of Lactobacillus bulgaricus by intracellular trehalose delivery via electroporation. Food Research International 127:108725. https://doi.org/10.1016/J.FOODRES.2019.108725

215. Fara A, Sabater C, Palacios J, et al (2020) Prebiotic galactooligosaccharides production from lactose and lactulose by Lactobacillus delbrueckii subsp. bulgaricus CRL450. Food Funct 11:5875–5886. https://doi.org/10.1039/D0FO00942C

216. Yamamoto E, Watanabe R, Ichimura T, et al (2021) Effect of lactose hydrolysis on the milk-fermenting properties of Lactobacillus delbrueckii ssp. bulgaricus 2038 and Streptococcus thermophilus 1131. J Dairy Sci 104:1454–1464. https://doi.org/10.3168/JDS.2020-19244

217. Zhang Y, Huang Z, Du C, et al (2009) Introduction of an NADH regeneration system into Klebsiella oxytoca leads to an enhanced oxidative and reductive metabolism of glycerol. Metab Eng 11:101–106. https://doi.org/10.1016/J.YMBEN.2008.11.001

218. Su L, Shen Y, Zhang W, et al (2017) Cofactor engineering to regulate NAD+/NADH ratio with its application to phytosterols biotransformation. Microb Cell Fact 16:1–11. https://doi.org/10.1186/S12934-017-0796-4/FIGURES/5

219. Schöning-Stierand K, Diedrich K, Fährrolfes R, et al (2020) ProteinsPlus: interactive analysis of protein–ligand binding interfaces. Nucleic Acids Res 48:W48–W53. https://doi.org/10.1093/NAR/GKAA235

220. Adasme MF, Linnemann KL, Bolz SN, et al (2021) PLIP 2021: expanding the scope of the protein–ligand interaction profiler to DNA and RNA. Nucleic Acids Res 49:W530–W534. https://doi.org/10.1093/NAR/GKAB294

221. Yang Y, Lin Y, Wang J, et al (2018) Sensor-regulator and RNAi based bifunctional dynamic control network for engineered microbial synthesis. Nat Commun 9:. https://doi.org/10.1038/S41467-018-05466-0

222. Mikkonen M, Vuoristo J, Alatossava T (1994) Ribosome binding site consensus sequence of Lactobacillus delbrueckii subsp. lactis bacteriophage LL-H. FEMS Microbiol Lett 116:315–320. https://doi.org/10.1111/J.1574-6968.1994.TB06721.X

223. Neves D, Liebal UW, Nies SC, et al (2023) Cross-Species Synthetic Promoter Library: Finding Common Ground between Pseudomonas taiwanensis VLB120 and Escherichia coli. ACS Synth Biol 12:2029–2040. https://doi.org/10.1021/ACSSYNBIO.3C00084

224. Bouffartigues E, Gicquel G, Bazire A, et al (2012) Transcription of the oprF gene of Pseudomonas aeruginosa is dependent mainly on the SigX sigma factor and is sucrose induced. J Bacteriol 194:4301–4311. https://doi.org/10.1128/JB.00509-12

225. Rampioni G, Bertani I, Pillai CR, et al (2012) Functional characterization of the quorum sensing regulator RsaL in the plant-beneficial strain Pseudomonas putida WCS358. Appl Environ Microbiol 78:726–734. https://doi.org/10.1128/AEM.06442-11

226. Sawers G, Wagner AF, Böck A (1989) Transcription initiation at multiple promoters of the pfl gene by E sigma 70-dependent transcription in vitro and heterologous expression in Pseudomonas putida in vivo. J Bacteriol 171:4930–4937. https://doi.org/10.1128/JB.171.9.4930-4937.1989

227. Callaghan JD, Stella NA, Lehner KM, et al (2020) Xylose-Inducible Promoter Tools for Pseudomonas Species and Their Use in Implicating a Role for the Type II Secretion System Protein XcpQ in the Inhibition of Corneal Epithelial Wound Closure. Appl Environ Microbiol 86:. https://doi.org/10.1128/AEM.00250-20

228. Hussain A, Ray MK (2024) Role of DEAD-box RNA helicases in low-temperature adapted growth of Antarctic Pseudomonas syringae Lz4W. Microbiol Spectr 12:. https://doi.org/10.1128/SPECTRUM.04335-22

229. Blanch-Asensio M, Tadimarri VS, Wilk A, Sankaran S (2024) Discovery of a high-performance phage-derived promoter/repressor system for probiotic lactobacillus engineering. Microb Cell Fact 23:1–13. https://doi.org/10.1186/S12934-024-02302-7/FIGURES/6

230. Atitey K, Loskot P, Rees P (2018) Determining the Transcription Rates Yielding Steady-State Production of mRNA in the Lac Genetic Switch of Escherichia coli. J Comput Biol 25:1023–1039. https://doi.org/10.1089/CMB.2018.0055

231. Gawin A, Valla S, Brautaset T (2017) The XylS/Pm regulator/promoter system and its use in fundamental studies of bacterial gene expression, recombinant protein production and metabolic engineering. Microb Biotechnol 10:702. https://doi.org/10.1111/1751-7915.12701

232. McCracken A, Turner MS, Giffard P, et al (2000) Analysis of promoter sequences from Lactobacillus and Lactococcus and their activity in several Lactobacillus species. Arch Microbiol 173:383–389. https://doi.org/10.1007/S002030000159/METRICS

233. Gomes ALC, Johns NI, Yang A, et al (2020) Genome and sequence determinants governing the expression of horizontally acquired DNA in bacteria. ISME J 14:2347. https://doi.org/10.1038/S41396-020-0696-1

234. Blanch-Asensio M, Tadimarri VS, Wilk A, Sankaran S (2024) Discovery of a high-performance phage-derived promoter/repressor system for probiotic lactobacillus engineering. Microb Cell Fact 23:1–13. https://doi.org/10.1186/S12934-024-02302-7/FIGURES/6

235. Zuo W, Yin G, Zhang L, et al (2025) Engineering artificial cross-species promoters with different transcriptional strengths. Synth Syst Biotechnol 10:49–57. https://doi.org/10.1016/J.SYNBIO.2024.08.003

236. Meier D, Rauch C, Wagner M, et al (2024) A MoClo-Compatible Toolbox of ECF Sigma Factor-Based Regulatory Switches for Proteobacterial Chassis. BioDesign Research 6:. https://doi.org/10.34133/BDR.0025/SUPPL_FILE/BDR.0025.F1.ZIP

237. Gaida SM, Sandoval NR, Nicolaou SA, et al (2015) Expression of heterologous sigma factors enables functional screening of metagenomic and heterologous genomic libraries. Nature Communications 2015 6:1 6:1–10. https://doi.org/10.1038/ncomms8045

238. Taniguchi H, Wendisch VF (2015) Exploring the role of sigma factor gene expression on production by Corynebacterium glutamicum: Sigma factor H and FMN as example. Front Microbiol 6:152776. https://doi.org/10.3389/FMICB.2015.00740/BIBTEX

239. Bervoets I, Van Brempt M, Van Nerom K, et al (2018) A sigma factor toolbox for orthogonal gene expression in Escherichia coli. Nucleic Acids Res 46:2133. https://doi.org/10.1093/NAR/GKY010

240. Winkler JD, Kao KC (2014) Complementation of a temperature sensitive Escherichia coli rpoD mutation using Lactobacillus sigma factors. bioRxiv 003152. https://doi.org/10.1101/003152

241. Tripathi L, Zhang Y, Lin Z (2014) Bacterial Sigma Factors as Targets for Engineered or Synthetic Transcriptional Control. Front Bioeng Biotechnol 2:. https://doi.org/10.3389/FBIOE.2014.00033

242. Garcia HG, Sanchez A, Boedicker JQ, et al (2012) Operator Sequence Alters Gene Expression Independently of Transcription Factor Occupancy in Bacteria. Cell Rep 2:150. https://doi.org/10.1016/J.CELREP.2012.06.004

243. Hammar P, Walldén M, Fange D, et al (2014) In vivo repressor kinetics exclude simple operator occupancy model for gene regulation

244. Yan Y, Zhang D, Zhou P, et al (2017) HDOCK: a web server for protein–protein and protein–DNA/RNA docking based on a hybrid strategy. Nucleic Acids Res 45:W365–W373. https://doi.org/10.1093/NAR/GKX407

245. Salentin S, Schreiber S, Haupt VJ, et al (2015) PLIP: fully automated protein–ligand interaction profiler. Nucleic Acids Res 43:W443–W447. https://doi.org/10.1093/NAR/GKV315

246. Spangler JR, Cooper DN, Malanoski AP, Walper SA (2023) Promoter Identification and Optimization for the Response of Lactobacillus plantarum WCFS1 to the Gram-Negative Pathogen-Associated Molecule N-3-Oxododecanoyl Homoserine Lactone. ACS Biomater Sci Eng 9:5111–5122. https://doi.org/10.1021/ACSBIOMATERIALS.1C01191

247. Ulmer A, Erdemann F, Mueller S, et al (2022) Differential Amino Acid Uptake and Depletion in Mono-Cultures and Co-Cultures of Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus in a Novel Semi-Synthetic Medium. Microorganisms 10:. https://doi.org/10.3390/MICROORGANISMS10091771/S1

248. Hartline CJ, Schmitz AC, Han Y, Zhang F (2021) Dynamic control in metabolic engineering: Theories, tools, and applications. Metab Eng 63:126. https://doi.org/10.1016/J.YMBEN.2020.08.015

249. Machado J, Rossi DM, Ayub MAZ (2024) Batch and fed-batch strategies of lactic acid production by Lactobacillus plantarum BL011 using soybean hull hydrolysates as substrate. Biomass Convers Biorefin 14:3249–3259. https://doi.org/10.1007/S13399-022-02544-8/TABLES/5

250. Abbasalizadeh S, Hejazi MA, Hajiabbas MP (2015) Kinetics of β-galactosidase Production by Lactobacillus bulgaricus During pH Controlled Batch Fermentation in Three Commercial Bulk Starter Media. Applied Food Biotechnology 2:39–47. https://doi.org/10.22037/AFB.V2I4.9512

251. Razeto A, Kochhar S, Hottinger H, et al (2002) Domain Closure, Substrate Specificity and Catalysis of d-Lactate Dehydrogenase from Lactobacillus bulgaricus. J Mol Biol 318:109–119. https://doi.org/10.1016/S0022-2836(02)00086-4

252. Ream M, Prather KLJ (2023) Engineered autonomous dynamic regulation of metabolic flux. Nature Reviews Bioengineering 2023 2:3 2:233–243. https://doi.org/10.1038/s44222-023-00140-7

253. Guo Y, Wang G, Chen H, et al (2022) Continuous Fermentation by Lactobacillus bulgaricus T15 Cells Immobilized in Cross-Linked F127 Hydrogels to Produce D-Lactic Acid. Fermentation 8:. https://doi.org/10.3390/FERMENTATION8080360

254. Salmazo GC, Dal Molin Filho RG, Robazza W da S, et al (2023) Modeling the growth dependence of Streptococcus thermophilus and Lactobacillus bulgaricus as a function of temperature and pH. Braz J Microbiol 54:323–334. https://doi.org/10.1007/S42770-023-00907-5

255. Xiao W, Wang RS, Handy DE, Loscalzo J (2018) NAD(H) and NADP(H) Redox Couples and Cellular Energy Metabolism. Antioxid Redox Signal 28:251–272. https://doi.org/10.1089/ARS.2017.7216

256. Liu J, Jiang X, Zheng Y, et al (2024) Expression, Characterization, and Immobilization of a Novel D-Lactate Dehydrogenase from Salinispirillum sp. LH 10-3-1. Processes 12:1349. https://doi.org/10.3390/PR12071349

257. Yi X, Kot E, Bezkorovainy A (1998) Properties of NADH oxidase from Lactobacillus delbrueckii ssp bulgaricus. J Sci Food Agric. https://doi.org/10.1002/(SICI)1097-0010(199812)78:4

258. Marty-Teysset C, De La Torre F, Garel JR (2000) Increased production of hydrogen peroxide by Lactobacillus delbrueckii subsp. bulgaricus upon aeration: involvement of an NADH oxidase in oxidative stress. Appl Environ Microbiol 66:262–267. https://doi.org/10.1128/AEM.66.1.262-267.2000

259. Trovão M, Schüler LM, Machado A, et al (2022) Random Mutagenesis as a Promising Tool for Microalgal Strain Improvement towards Industrial Production. Mar Drugs 20:440. https://doi.org/10.3390/MD20070440/S1

260. Ribeiro O, Magalhães F, Aguiar TQ, et al (2013) Random and direct mutagenesis to enhance protein secretion in Ashbya gossypii. Bioengineered 4:322–331. https://doi.org/10.4161/BIOE.24653

261. Hanif A, Ejaz U, Hasan KA, et al (2023) Two-way strategy for enhanced pectinase production: Random mutagenesis and utilization of a halophytic biomass. Biocatal Agric Biotechnol 47:102619. https://doi.org/10.1016/J.BCAB.2023.102619

262. Naeem M, Sadia B, Awan FS, Zia MA (2018) Enhanced Production of Streptokinase by UV-and Ethidium Bromide-Treated Streptococus equisimilis Mutant. https://doi.org/10.17582/journal.pjz/2018.50.2.655.661

263. Nasri F, Zakizadeh H, Vafaee Y, Mozafari AA (2022) In vitro mutagenesis of Chrysanthemum morifolium cultivars using ethylmethanesulphonate (EMS) and mutation assessment by ISSR and IRAP markers. Plant Cell Tissue Organ Cult 149:657–673. https://doi.org/10.1007/S11240-021-02163-7/FIGURES/5

264. Macdonald Miller S, Abbriano RM, Herdean A, et al (2023) Random mutagenesis of Phaeodactylum tricornutum using ultraviolet, chemical, and X-ray irradiation demonstrates the need for temporal analysis of phenotype stability. Scientific Reports 2024 13:1 13:1–10. https://doi.org/10.1038/s41598-023-45899-2

265. Erkorkmaz BA, Kırtel O, Abaramak G, et al (2022) UV and chemically induced Halomonas smyrnensis mutants for enhanced levan productivity. J Biotechnol 356:19–29. https://doi.org/10.1016/J.JBIOTEC.2022.07.005

266. Fonseca DR, Loppnow MB, Day LA, et al (2023) Random transposon mutagenesis identifies genes essential for transformation in Methanococcus maripaludis. Molecular Genetics and Genomics 298:537–548. https://doi.org/10.1007/S00438-023-01994-7/FIGURES/5

267. Yin L, Ruan Q, Fu Y (2013) African Journal of Microbiology Research Strain improvement of Rhizopus oryzae for over-production of lactic acid by random mutations. 7:2970–2975. https://doi.org/10.5897/AJMR2013.5425

268. Suntornsuk W, Hang YD (1994) Strain improvement of Rhizopus oryzae for production of l(+)-lactic acid and glucoamylase. Lett Appl Microbiol 19:249–252. https://doi.org/10.1111/J.1472-765X.1994.TB00956.X

269. Jiang A lian, Hu W, Li W jian, et al (2018) Enhanced production of l-lactic acid by Lactobacillus thermophilus SRZ50 mutant generated by high-linear energy transfer heavy ion mutagenesis. Eng Life Sci 18:626. https://doi.org/10.1002/ELSC.201800052

270. Lin H, Chen X, Yu L, et al (2012) Screening of Lactobacillus rhamnosus strains mutated by microwave irradiation for increased lactic acid production. Afr J Microbiol Res 6:6055–6065. https://doi.org/10.5897/AJMR12.434

271. Karnaouri A, Asimakopoulou G, Kalogiannis KG, et al (2020) Efficient d-lactic acid production by Lactobacillus delbrueckii subsp. bulgaricus through conversion of organosolv pretreated lignocellulosic biomass. Biomass Bioenergy 140:105672. https://doi.org/10.1016/J.BIOMBIOE.2020.105672

272. Yang M, An Y, Zabed HM, et al (2019) Random mutagenesis of Clostridium butyricum strain and optimization of biosynthesis process for enhanced production of 1,3-propanediol. Bioresour Technol 284:188–196. https://doi.org/10.1016/J.BIORTECH.2019.03.098

273. Mithun S, Dipak V (2016) MUTAGENESIS OF LACTOBACILLUS SPECIES FOR THE GENERATION OF A MUTANT WITH HYPER-PRODUCING DEXTRANSUCRASE ACTIVITY. 777 Mithun et al World Journal of Pharmaceutical Research 5:

274. Irianto VS, Demirkan E, Cetinkaya AA (2024) UV mutagenesis for lipase overproduction from Bacillus cereus ATA179, nutritional optimization, characterization and its usability in the detergent industry. Prep Biochem Biotechnol. https://doi.org/10.1080/10826068.2023.2299441

275. Yu L, Li F, Ni J, et al (2024) UV-ARTP compound mutagenesis breeding improves macrolactins production of Bacillus siamensis and reveals metabolism changes by proteomic. J Biotechnol 381:36–48. https://doi.org/10.1016/J.JBIOTEC.2023.12.011

276. Sharma A, Mukherjee S, Reddy Tadi SR, et al (2021) Kinetics of growth, plantaricin and lactic acid production in whey permeate based medium by probiotic Lactobacillus plantarum CRA52. LWT 139:110744. https://doi.org/10.1016/J.LWT.2020.110744

277. Pandey KK, Sood SK, Verma SK, et al (2019) Bioutilization of paneer whey waste for production of paneer making powder containing pediocin PA-1 as a biopreservative to enhance shelf life of paneer. LWT 113:108243. https://doi.org/10.1016/J.LWT.2019.06.010

278. Granget C, Manikandan NA, Amulya K, et al (2024) Brewer's spent grain as a self-sufficient feedstock for homofermentative production of optically pure L-lactic acid using Lactobacillus rhamnosus. Environ Technol Innov 103582. https://doi.org/10.1016/J.ETI.2024.103582

279. Kumar S, Yadav N, Nain L, Khare SK (2020) A simple downstream processing protocol for the recovery of lactic acid from the fermentation broth. Bioresour Technol 318:124260. https://doi.org/10.1016/J.BIORTECH.2020.124260

280. Vermeulen N, Keeler WJ, Nandakumar K, Leung KT (2008) The bactericidal effect of ultraviolet and visible light on Escherichia coli. Biotechnol Bioeng 99:550–556. https://doi.org/10.1002/BIT.21611

281. Goodarzi A UV - Induced Mutagenesis in Lactic Acid Bacteria

282. Williams PD, Eichstadt SL, Kokjohn TA, Martin EL (2007) Effects of ultraviolet radiation on the Gram-positive marine bacterium Microbacterium maritypicum. Curr Microbiol 55:1–7. https://doi.org/10.1007/S00284-006-0349-2/FIGURES/4

283. Ohnishi J, Mizoguchi H, Takeno S, Ikeda M (2008) Characterization of mutations induced by N-methyl-N′-nitro-N-nitrosoguanidine in an industrial Corynebacterium glutamicum strain. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 649:239–244. https://doi.org/10.1016/J.MRGENTOX.2007.10.003

284. Trovão M, Schüler LM, Machado A, et al (2022) Random Mutagenesis as a Promising Tool for Microalgal Strain Improvement towards Industrial Production. Mar Drugs 20:. https://doi.org/10.3390/MD20070440/S1

285. Vu VH, Pham TA, Kim K (2009) Fungal Strain Improvement for Cellulase Production Using Repeated and Sequential Mutagenesis. Mycobiology 37:267. https://doi.org/10.4489/MYCO.2009.37.4.267

286. Sudi IY, De N, Ali-Dunkrah U (2008) Mutagenesis and Selection of Lactobacillus bulgaricus and Streptococcus thermophilus for Potential use as Starter Culture. J Am Sci 4:1545–1003

287. Zhai Z, An H, Wang G, et al (2015) Functional role of pyruvate kinase from Lactobacillus bulgaricus in acid tolerance and identification of its transcription factor by bacterial one-hybrid. Sci Rep 5:. https://doi.org/10.1038/SREP17024

288. Bai Y-H, Jiang Y-F, Jiang Y-S (2014) Renal Failure Lactobacillus bulgaricus mutants decompose uremic toxins Lactobacillus bulgaricus mutants decompose uremic toxins. Ren Fail 36:790–794. https://doi.org/10.3109/0886022X.2014.890111

289. Sesti-Costa R, Negrão C von Z, Shimizu JF, et al (2022) UV 254 nm is more efficient than UV 222 nm in inactivating SARS-CoV-2 present in human saliva. Photodiagnosis Photodyn Ther 39:103015. https://doi.org/10.1016/J.PDPDT.2022.103015

290. Wang K, Lin X, Wang T, et al (2023) Synergistic effects of low-dose arsenic and N-methyl-N′-nitro-N-nitrosoguanidine co-exposure by altering gut microbiota and intestinal metabolic profile in rats. Ecotoxicol Environ Saf 263:115195. https://doi.org/10.1016/J.ECOENV.2023.115195

291. Burgos-Rubio CN, Okos MR, Wankat PC (2000) Kinetic Study of the Conversion of Different Substrates to Lactic Acid Using Lactobacillus bulgaricus. Biotechnol Prog 16:305–314. https://doi.org/10.1021/BP000022P

292. Li X hua, Yang H jun, Roy B, et al (2010) Enhanced cellulase production of the Trichoderma viride mutated by microwave and ultraviolet. Microbiol Res 165:190–198. https://doi.org/10.1016/J.MICRES.2009.04.001

293. Chervaux C, Ehrlich SD, Maguin E (2000) Physiological study of Lactobacillus delbrueckii subsp. bulgaricus strains in a novel chemically defined medium. Appl Environ Microbiol 66:5306–5311. https://doi.org/10.1128/AEM.66.12.5306-5311.2000/ASSET/6E691909-B629-4323-8AE4-67F5552B1655/ASSETS/GRAPHIC/AM1200782003.JPEG

294. Zheng H, Liu E, Shi T, et al (2016) Strand-specific RNA-seq analysis of the Lactobacillus delbrueckii subsp. bulgaricus transcriptome. Mol Biosyst 12:508–519. https://doi.org/10.1039/C5MB00547G

295. Chervaux C, Ehrlich SD, Maguin E (2000) Physiological Study of Lactobacillus delbrueckii subsp. bulgaricus Strains in a Novel Chemically Defined Medium. Appl Environ Microbiol 66:5306. https://doi.org/10.1128/AEM.66.12.5306-5311.2000

296. Kadam SR, Patil SS, Bastawde KB, et al (2006) Strain improvement of Lactobacillus delbrueckii NCIM 2365 for lactic acid production. Process Biochemistry 41:120–126. https://doi.org/10.1016/J.PROCBIO.2005.06.007

297. Marziyeh A, Masood B, Morteza K (2014) Effect of Temperature and pH on Formulating the Kinetic Growth Parameters and Lactic Acid Production of Lactobacillus bulgaricus. 49–56

298. Cui JN, Hu W, Liu YX, et al (2023) Isolation and Screening of High-Yielding α-Amylase Mutants of Bacillus subtilis by Heavy Ion Mutagenesis. Appl Biochem Biotechnol 195:68–85. https://doi.org/10.1007/S12010-022-04097-Y/TABLES/3

299. Yang M, An Y, Zabed HM, et al (2019) Random mutagenesis of Clostridium butyricum strain and optimization of biosynthesis process for enhanced production of 1,3-propanediol. Bioresour Technol 284:188–196. https://doi.org/10.1016/J.BIORTECH.2019.03.098

300. Li C, Zhang GF, Mao X, et al (2016) Growth and acid production of Lactobacillus delbrueckii ssp. bulgaricus ATCC 11842 in the fermentation of algal carcass. J Dairy Sci 99:4243–4250. https://doi.org/10.3168/JDS.2015-10700

301. Yankov D (2022) Fermentative Lactic Acid Production From Lignocellulosic Feedstocks: From Source to Purified Product. Front Chem 10:823005. https://doi.org/10.3389/FCHEM.2022.823005/BIBTEX

302. WHEATER DM (1955) The characteristics of Lactobacillus acidophilus and Lactobacillus bulgaricus. J Gen Microbiol 12:123–132. https://doi.org/10.1099/00221287-12-1-123/CITE/REFWORKS

303. Burgos-Rubio CN, Okos MR, Wankat PC (2000) Kinetic Study of the Conversion of Different Substrates to Lactic Acid Using Lactobacillus bulgaricus. Biotechnol Prog 16:305–314. https://doi.org/10.1021/BP000022P

304. Ghasemi M, Najafpour G, Najafpour G, et al (2009) Effect of different media on production of lactic acid from whey by Lactobacillus bulgaricus. Afr J Biotechnol 8:81–084

305. Granget C, Manikandan NA, Amulya K, et al (2024) Brewer's spent grain as a self-sufficient feedstock for homofermentative production of optically pure L-lactic acid using Lactobacillus rhamnosus. Environ Technol Innov 34:103582. https://doi.org/10.1016/J.ETI.2024.103582

306. Benthin S, Villadsen J (1995) Different inhibition of Lactobacillus delbrueckii subsp. bulgaricus by D- and L-lactic acid: effects on lag phase, growth rate and cell yield. Journal of Applied Bacteriology 78:647–654. https://doi.org/10.1111/J.1365-2672.1995.TB03111.X

307. Carvalho AS, Silva J, Ho P, et al Effects of Various Sugars Added to Growth and Drying Media upon Thermotolerance and Survival throughout Storage of Freeze-Dried lactobacillus delbrueckii ssp. bulgaricus

308. de Lima CJB, Coelho LF, Contiero J (2010) The Use of Response Surface Methodology in Optimization of Lactic Acid Production: Focus on Medium Supplementation, Temperature and pH Control. Food Technol Biotechnol 48:175–181

309. Willem Schepers A, Thibault J, Lacroix C (2002) Lactobacillus helveticus growth and lactic acid production during pH-controlled batch cultures in whey permeate/yeast extract medium. Part I. multiple factor kinetic analysis. Enzyme Microb Technol 30:176–186. https://doi.org/10.1016/S0141-0229(01)00465-3

310. Reddy Tadi SR, EVR A, Limaye AM, Sivaprakasam S (2017) Enhanced production of optically pure D (–) lactic acid from nutritionally rich Borassus flabellifer sugar and whey protein hydrolysate based–fermentation medium. Biotechnol Appl Biochem 64:279–289

311. Zhang G, Liu L, Li C (2020) Effects of ccpA gene deficiency in Lactobacillus delbrueckii subsp. bulgaricus under aerobic conditions as assessed by proteomic analysis. Microb Cell Fact 19:1–12. https://doi.org/10.1186/S12934-020-1278-7/FIGURES/5

312. Labrou NE (2010) Random Mutagenesis Methods for In Vitro Directed Enzyme Evolution. Curr Protein Pept Sci 11:91–100. https://doi.org/10.2174/138920310790274617

313. Zeng C, Zhao R, Ma M, et al (2017) Mutagenesis and characterization of a Bacillus amyloliquefaciens strain for Cinnamomum camphora seed kernel oil extraction by

aqueous enzymatic method. AMB Express 7:154. https://doi.org/10.1186/S13568-017-0451-9

314. Prasirtsak B, Thitiprasert S, Tolieng V, et al (2019) D-Lactic acid fermentation performance and the enzyme activity of a novel bacterium Terrilactibacillus laevilacticus SK5–6. Ann Microbiol 69:1537–1546. https://doi.org/10.1007/S13213-019-01538-8/TABLES/2

315. Zhai Z, An H, Wang G, et al (2015) Functional role of pyruvate kinase from Lactobacillus bulgaricus in acid tolerance and identification of its transcription factor by bacterial one-hybrid. Sci Rep 5:. https://doi.org/10.1038/SREP17024

316. Clarke AR, Evington JRN, Dunn CR, et al (1986) The molecular pathway by which fructose 1,6-bisphosphate induces the assembly of a bacterial lactate dehydrogenase. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology 870:112–126. https://doi.org/10.1016/0167-4838(86)90014-2

317. Le Bras G, De La Torre F, Branny P, Garel JR (1998) Enzymatic and genetic régulation of glycolysis in Lactobacillus delbrueckii subsp. bulgaricus. Lait 78:85–90. https://doi.org/10.1051/LAIT:1998111

318. Chawong K, Rattanaphanee P (2011) n-Butanol as an Extractant for Lactic Acid Recovery. International Journal of Chemical and Molecular Engineering 5:747–750. https://doi.org/10.5281/ZENODO.1333162

319. Granget C, Manikandan NA, Amulya K, et al (2024) Brewer's spent grain as a self-sufficient feedstock for homofermentative production of optically pure L-lactic acid using Lactobacillus rhamnosus. Environ Technol Innov 34:103582. https://doi.org/10.1016/J.ETI.2024.103582

320. Hu Y, Kwan TH, Daoud WA, Lin CSK (2017) Continuous ultrasonic-mediated solvent extraction of lactic acid from fermentation broths. J Clean Prod 145:142–150. https://doi.org/10.1016/J.JCLEPRO.2017.01.055

321. Jaramillo L, Santos D, Borges E, et al (2018) Low-cost effective culture medium optimization for d-lactic acid production by Lactobacillus coryniformis subsp. torquens under oxygen-deprived condition. Ann Microbiol 68:547–555. https://doi.org/10.1007/S13213-018-1362-Y/METRICS

322. Cingadi S, Srikanth K, E.V.R A, Sivaprakasam S (2015) Statistical optimization of cassava fibrous waste hydrolysis by response surface methodology and use of hydrolysate based media for the production of optically pure d-lactic acid. Biochem Eng J 102:82–90. https://doi.org/10.1016/J.BEJ.2015.02.006

323. Pekkoh J, Lomakool S, Chankham J, et al (2024) Maximizing biomass productivity of cyanobacterium Nostoc sp. through high-throughput bioprocess optimization and application in multiproduct biorefinery towards a holistic zero waste. Biomass Convers Biorefin 14:327–347. https://doi.org/10.1007/S13399-021-02285-0/FIGURES/5

Research Outcome

List of Publications

Research outcomes of thesis work

- **Mukherjee, P**., Raj, N. & Sivaprakasam, S. Harnessing valorization potential of whey permeate for D-lactic acid production using lactic acid bacteria. *Biomass Conversion and Biorefinery.* **13**, 15639–15658 (2023). https://doi.org/10.1007/s13399-023-05038-3

- **Mukherjee, P**., Pal, S. & Sivaprakasam, S. Optimization of D-lactic acid biosynthesis from diverse carbon sources in mutant *Lactobacillus delbrueckii* subsp. *bulgaricus* via random mutagenesis. *Syst Microbiol and Biomanuf* (2024). https://doi.org/10.1007/s43393-024-00316-1

- **Mukherjee, P** and Sivaprakasam, S. Engineering the D-lactic acid responsive promoter/repressor system as dynamic metabolic engineering tool in Lactobacillus delbrueckii subsp. bulgaricus for controlled D-lactic acid biosynthesis." *Enzyme and Microbial Technology* (2025): 110606 https://doi.org/10.1016/j.enzmictec.2025.110606

- **Mukherjee, P** and Sivaprakasam, S. "Metabolic engineering of Lactobacillus delbrueckii subsp. bulgaricus VI104 as a D-lactic acid cell factory through strategic pathway optimization for enhanced biosynthesis" (Under review)

Research outcome from collaborative work

- **Mukherjee, P**., Pal, S., Sivaprakasam, S. (2024). Process Parameter Controls for Efficient Enzymatic Hydrolysis of Cellulosic Biomass. In: Bisaria, V. (eds) Handbook of Biorefinery Research and Technology. Springer, Dordrecht. https://doi.org/10.1007/978-94-007-6724-9_77-1

- Gali, K.K., **Mukherjee, P.,** Katiyar, V., Sivaprakasam, S. (2022). Process Efficacy in Cassava-Based Biorefinery: Scalable Process Technology for the Development of Green Monomer D-Lactic Acid. In: Verma, P. (eds) Thermochemical and Catalytic Conversion Technologies for Future Biorefineries. Clean Energy Production Technologies. Springer, Singapore. https://doi.org/10.1007/978-981-19-4316-4_5

- Nirmal Sarkar, **Payal Mukherjee**, Ayigari Ramesh, Senthilkumar Sivaprakasam "Comparative Analysis of Functional Expression and Characterization of D-Lactate Dehydrogenase Homologs in Lactobacillus delbrueckii subsp. bulgaricus for Enhanced D-Lactic Acid Production" (Under preparation)

List of conferences

- Attended 'National Conference on Nucleic acid Science and Technology' (NCNST 2021), CSIR IMMT, Bhubaneswar, India. (As Attendee; Mode-online).

- **Payal Mukherjee**, Senthilkumar Sivaprakasam "Harnessing valorisation potential of whey permeate for enhanced D-lactic acid production: A metabolic engineering and process intensification perspective "International Conference on Biotechnology for Resource Efficiency, Energy, Environment, Chemicals and Health (BREEECH 2021)', Dehradun, India (Hybrid mode-Oral presentation).

- **Payal Mukherjee**, Senthilkumar Sivaprakasam "Construction of galactose metabolism in probiotic *Lactobacillus delbreuckii* subsp. *bulgaricus* for enhanced D-lactic acid production from whey permeate" North-East Research Conclave (NERC), IIT Guwahati, India, May 20-22, 2022 (Poster presentation).

- **Payal Mukherjee**, Senthilkumar Sivaprakasam "Improvement in D-lactic acid production in *Lactobacillus delbreuckii* subsp. *bulgaricus* mutants generated using random mutagenesis approach and optimization of

fermentation conditions "Research and Industrial Conclave (RIC) 2023, Indian Institute of Technology Guwahati, India, May 14-16, 2023. (Poster presentation).

- **Payal Mukherjee**, Senthilkumar Sivaprakasam "Sensor regulator based dynamic control network for engineered *Lactobacillus delbreuckii* subsp.*bulgaricus* for enhanced D-lactic acid (DLA) production" Metabolic engineering conference (ME15), Marina Bay Sands, Singapore, June 11-15, 2023 (Poster presentation).

- **Payal Mukherjee**, Senthilkumar Sivaprakasam "Engineering the D-lactic acid responsive promoter/repressor system as dynamic metabolic engineering tool in *Lactobacillus delbrueckii* subsp.*bulgaricus* for controlled D-lactic acid biosynthesis" Bioprocessing India (BPI) - 2024, IIT Madras, Chennai, India, December 15-17, 2024 (Poster presentation).

Appendix

A. Molecular biology protocols

The molecular biology protocols employed in this research were based on the methods outlined in the 2001 edition of *Molecular Cloning* by Sambrook and Russell. Any modifications to these standard procedures are detailed below.

1. Genomic DNA isolation from Gram positive bacteria

Genomic DNA was extracted from *L.bulgaricus* and *L.helveticus* using the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich) according to the manufacturer's instructions.

2. Amplification of DNA by Polymerase Chain Reaction (PCR)

Q5 high-fidelity polymerase (NEB), Phusion high-fidelity polymerase (NEB) was used to amplify all the DNA fragments. All the amplifications were performed in PCR (M/s Applied Biosystems, Massachusetts, USA) with a reaction volume of 50 µL. The conditions used for performing PCR were as per the manufacturer's instructions.

3. Agarose gel electrophoresis

For analytical separation of DNA fragments, agarose gels consisting of 0.8% (w/v) agarose in 1X TAE buffer were prepared and EtBr was added to at a final concentration, 0.25 µg/ml.. The DNA samples were mixed with 6 x DNA loading dye to assist loading and to indicate the progress of the samples in the gel. MassRuler™ DNA Ladder Mix (Thermo Scientfic; USA) were used as size standards according to the manufacturer's instructions. A voltage of 90 V was applied. The DNA fragments migrate was examined using Gel doc (Bio-Rad; Munich, Germany). Composition of the solutions used for agarose gel electrophoresis are given in Table S1

Table S1: Composition of the solutions used for agarose gel electrophoresis

50 X TAE Buffer	Tris base	24.2g
	Glacial acetic acid	5.71 mL
	0.5M EDTA (pH 8)	10 mL
	MilliQ water	make up to 100ml
6X gel loading dye	Glycerol	1.5 mL (100% glycerol)

	EDTA	1.32 mL of 0.5M EDTA
	Tris	0.198 ml of 1M Tris (pH 8.0)
	SDS	0.102 mL OF 10% SDS
	Bromophenol Blue	0.009 g
	MilliQ water	make up to 10 mL
EtBr Solution (10 mL)	Ethidium bromide	10mg
	MilliQ water	make up to 1 mL

4. Purification of PCR products and plasmid fragments

After PCR or plasmid digestion, The DNA fragment of interest was excised from the gel and purified using the PCR cleanup and gel extraction Kit (Macherey Nagel, Düren, Germany) as per the manufacturer's instructions.

5. Protocol for isothermal assembly

The amplified genes and linearized plasmids were cloned using isothermal assembly. The DNA fragments need to be assembled were designed in such way that they have 15 bp terminal sequence overlaps. This assembly technique uses T5 exonuclease to generate complementary sticky ends, which then annealed and repaired by Taq DNA ligase and DNA polymerase respectively. An assembly master mixture was prepared by combining 32 µl of 5x ISO reaction buffer (1.5 mL. 2 M Tris-HCl pH 7.5, 150 µl 2 M $MgC1_2$, 60 µl of each 100 mM dNTPs, 300 µl IM DTT, 1.5 g PEG-8000, 300 µl 100 mM NAD and sterile water upto 6 mL), 0.64 µl of 10,000 U/mL. T5 exonuclease (NEB), 1.2 µl of 2,000 U/mL. Q5 high-fidelity polymerase (NEB), 0.16 µl of 40,000 U/mL Taq DNA ligase (NEB), and water up to a final volume of 120 µl. 15 µl of this mastermix was aliquoted and stored in -20 °C. Equimolar amounts of each DNA fragments (90 and 45 ng of linearized DNA were used in assembly reactions. 5 µl of DNA need to be assembled was added to 15 µl of assembly master mixture. The reaction mixture was incubated at 50 °C for 60 minutes. Later, 20 µl of reaction mixture was transformed into *E. coli*.

6. Plasmid DNA isolation

High quality plasmid DNA for the transformation of *L.bulgaricus* was prepared from *E. coli* TOP 10 carrying the corresponding plasmid using the mini Prep Kit from QIAgen (QIAgen: Hilden; Germany) according to the manufacturer's instructions. MilliQ water was used for elution of the DNA from the columns.

7. *E. coli* transformation using $CaCl_2$ method

Transformation of *E. coli* was performed by heat shock method according to the Sambrook and Russell's Molecular cloning manual.

Overnight *E.coli* TOP10 culture was used to inoculate 100 mL of low salt LB medium and incubated at 37 °C. When OD_{600} reaches to 0.4, culture was transferred into sterile 50 ml. centrifuge tubes. Culture was kept on ice for 10 min and centrifuged at 4100 rpm for 10 min at 4 °C. Supernatant was decanted completely, and the pellet was resuspended by swirling in 30 ml. ice cold MgCl-CaCl, solution (80 mM MgCl, and 20 mM $CaCl_2$). Cells were recovered by centrifuging at 4100 rpm for 10 min at 4 °C. Supernatant was decanted, and pellet was resuspended by swirling in 2 ml. of ice cold 0.1 M $CaCl_2$ solution for each 50 mL initial culture. Glycerol was added at a final concentration of 15 % (v/v) and mixed smoothly. 150 µl of the the competent cells were transferred into sterile 1.5 mL Eppendorf tubes and preserved at -80 °C freezer for future use.

For transformation, TOP 10 competence cells were taken from -80 °C freezer and placed on ice; allowed to thaw for 15 minutes. Recombinant plasmid DNA was added to cells and incubated in ice for 15 min with intermittent shaking for every 5 minutes. Mixture of competent cells and plasmid DNA is placed at 42 °C for 1 min; immediately cells were then placed back in ice and incubated for 1 minute. 1 mL of sterile LB media was added and incubated at 37 °C for 60 min at 180 rpm. After incubation, cells were centrifuged at 5000 rpm for 10 min at room temperature, resulting pellet was cultured in LB agar media containing 100 µg/mL of Ampicillin.

8. Preparation of cell free extract from recombinant *L. bulgaricus*

The recombinant strains obtained were streaked on LB agar plates containing the appropriate antibiotic. The cultures were centrifuged at 6000 rpm for 5 minutes in Falcon tubes, and the supernatant was discarded. The resulting cell pellet was suspended in 25 mL of lysis buffer containing 500 mM NaCl, 10 mM Imidazole, 5% Glycerol, and 50 mM Tris-HCl (pH 8). Subsequently, 25 µL of lysozyme (100 mg/mL stock) was added, and the mixture was kept on

ice for 20 minutes. Before sonication, the contents were mixed gently, and the resuspended cells were sonicated at 50% amplitude with 10-second on and 30-second off pulses, repeated four times. The sonicated sample was centrifuged at 10,000 rpm for 1 hour at 4°C, and the supernatant was stored at -60°C for further use. Protein expression was analyzed by running the samples on a 12% SDS-PAGE gel.

9. Protein expression analysis using SDS-PAGE

Samples were prepared by mixing 30 µL of protein sample with 10 µL of 4X SDS-sample loading buffer and heating the mixture at 95°C for 5 minutes. The plates were assembled according to the manufacturer's instructions, and the resolving gel (12%) was cast using the following composition:

Table S2: Resolving gel composition

Component	Volume (mL)
Water	1.6
30% Acrylamide	2.0
1.5 M Tris (pH 8)	1.3
10% SDS	0.05
10% APS	0.05
TEMED	0.002
Total	5

The acrylamide solution was poured between the glass plates, leaving enough space for the stacking gel. Once the resolving gel had polymerized, the stacking gel mixture was prepared using the following composition:

Table S3: Stacking gel composition

Component	Volume (mL)
Water	1.4
30% Acrylamide	0.34

1.5 M Tris (pH 8)	0.25
10% SDS	0.02
10% APS	0.02
TEMED	0.002
Total	2

The stacking gel was poured over the polymerized resolving gel, and a comb was inserted. After the stacking gel had solidified, the gel was mounted into the electrophoresis apparatus, and 10X Tris-Glycine electrophoresis buffer was added to the upper and lower reservoirs. Protein samples were prepared by mixing 10 µL of the sample with 7 µL of gel loading dye, followed by heating at 95°C for 5 minutes. After electrophoresis, the gel was carefully removed and stained using Coomassie Brilliant Blue R-250. The staining solution was prepared by dissolving 0.3 g of Coomassie Brilliant Blue R-250 in 200 mL of a 5:4:1 solution of water, methanol, and glacial acetic acid. The gel was immersed in the staining solution and incubated at room temperature for 4 hours. For destaining, the gel was soaked in the same 5:4:1 water, methanol, and glacial acetic acid solution for approximately 6 hours.

B. Sequences used in this study:

Galactokinase (*GalK*)

>CP002081.1:c1643646-1642480 Lactobacillus helveticus CNRZ32, complete genome

ATGAATAAAGAAGAACTACTTAAGAAATATGAAGCAACTTCTAACGAAAAGGCAAAGGATGTATTTTTCT
CACCAGGTCGAATTAATGTAATTGGTGAACATACTGACTACAACGGTGGAAACGTCTTTCCAGCCGCAAT
CAGTTTAGGTGTTTATGGAGTCTATGGTCCAAGAGACGACAAAAAGGTACGCCTTTACTCAGGCAACGTT
GACGGCGATATCGTTGAATTTGACATTGATGACACAAATGTTGAAAAAGATGAAGACCGTTTCTGGGCTA
ACTACTTTAAGGGCATGATCACTTATTTGCGTGAAAAGTATGATGGTATTGATCATGGCTTTAACTTGTA
CATCGAAGCTAACTTGCCATCAGGTTCAGGTCTTTCATCCAGTGCAGCCATTGAAATGCTAATGGGAATC
ATTTTAAAAGATGAATTTAACTTAGATGTTGACCGTGTGAGTTTAGCCAAGATGGGCCAAAGAACTGAAA
ATGAATTTATTGGCTTGAATTCAGGAATTATGGATCAATTTGCCTGCATTATGGGTAAAAAAAATAGTGC
TATTTTCCTAGATTGCAACACCTTAAAGTATGAATACTTACCACTTGCATTAGGTGATTACGAAATCATC
ATCATGGCTACTAATAATCCACATACTTTAGCTGATTCTGCTTACAACAATAGAGTTGCTGAATGTGGTC
GTGCACTTAAGAAGTTGCAACAAAAGCTTGATATTAAGGCATTAGGTGAGCTTGATAACGATACTTTTGA
TGAATATTCTTACCTCATTAATGATGAAACAGAAATCAAGCGTGCTCGTCATGCAGTTAGCGAAAACCAA
AGAACTTTGCGTGCAACTCAAGCTATGAAGGATGGCGATCTTGAAAAGCTTGGTCGTTTGATTAATGCTT
CTCATGAATCTCTTCACTACGATTATGAAGTAACTGGTAAGGAACTTGATACTTTGGCAGAGGCTTCATG
GAAACAACCTGGCGTGCTCGGTGCCAGAATGATCGGCGGCGGTTTCGGTGGTAGTGCTATTGCGATCGTT
AAGAAATCTGAAGCTGAAAACTTTAAGAAGAACGTCGGCAAGATTTATCGTGATGCAGTTGGTTATGATG
CAAGCTTCTATGATGCAGAAATCGTTGACGGTACTAAGAGAATTTAA

galactose-1-phosphate uridylyltransferase (*GalT*)

>CP002081.1:c682636-681833 Lactobacillus helveticus CNRZ32, complete genome
ATGGACAAAAATCCTTTAGTATATAAATATTCAATCGGTAAACGTAAGCCTTATGATTATGATTACGGCA
ATCGCCAGGGTAACCAAAATGCTGGTTGTCCCTTTTGCGATGTCGCACATTTAACTAATATCTATGAAAA
AGATGGGGACAAGATTTGGCTAAAGAACAAATATCCAACTTTGCAGGATACTGACCAAACTATTTTGATT
GAATCAAGCGATCATCAAGGCGATATTTCAACTTATACCCGTGAAGACAATCAAGAATTAATGAAGTTTG
GATTGAAATGTTTTCAAAAAATGAATAATTCAGGACGCTACGAAAGCGTGCTCTGGTATAAAAACTTTGG
TCCTAAATCCGATGGTTCTTTGACGCATCCCCACATGCAGATTGTTGGTCTCTACCATAAAGATGGCTAT
CGCGATATTGGCCTCGACAATTTCAAAGGCTTTGAAGTTGGACGATCAGGATCAGTAGAAATGAATCTTT
CAGCTTATCCAGTTCAAGGCTATCAAGAGGTAAATATTTTAACTCAAGATAATACCAATTTAGAAACGTG
GGCAGATTTAATCCAAAAAGGTGCGCAATATATGCGTTCCGTTTTGTCACACGGTGTGGATTCATACAAC
TTATTCTTTTATCCGATTAATGATTGCAAAGGCACTTGCTGCAAGATTATTCCTCGTTTTTATGCTTCAC
CATATTTTGTTGGCTATAAGATCTCACAAGTCGATGATTCCGATACTCTTAAATGGGAAGCAGAGCGACT
TAAAGGCTTTGTCAATGGCGGTATTTTACATTAA

pyruvate kinase (*pyk*)

>CR954253.1:728462-730231 Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 complete genome
ATGAAAAAAACGAAGATTGTTAGTACTTTAGGGCCAGCTTCAGACGATATTGAAACTATTACCAAGTTAG
CCGAAGCAGGCGCAAACGTATTCCGTTTCAACTTCTCACACGGTAACCACGAAGAACACTTGGCAAGAAT
GAACATGGTTCGTGAAGTTGAAAAGAAGACTGGCAAGCTTTTGGGCATCGCTTTGGACACCAAGGGTGCT
GAAATCAGAACCACTGACCAAGAAGGCGGCAAGTTCACTATCAACACTGGTGACGAAATCCGCGTGTCAA
TGGACGCAACCAAGGCCGGCAACAAGGACATGATCCACGTTACCTACCCAGGTCTGTTCGACGACACTCA
CGTAGGCGGCACTGTATTGATCGACGACGGTGCTGTTGGTTTGACTATCAAGGCCAAGGACGAAGAAAAG
CGCGAATTGGTTTGTGAAGCTCAAAACACTGGTGTCATCGGCTCAAAGAAGGGTGTTAACGCTCCAGGTG
TTGAAATCCGCCTCCCAGGGATTACTGAAAAGGACACTGACGACATCCGCTTTGGTTTGAAGCACGGTAT
TAACTTCATCTTTGCTTCATTTGTACGTAAGGCTCAAGACGTTCTTGACATTCGCGCACTTTGCGAAGAA
GCTAACGCATCATACGTTAAGATCTTCCCAAAGATTGAATCACAAGAAGGTATTGACAACATCGACGAAA
TCTTGCAAGTTTCAGATGGTTTGATGGTTGCCCGTGGTGACATGGGTGTTGAAATCCCATTCATCAACGT
GCCATTTGTTCAAAAGACTTTGATCAAGAAGTGCAACGCTTTGGGCAAGCCAGTTATCACTGCTACTCAA
ATGCTGGACTCAATGCAAGAAAACCCACGTCCAACCCGTGCCGAAGTAACTGACGTTGCTAACGCCGTTC
TTGACGGTACTGACGCAACTATGCTGTCAGGTGAATCAGCAAACGGTTTGTACCCAGTACAATCAGTTCA
AGCTATGCACGACATCGATGTTCGGACTGAAAAGGAATTGGACACCCGGAACACTCTGGCTCTGCAACGC
TTTGAAGAATACAAGGGCTCAAACGTTACTGAAGCTATCGGTGAATCAGTTGTCCGCACTGCTCAAGAAC
TGGGCGTTAAGACTATCATCGCTGCTACTAGCTCCGGCTACACAGCTCGTATGATCTCCAAGTACCGTCC
AGACGCAACCATCGTTGCCTTGACTTTCGACGAAAAGATCCAACACTCATTGGGTATCGTTTGGGGCGTT
GAACCAGTTTTGGCAAAGAAACCTTCAAACACTGACGAAATGTTCGAAGAAGCTGCCCGCGTAGCTAAGG
AACACGGTTTCGTTAAGGATGGCGACCTGGTAATCATCGTTGCCGGCGTACCATTCGGCCAATCAGGTAC
TACTAACTTGATGAAGCTGCAAATCATCGGCAACCAACTTGCTCAAGGTTTGGGCGTAGGCACTGGCTCA
GTTATCGGCAAGGCTGTTGTTGCGAACAGCGCTGAAGAAGCCAACGCTAAGGTTCACGAAGGCGACATCC
TGGTAGCTAAGACTACTGACAAGGACTACATGCCAGCTATCAAGAAGGCCAGCGGTATGATCGTTGAAGC
TTCCGGCTTGACCAGCCACGCAGCTGTTGTCGGCGTATCACTCGGCATTCCAGTTGTTGTCGGTGTTGCT
GACGCAACTTCAAAGATCGCTGACGGCTCAACTTTGACTGTTGACGCACGTCGCGGCGCAATTTACCAAG
GTGAAGTTTCAAACCTGTAA

d-lactate dehydrogenase (Ldb0101)

>CR954253.1:c82171-81170 Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 complete genome
ATGACTAAAATTTTTGCTTACGCAATTCGTGAAGATGAAAAGCCATTCTTGAAGGAATGGGAAGACGCTC
ACAAGGACGTCGAAGTTGAATACACTGACAAGCTTTTGACCCCAGAAACTGTTGCTTTGGCAAAGGGTGC
TGACGGTGTTGTTGTTTACCAACAACTTGACTACACCGCTGAAACTCTGCAAGCTTTGGCAGACAACGGC
ATCACTAAGATGAGCCTGCGTAACGTTGGTGTTGACAACATCGACATGGCTAAGGCTAAGGAACTTGGCT
TCCAAATCACCAACGTTCCAGTTTACTCACCAAACGCCATCGCAGAACACGCTGCTATCCAAGCTGCCCG

CATCCTGCGTCAAGACAAGGCTATGGACGAAAAGGTTGCCCGTCACGACTTGCGTTGGGCACCAACTATC
GGCCGTGAAGTTCGCGACCAAGTTGTTGGTGTTATAGGTACTGGCCACATCGGTCAAGTCTTCATGCAAA
TCATGGAAGGCTTCGGCGCTAAGGTTATCGCTTACGACATCTTCCGCAACCCAGAATTGGAAAAGAAGGG
CTACTACGTAGACTCACTTGACGACCTGTACAAGCAAGCTGACGTTATTTCCCTGCACGTTCCTGACGTT
CCAGCTAACGTTCACATGATCAACGACGAGTCAATCGCTAAAATGAAGCAAGACGTAGTTATCGTTAACG
TATCACGTGGTCCATTGGTTGACACTGACGCGGTTATCCGTGGTTTGGACTCAGGCAAGATCTTCGGTTA
CGCAATGGACGTTTACGAAGGTGAAGTTGGCATCTTCAACGAAGACTGGGAAGGCAAGGAATTCCCAGAC
GCACGTTTAGCTGACTTAATCGCTCGTCCAAACGTTCTGGTAACTCCACACACTGCTTTCTACACTACTC
ACGCTGTTCGCAACATGGTAGTTAAGGCCTTCGACAACAACCTTGAATTGGTTGAAGGCAAGGAAGCTGA
AACTCCAGTTAAGGTTGGCTAA

Ldb1010 (*dldh* homolog)

>CR954253.1:c869798-868797 Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 complete genome

ATGACTAAAATTGCCATGTATAATGTCAGCCCGATCGAAGTGCCTTACATTGAAGACTGGGCTAAGAAAA
ACGATGTCGAAATTAAGACGACCGACCAGGCCTTGACCAGCGCGACTGTAGATTTAGCTGAAGGCTGCAG
CAGCGTTTCCCTTAAGCCACTTGGCCCGGTTGACGAAGAAGTTGTTTACCAAAAATTGAGCGAATACGGG
GTCAAGTGCATCGGCCTCAGAATCGTTGGCTTCAACACCATCAACTTCGACTGGACCAAGAAGTACAACT
TGCTGGTCACCAACGTTCCGGTCTACTCCCCGCGGGCGATCGCTGAAATGACGGTTACCCAGGCCATGTA
CCTCCTGCGCAAGATCGGGGAATTCCGCTACCGTATGGACCATGACCATGACTTTACTTGGCCAAGTAAC
TTGATCAGCAATGAAATCTACAACCTGACTGTCGGCTTGATCGGGGTCGGCCACATCGGCAGCGCCGTGG
CAGAAATCTTCTCAGCCATGGGGGCCAAGGTCATCGCCTATGACGTGGCTTACAACCCGGAATTTGAACC
ATTTTTGACCTACACCGACTTTGACACGGTCTTGAAAGAAGCTGACATCGTCTCCCTCCACACTCCCCTC
TTCCCATCAACGGAAAACATGATCGGGGAAAAGCAGCTGAAGGAAATGAAGAAGTCTGCCTACTTGATCA
ACTGTGCCCGGGGCGAATTAGTCGACACTGGAGCCTTGATCAAGGCCTTGCAAGATGGCGAAATCGCCGG
TGCCGGCCTGGACACTTTGGCTGGGGAATCCAGCTACTTTGGCCACACTGGCCTGACGGACAGCGAAATC
CCAGAAGACTACAAGACCCTGGCCAAGATGCCAAACGTTGTCATTACTCCGCACTCAGCCTTCTACACGG
AAACTTCCATCCGCAACATGGTGCAGATCTGCTTGACTGACCAATTGACCATCGCCAAGGGCGGCCGGCC
AAGAAGCATCGTTAACCTGTAA

6-phosphofructokinase (*pfk*)

>CR954253.1:727463-728422 Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 complete genome

ATGAAACGGATTGGTATTTTGACTAGCGGCGGTGACGCCCCTGGTATGAATGCAGCCGTAAGAGCCGTAA
CCAGAGTCGCGATCGCGAACGGTTTGGAAGTTTTCGGTATCAGATACGGTTTTGCAGGTTTGGTTGCGGG
CGACATTTTCCCATTGGAAAGTGAAGACGTAGCCCACTTGATCAATGTTTCCGGTACCTTCCTCTACTCT
GCGCGTTATCCTGAATTTGCAGAAGAAGAAGGACAGCTGGCTGGTATTGAGCAATTGAAGAAGCACGGCA
TCGATGCTGTTGTCGTTATTGGTGGGGATGGTTCCTACCATGGCGCCCTGCAGCTGACCCGCCACGGCTT
CAACTCAATTGGCCTGCCAGGCACGATCGACAACGATATCCCTTACACGGATGCCACGATCGGCTATGAC
ACGGCCTGCATGACGGCAATGGACGCGATCGACAAGATCCGTGACACTGCTTCTAGCCACCACCGCGTCT
TCATTGTCAACGTAATGGGCCGCAACTGTGGTGACATCGCTATGCGCGTCGGCGTAGCCTGCGGGGCGGA
CGCGATCGTCATTCCTGAAAGACCATATGATGTCGAAGAAATCGCCAACCGGCTCAAGCAAGCCCAGGAA
AGCGGCAAGGACCACGGTTTGGTAGTTGTTGCTGAAGGCGTAATGACCGCTGACCAATTCATGGCTGAAC
TGAAGAAGTATGGTGACTTCGACGTCCGGGCCAATGTTTTGGGTCACATGCAGCGGGGCGGCACGCCAAC
TGTTTCTGACCGGGTGCTGGCTTCCAAGCTGGGCAGCGAAGCCGTTCACCTTCTTTTGGAAGGCAAGGGC
GGCCTGGCTGTCGGCATTGAAAACGGCAAGGTTACTTCACACGACATCCTTGATTTATTTGATGAGTCTC
ACAGGGGTGACTATGACTTGTTAAAGCTCAACGCAGATTTATCAAGATAG

Phosphoglycerate kinase (*pgk*)

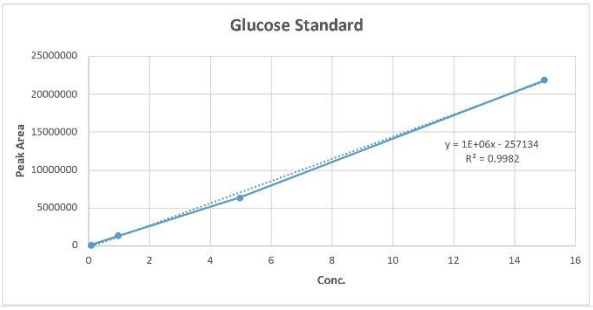
>CR954253.1:541728-542939 Lactobacillus delbrueckii subsp. bulgaricus ATCC 11842 complete genome

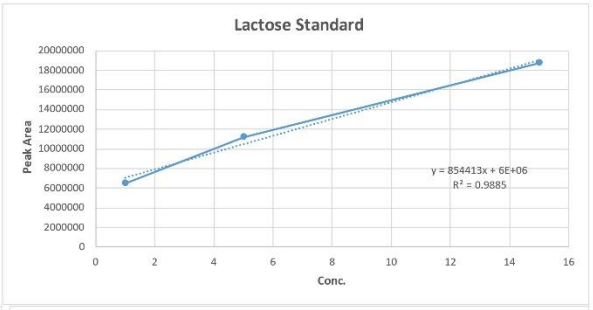
ATGGCTAAATTGATTGTTTCTGACGTAGACGTTAAGGACAAGAAGGTTTTGGTTCGCGTTGACTTCAACG

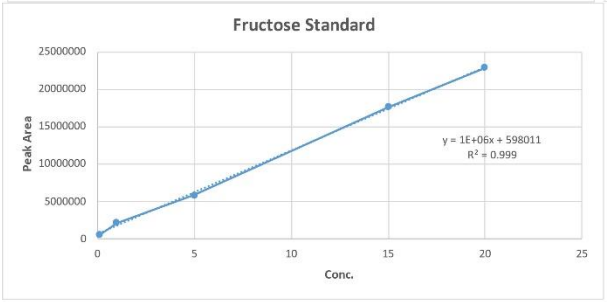
TGCCGATTAAGGACGGCGTTATCGGCGACGACAACCGTATCGTGGCTGCTTTGCCAACTATCAAGTACAT
CATCGAAAACGGCGGCAAGGCTATCTTGCTTTCCCACCTTGGCCGGATCAAGAGCGATGAAGACAAGAAG
AGCTTGAGCTTGGCTCCAGTTGCCAAACGTTTGGGCGAATTGCTTGAAAAGCCTGTAACTTTCGTACCTT
CAAACGAAGGCAAGGAAGTTGAAGACGCCATCAACAACATGAAGGACGGCGACGTAGTTGTTTTGGAAAA
CACCCGTTTCCAAGACATCGACAACGACTTCGGCAAGCGTGAATCAAAGAACGACCCTAAGCTGGGCGAA
TACTGGGCATCATTGGGTGACGTTTTCGTAAACGACGCCTTCGGTACTGCTCACAGAAGCCACGCTTCAA
ACGTTGGTATCGCTACGGCCATGAAGGCTGCCGGCAAGCCAGCAGCTGCTGGTTTCCTGCTTGAAAAGGA
AATCAAGTTCCTGGGCAACGCTGTTGCTAACCCAGTTCACCCATTCGTAACTATTCTTGGCGGGGCTAAG
GTTTCTGACAAGATCGGCGTTATCACCAACTTGATTCCAAAGGCTGACCACATCATCATCGGTGGTGGTA
TGGCTTACACCTTCCTTAAGGCTCAAGGCCACAATATCGGCAAGTCCCTGGTTGAAGACGACAAGGTTGA
ATTTGCCAAGGAATTGTTGGAAAAGGCTGGCGACAAGCTGGTTCTGCCAATCGACAACGTAGCCGCAACT
GAATTCAACAACGATGCTGCTTCAGAAGTTGTTGGCCAAGACATCCCAGACAACGAAATGGGCTTGGACA
TCGGTCCTAAGACTATTGAACTCTTCAAGAAGACTCTTGAAGGTGCCAAGACTGTTGTTTGGAACGGGCC
AATGGGCGTCTTCGAAATGCCAAACTTCGCCAAGGGTACTTTGGAAGTTGGCCGTGCTTTGGCTGACTTG
CCAGACGCTACTACTATTGTCGGCGGTGGTGACTCAACTGCTGCTGCTAAGCAATTGGGGATCGCTCCTA
AGTTGACTCACATCTCAACTGGTGGTGGTGCATCTCTTGAATACCTTGAAGGTAAGGAATTGCCAGGTAT
CGCTTGCGTTTCAGACAAGTAG

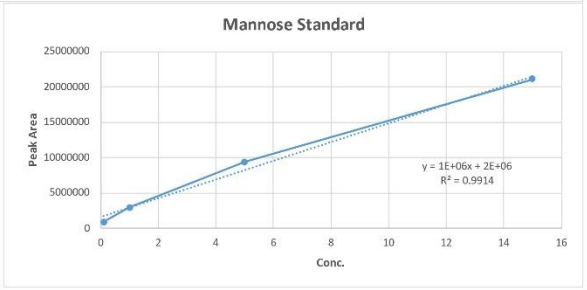
C. HPLC Standards:

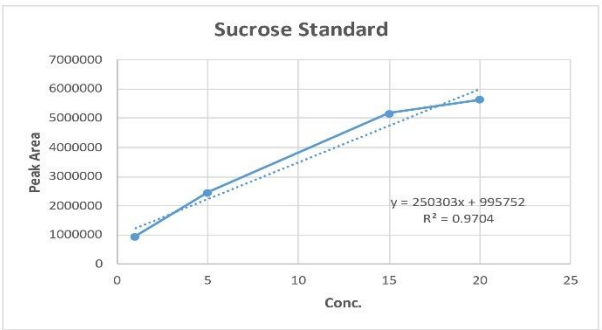
HPLC standards for sugars detected in RID-detector.

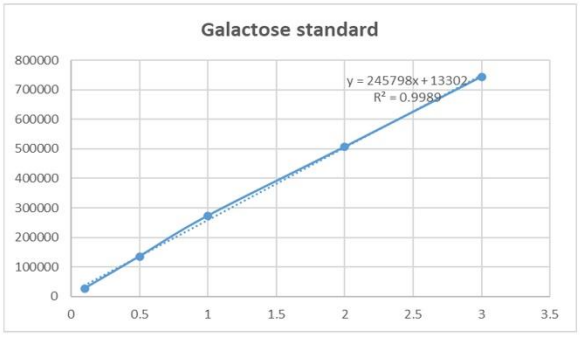












HPLC standards for organic acids detected in UV-detector (210nm)