Effect of Substrate Concentration & Elevated CO_2 Partial Pressure on the Odd & Even Carboxylate Formation

A thesis submitted to Delft University of Technology in partial fulfilment of the requirements for the degree of

Master of Science in Civil Engineering

(Environmental Engineering)

By

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Date: 30th June 2023

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"When a man takes an oath, he's holding his own self in his own hands. Like water (he cups his hands) and if he opens his fingers then, he needn't hope to find himself again. Some men aren't capable of this, but I'd be loathe to think of me as one of them."

Sir Thomas More, Robert Bolt, 'A Man for All Seasons'

"The only thing necessary for the triumph of evil is for good men to do nothing."

John Stuart Mill

Acknowledgement

If I have seen further, it is by standing upon the shoulders of giants [1]. As such it behooves me to express my sincere gratitude to the giants who have helped me look past the foggy mountains of pseudoscience so that I may march towards the lush meadows of logic and critical thinking. First and foremost, I would like to thank Dr. Robert Hoelzle who has been an embodiment of everything a good scientist should aspire to be. You have taught me everything I know about mixed culture fermentation and in the three years since I first read your PhD dissertation it continues to reign as arguably the best literature in our field. It goes without saying that my thesis in its current form would not have existed if it wasn't for the time you made to help and support me as I made my way through the maze of academia. Secondly, I must express my sincere appreciation to Dr. Robert Robergs and his colleagues for inspiring me with their work and coming to my aide when I requested assistance during my scientific journey. I would also like to welcome this opportunity to thank Prof. dr. Douglas Montgomery for his contagious passion for design of experiments and education. I dearly cherish our "fireside chats" and the advice you gave me on how to approach research in a way guided by logic and statistical reasoning rather than "feelings".

There were a multitude of other individuals who provided valuable assistance and insight into chromatographic methods which were instrumental in shaping the chromatographic methods I adopted in my research. Therefore, I must thank these strangers on the internet for their patience and support which helped me develop my chromatographic skills. I would also like to express my gratitude to Prof. dr. Mark van Loosdrecht, Prof. dr. David Weissbrodt and Dr. ir. Michele Laureni for their academic supervision and the personal support they have provided throughout the duration of my MSc. thesis. Additionally, my acknowledgement would not be complete without thanking Dr. S. V. Mohan and his team in IICT-Hyderabad for their continuous support and for giving me the opportunity to conduct my experiments in their laboratory.

I would be remiss in my duties if I didn't acknowledge the unfailing support and unconditional love my parents have provided throughout my journey. From the day I first started walking to this very moment you thought me to get up every time fell down. Through the brightest days and the blackest nights, you believed in me even when I stopped believing in myself. There are no words to express my unending gratitude for the constant source of encouragement you have been.

Last but certainly not the least, I would like thank Sal Khan and the entire Khanacademy team for their selfless commitment to the cause of education for everyone. In the fifteen years since I first came across your work, Khanacademy continues to shape my life in best way possible. Through your work you have taught me to prioritize mastery instead of memorization. Through your work you gave me the courage to pursue science when everyone was telling them not to. Through your work you taught me to love learning when my disabilities were telling me not to. It is because of your work that I can proudly say that I have earned this MSc. degree. It is because of your work and sacrifices that I've become the person I am today. Because of you, Sal [2].

This MSc. thesis is dedicated to Daisy (good girl) Chowdhary

Abstract

Of all the greenhouse gases (GHGs), carbon dioxide (CO_2) has been the target of most climate recovery efforts as it is the most abundantly emitted GHG by mass. In fact, in 2015 a legally binding international treaty was adopted by 196 parties in Paris, France to constrain the anthropogenic warming to 1.5-2.0˚C above the pre-industrial level. In order to meet this goal, a carbon budget was formulated as an estimate of the amount of carbon that can be emitted while limiting the anthropogenic warming to prescribed levels. However, the global CO_2 emissions from industries are rapidly depleting this budget. Therefore, to mitigate the effects of climate change, CO_2 emissions must be reduced by employing alternative commodities that can replace petrochemical resources. In this context, mixed culture fermentation presents an opportunity for redefining CO_2 and waste streams as raw material for production of commodities traditionally derived from petrochemical resources. Previous studies by on this topic have indicated a potential association between elevated CO_2 levels (pCO_2) and butyrate formation from mixed culture fermentation. However, the cellular mechanism underlying this association are still poorly understood. Therefore, the principal objective of this research was to investigate the effects of initial substrate concentrations (g/L) and elevated pCO_2 (bar) conditions on selectivity (mol_i/mol_{total}) of biomolecules produced from anaerobic conversion of glucose. For this purpose, a between-subject mixed factorial experimental design was developed to gauge the main and interaction effects of initial substrate concentrations (g/L) and elevated pCO_2 (bar) conditions on selectivity of biomolecules. The principal findings of this research indicate that a strong positive relationship exists between the pCO_2 and butyrate formation as the application of CO_2 in reactor (EPBs) headspace resulted in higher butyrate selectivity compared to the control reactors (APBs). However, contrary to the conclusions reached by previous studies it was found that increasing the initial substrate concentration steered the product formation towards lactate and not butyrate. Whereas the highest recorded butyrate selectivity for EPBs was 30.41% for experimental condition with 5 g/L substrate concentration and 4 bar pCO_2, the highest recorded butyrate selectivity for APBs was only 11.72% for 10 g/L substrate concentration and atmospheric pressure conditions. Conversely, the highest recorded lactate selectivity for EPBs was 15.13% for 20 g/L substrate and 3 bar pCO_2 while the highest recorded lactate selectivity for APBs was 47.95% for 25 g/L substrate concentration and atmospheric pressure conditions. As a result of these investigations, theories concerning formation of butyrate and lactate were proffered in context of the role of CO_2 in mixed culture fermentation. By confronting the existing understanding regarding product formation with new evidence this investigation seeks to advance theories concerning mixed culture fermentation.

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Abbreviations & Acronyms

GHG	Greenhouse Gases
pCO_2	Partial pressure of CO_2
pH_2	Partial Pressure of molecular hydrogen (H_2)
AD	Anaerobic Digestion
MCF	Mixed culture fermentation
ECC	Even-carbon carboxylates
OCC	Odd-carbon carboxylates
APB	Atmospheric Pressure Bioreactor
EPB	Elevated Pressure Bioreactor
GC	Gas Chromatography
HPLC	High-pressure liquid chromatography
COD	Chemical oxygen demand
sCOD	Soluble chemical oxygen demand
tCOD	Total chemical oxygen demand
VFA	Volatile fatty acids
tVFA	Total volatile fatty acids

1. Introduction

1.1 Background Knowledge

The anthropogenic driven increase in concentrations of greenhouse gases (GHG) in the atmosphere is a growing concern worldwide due to their association with climate change [3-12]. This accrual of GHGs in atmosphere results in absorption and/or redirection of the outgoing infrared radiations back towards the climate change [13]. There is a growing body of literature which suggests that climate change is intricately linked to the extreme weather events, food insecurity and economic disruption which may trigger mass human migration and geopolitical conflicts [14]. Therefore, there is an increasing interest in mitigating GHG emissions as a part of climate recovery efforts.

Of all the GHGs, CO_2 has been the target of most climate recovery efforts as it is the most abundantly emitted GHG by mass [10]. In fact, in 2015 a legally binding international treaty was adopted by 196 parties in Paris, France to constrain the anthropogenic warming to 1.5-2.0˚C above the pre-industrial level [12] . In order to meet this goal, a carbon budget was formulated as an estimate of the amount of carbon that can be emitted while limiting the anthropogenic warming to prescribed levels [11]. According to the IPCC report for 2021, the remaining carbon budget from 2020 is estimated to be 400 $GtCO_2$ and 1150 $GtCO_2$ respectively [11]. However, the global CO_2 emissions from industries are rapidly depleting this budget [11]. Therefore, to mitigate the effects of climate change, carbon dioxide emissions must be reduced by employing alternative resources that can replace petrochemical resources.

Amongst the industries most reliant upon fossil fuels is the chemical industry which consumes fossil fuels for production of various chemicals. Thus, in order to mitigate the effects of climate change and reduce CO_2 emissions, an alternative to petrochemical resources must be employed in the chemical industry. The development of circular bioeconomy in response to the overreliance on petrochemical resources provides a conceptual framework for transitioning from the current linear economy based on petrochemical resources to a circular economy based on renewable natural capital [3, 5, 7, 8]. A successful transition to such circular bioeconomy requires suitable renewable natural capital which can sustain current economic demands and support future growth of industries currently dependent on petrochemical feedstocks [3, 5, 7]. In this context, biorefineries present an opportunity for redefining waste streams as raw material for production of commodities traditionally derived from petrochemical resources [3, 5, 7, 8].

An emerging part of biorefineries is the biomolecule conversion technologies where the feedstocks are microbiologically converted into products [6, 8]. Conventionally,

the production of these biomolecules is undertaken using pure cultures, however this type of production is limited by the operational costs of sterilization [16-18]. In contrast to the production of biomolecules using pure culture, the use of undefined mixed or open cultures to ferment the heterogeneous organic feedstock circumvents the need for expensive sterilization while allowing for robustness and flexibility [16-18]. However, the formation of undesirable byproducts due to the competing microbial and metabolic pathways involved in the fermentation of organic feedstock leads poses challenges in downstream processing, thus limiting the industrial applicability of mixed culture fermentation [19]. Hence, to make mixed culture fermentation (MCF) market-competitive it is imperative to understand the process conditions and their influence on the product spectrum at the biomechanistic level to effectively steer end-product formation towards the production of specific biomolecules. In this context, elevated CO_2 partial pressure (pCO_2) has been suggested to be instrumental in modulating fermentative pathways towards selective biomolecules [19].

1.2 Research Scope

Previous research on the effects of pCO_2 were mostly conducted in context of biogas production rather than biomolecule formation. Evidence from such investigations has established that elevated pCO_2 promotes carboxylate accumulation in AD [20, 21]. These observations have been attributed to the inhibitory effects of pCO_2 on acetogens and methanogens which oxidize the carboxylates to produce biogas. Interestingly, investigations on ethanol conversion have reported that increased CO_2 loading rates significantly stimulated its oxidation compared to control conditions [22]. However, these observed effects of pCO_2 on alcohol oxidation have yet to be confirmed in replication studies.

In contrast to its effect on biomolecule oxidation, remarkably few studies have explored the effects of pCO_2 on biomolecule formation. Perhaps the best-known investigations on this field were conducted by Arslan et. al who fermented different waste streams under 2 bar pCO_2 and after one week observed that the carbohydrate-rich streams of up to 13.5 gCOD/L concentrations had higher butyrate selectivity and lower total carboxylate concentrations than control conditions [19, 23, 24]. Additionally, the degree of hydrolysis for carbohydrate-rich waste stream under 2 bar pCO_2 was also found to be lower than control condition after the first week which might explain the why the total carboxylate concentration was low [19, 23, 24]. Interestingly, while the degree of hydrolysis was similar for all cases after four weeks, the carbohydrate-rich waste stream under 2 bar pCO_2 had higher total carboxylate concentrations compared to control [19, 23, 24]. The authors also remarked that the control conditions had higher butyrate selectivity than 2 bar pCO_2 for carbohydrate-rich streams of 23 gCOD/L concentration. However, the results should be interpreted with caution as the true effect of elevated pCO_2 conditions on

biomolecule formation may have been masked by its inhibitory effect on hydrolysis. Furthermore, these investigations also suffer from poor internal validity as the batch of carbohydrate-rich waste up to 13.5 gCOD/L had different compositions than the batch used for experimental conditions with 23 gCOD/L concentration [23, 24].

In this research, the effect of elevated pCO_2 was investigated using synthetic substrates to provide consistency in all tests. Additionally, the choice of synthetic substrate was restricted to monosaccharides to isolate the effect of elevated pCO_2 on biomolecule formation from its effect on hydrolysis.

1.3 Research Objective & Question

1.3.1 Research Objective

The main objective of this research was to investigate the effect of initial substrate concentrations (g/L) and elevated pCO_2 conditions on the product spectrum of glucose fermentation under mesophilic conditions. The sub-objective of this research was to reevaluate the findings of Arslan et al. by replicating their studies across experimental conditions differing from those utilized in the original studies.

1.3.2 Research Question

What is the effect of initial substrate concentrations (g/L) and elevated pCO_2 (bar) conditions on selectivity (mol_i/mol_{total}) of biomolecules produced from anaerobic conversion of glucose?

> H_1 (1.3.2-a): With an increase in initial substrate concentration for fixed elevated pCO_2 conditions, the selectivity of odd-carbon carboxylate will increase while the selectivity of even-carbon carboxylates will decrease.
>
> H_1 (1.3.2-b): With increase in pCO_2 levels for fixed initial substrate concentrations, the selectivity of even-carbon carboxylate will increase while the selectivity of odd-carbon carboxylates will decrease.

1.4 Thesis Outline

Chapter 2 provides literature review for mixed culture fermentation and the effects of key process parameters on the product spectrum of glucose fermentation. Chapter 3 describes the methodology applied in this experimental research. In Chapter 4, the results obtained from the experiments conducted for this thesis would be presented and discussed. Finally, the conclusions reached through the analysis of results would be presented along with recommendations for supplementary research.

2. Literature Review

2.1 Anaerobic Digestion

Anaerobic digestion (AD) is the multistage conversion of organic matter to biogas mediated by consortia of phylogenetically diverse microorganisms which can be functionally categorized into fermentative, acetogenic and methanogenic microorganisms [25]. The first stage of the AD process is defined by enzymatic conversions of complex organic polymers to smaller soluble molecules such as simple saccharides, amino acids, and long-chain carboxylates (LCC) - this stage is collectively called hydrolysis. Subsequently, during the fermentation stage, the smaller dissolved molecules are degraded into a variety of by-products such as organic acids, solvents, and gases, which are further degraded into acetate, carbon dioxide (CO_2) and hydrogen gas (H_2) by acetogens [16, 17, 26, 27]. In the final stage of the AD process, acetate, CO_2, and H_2 are consumed by the acetolactic and hydrogenotrophic methanogens to produce biogas [25, 26, 28].

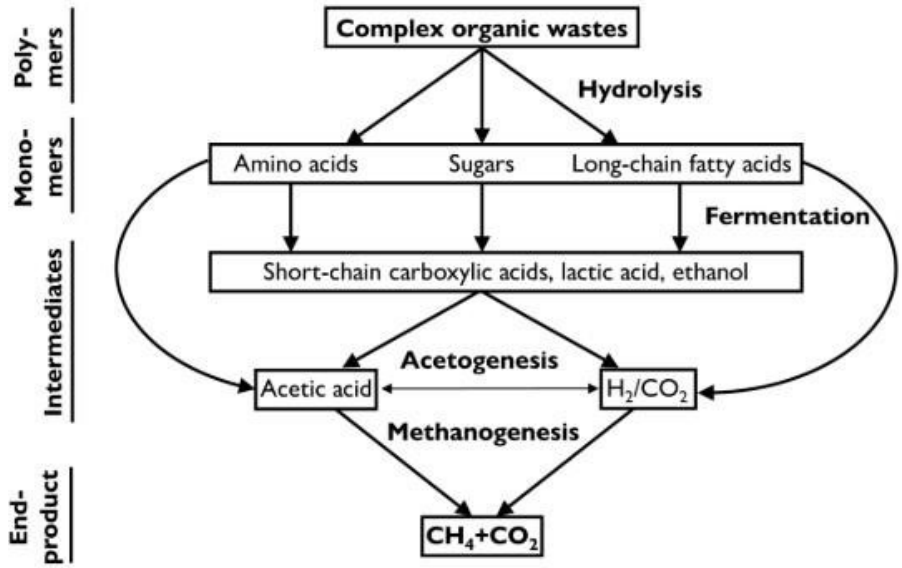

Figure 1: Stages of Anaerobic Digestion [29]

Conventionally, AD process technology has been implemented for energy recovery through the biogas harnessed during the treatment of municipal and industrial waste streams [16]. Such implementation relies upon mixed culture fermentation (MCF) for mineralization of the organic carbon content in waste or residual streams into methane (CH_4), thus providing the opportunity to close the carbon cycle. However, AD has increasingly garnered interest as a potential medium for carbon recovery due to the prospect of biological production of alternative higher-value

compounds such as short-chain carboxylates (SCC), alcohols, and H_2 in non-sterile conditions during the intermediate stages of AD [16-18]. In the context of carbon recovery, the interest in AD is a function of the robustness and diversity of mixed culture microbiome which allows the AD process to convert a broad range of feedstocks into biochemical products of higher value. Nevertheless, a common challenge in pursuit of biochemical production is the formation of undesirable byproducts due to the competing microbial and metabolic pathways involved in MCF of organic feedstock which limit the industrial applicability of MCF. Thus, to make MCF market-competitive it is imperative to understand the fermentation process mechanism at pathway level in order to efficiently steer the end-product formation towards the production of specific biochemicals.

2.2 Mixed Culture Fermentation

Microbial production of biochemicals is primarily the end-result of fermentation. In general, fermentation refers to metabolism of organic feedstock in absence of external electron acceptors such as O_2, SO_4^{3-}, or NO_3^- [30]. In absence of external electron acceptors, these redox reactions are mediated by electron carriers such as nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD), and ferredoxin (Fd) which transfer electrons between electron donors (ED) and electron acceptors (EA) through a process called substrate-coupled electron transfer [30]. Whereas the reduction of EAs serves as the primary electron sink, the oxidation of EDs in this process releases the cellular potential energy required to drive the synthesis of adenosine triphosphate (ATP) from adenosine diphosphate (ADP) and inorganic phosphate (P_i) [30]. Such formation of ATP through the transfer of phosphoryl group from a substrate is called substrate-level phosphorylation (SLP) [30].

For mixed culture fermentation systems, the catabolism of carbohydrates may proceed through one of the following three metabolic pathways: (1) Embden-Meyerhof Pathway (EMP); (2) the Entner-Doudoroff pathway (EDP) and, (3) the phosphoketolase pathway (PKP) [31]. Regardless of the metabolic pathway used, the catabolism of most saccharides eventually converges at the glyceraldehyde-3-phosphate node before being oxidized to pyruvate. For glucose as a carbon source, these pathways can be summarized as follows:

$$\text{EMP: Glucose} + 2\cdot NAD^+ + 2\cdot ADP + 2P_i \rightarrow 2\cdot \text{Pyruvate} + 2\cdot NADH + 2\cdot H^+ + 2\cdot ATP + 2\cdot H_2O \quad (1)$$

$$\text{EDP: Glucose} + NAD^+ + NADP^+ + ADP + P_i \rightarrow 2\cdot \text{Pyruvate} + NADH + NADPH + ATP \quad (2)$$

$$\text{PKP: Glucose} + 2\cdot NAD^+ + ADP + P_i \rightarrow \text{Pyruvate} + \text{Acetyl} - \text{Pi} + CO_2 + 2\cdot NADH + ATP \quad (3)$$

In this study, it was assumed that the metabolism of glucose proceeds through the EMP pathway and as such the literature review and discussions would be focused on the associated pathways.

2.2.1 Glycolysis

The EMP begins with glycolysis which refers to a series of enzyme catalyzed reactions in which a glucose molecule is converted to two molecules of pyruvate [32]. During these sequential reactions, part of the free energy released from glucose is conserved in the form of ATP and NADH [32]. The glycolytic breakdown of organic feedstock is the sole source of metabolic energy for some cell types [32]. This breakdown of glucose into two molecules of pyruvate occurs in two phases: (1) the preparatory phase, and (2) the payoff phase [32].

2.2.1.1 Preparatory Phase

The preparatory phase of glycolysis constitutes the first five reactions in which glucose is converted to glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) [32]. This phase begins with an irreversible reaction catalyzed by hexokinase in which glucose is activated through phosphorylation of its C-6 carbon to yield glucose-6-phosphate (G6P) using ATP as the phosphoryl donor [32]. This reaction causes a covalent release of H^+ [32-34]. In the second step, the G6P is reversibly isomerized to fructose-6-phosphate (F6P) which is subsequently phosphorylated at C-1 to yield D-fructose 1,6-biphosphate (F1,6P) in an essentially irreversible priming reaction catalyzed by phosphofructosekinase-1 (PFK-1) [32]. This reaction is heavily ~H^+ releasing at pH 7.0 and involves transfer of α phosphate from ATP to F6P [33, 34]. The ~H^+ exchange associated with this reaction is progressively altered towards zero as pH approaches 6.0 [33, 34]. In the penultimate reaction of preparatory phase, F1,6P is cleaved into two different triose phosphates, glyceraldehyde-3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), in a reaction with strongly positive standard free-energy change in direction of F1,6P and minimal H^+ exchange [33, 34]. Finally, the fifth reaction of glycolysis involves interconversion of trios phosphate as only G3P can be directly degraded in the payoff phase of glycolysis [32]. Thus, in this reaction catalyzed by trios phosphate isomerase, DHAP is rapidly and reversibly converted to G3P with minimal ~H^+ exchange [33, 34].

2.2.1.2 Payoff Phase

During glycolysis, the gain in energy comes in the payoff phase formation of four ATPs from ADPs [32]. Additionally, in this phase the energy released from reactions is conserved through the formation of two NADH molecules per molecule of glucose [32]. In the first reaction of the payoff phase, G3P is oxidized and phosphorylated by NAD^+ and inorganic phosphate (P_i) respectively to yield 1,3-biphosphoglycerate

(1,3BPG) [32]. During this reaction, it is the aldehyde group of G3P which is oxidized to form a carboxylate anhydride with phosphoric acid and the NAD^+ is reduced by one electron and one proton while covalently releasing one H^+ [32]. For this reaction, increase in cellular acidosis causes a change from HPO_4^{-2} to $H_2PO_4^{-1}$ due to increased $\sim H^+$ association to P_i which results in $\sim 2H^+$ release for an increased proportion of 1,3BPG as the pH falls [33]. The 1,3BPG thus formed is then used as a substrate in a reversible yet endergonic reaction catalyzed by phosphoglycerate kinase where the high-energy phosphoryl group from carboxyl group of 1,3BPG is transferred to ADP to form ATP and 3-phosphoglycerate (3PG) [32]. The $\sim H^+$ exchange for this reaction is remarkably negative due to the greater H^+ association of 1,3BPG and ADP compared to the products resulting in a net $\sim H^+$ release [33, 34]. The first two reactions of the payoff phase together constitute an energy coupling reaction yield an overall exergonic reaction with 1,3BPG as the common intermediate [[32]]. The third reaction of the payoff phase involves simple phosphoryl group transfer between the C-2 and C-3 of glycerate to yield 2-Phosphoglycerate (2PG) with minimal $\sim H^+$ exchange [33, 34]. In the penultimate reaction of the payoff phase, enolase promotes reversible dehydration of 2PG to remove a water molecule and yield phosphoenolpyruvate [33, 34]. Finally, in the last reaction of the payoff phase and glycolysis, the phosphoryl group of PEP is transferred to ADP via pyruvate kinase catalysis to yield pyruvate in its enol form which tautomerizes rapidly and non-enzymatically to its keto form to conclude glycolysis [32].

2.2.2 Pyruvate Oxidation

The cytosolic formation of pyruvate from glycolysis represents a key node in the metabolism of organic feedstock. The pyruvate thus formed may either be reduced to ethanol or lactate to regenerate NAD^+ for continuing ATP production by glycolysis or it may be oxidized to CO_2 and acetyl-CoA [30]. Pyruvate oxidation to acetyl-CoA may be induced by one of the following three enzymes: (1) pyruvate dehydrogenase complex (Pdh); (2) pyruvate-formate lyase (Pfl), and (3) Pyruvate-Fd Oxidoreductase (Pfo) [30].

Pdh is a highly ordered multienzyme complex located in the cytosol of bacteria in which a series of biochemical intermediates remain bound to enzyme molecules as the pyruvate is converted to acetyl-CoA [32]. The overall reaction catalyzed by Pdh is an irreversible oxidative decarboxylation reaction in which the carboxyl group of pyruvate is released as a CO_2 molecule and a bound FAD is reduced [30, 32]. These reactions begin with the rate limiting reaction in which the CO_2 from C-1 of pyruvate is liberated while the rest of the molecule is attached to thiamine pyrophosphate (TPP) as hydroxyethyl group [32]. The hydroxyethyl group is then oxidized to the level of acetate while two electron are removed to reduce the lipoyl group to two thiol (-SH) groups [32]. The acetyl moiety thus produced is first esterified to one of

the lipoyl -SH group and subsequently transesterified to CoA to form acetyl-CoA [32]. Finally, in the remaining reactions catalyzed by Pdh, electrons are transferred to regenerate the oxidized form of the lipoyl group to prepare the complex for the next round of oxidation [32].

$$2 \cdot H_2 + CO_2 \rightarrow CH_2O_2 \quad (4)$$

In contrast to the Pdh, the Pfl generates formate instead of CO_2 and H_2 by oxidizing pyruvate through direct replacement of its carboxyl group with -CoA [30]. The formate thus produced can be oxidized to CO_2 at low pH by reduction of NAD^+ through the enzyme formate dehydrogenase (Fdh) [30]. However, the generation of NADH through this way is associated with formate oxidation instead of pyruvate oxidation [30]. Pfo on the other hand works similarly to Pdh by producing CO_2 but reducing Fd instead of FAD [30].

2.2.3 Acetyl-CoA Branches

The fermentative pathways branching from the acetyl-CoA derived from pyruvate lead to some of the most common acetyl-CoA derived products in mixed culture fermentation such as acetate, butyrate, ethanol, butanol, acetone and isopropanol [30]. This is likely due to their due to their ATP generating capacity through SLP [30]. These products enable their corresponding pathways to effectively regulate energy production and electron removal [30].

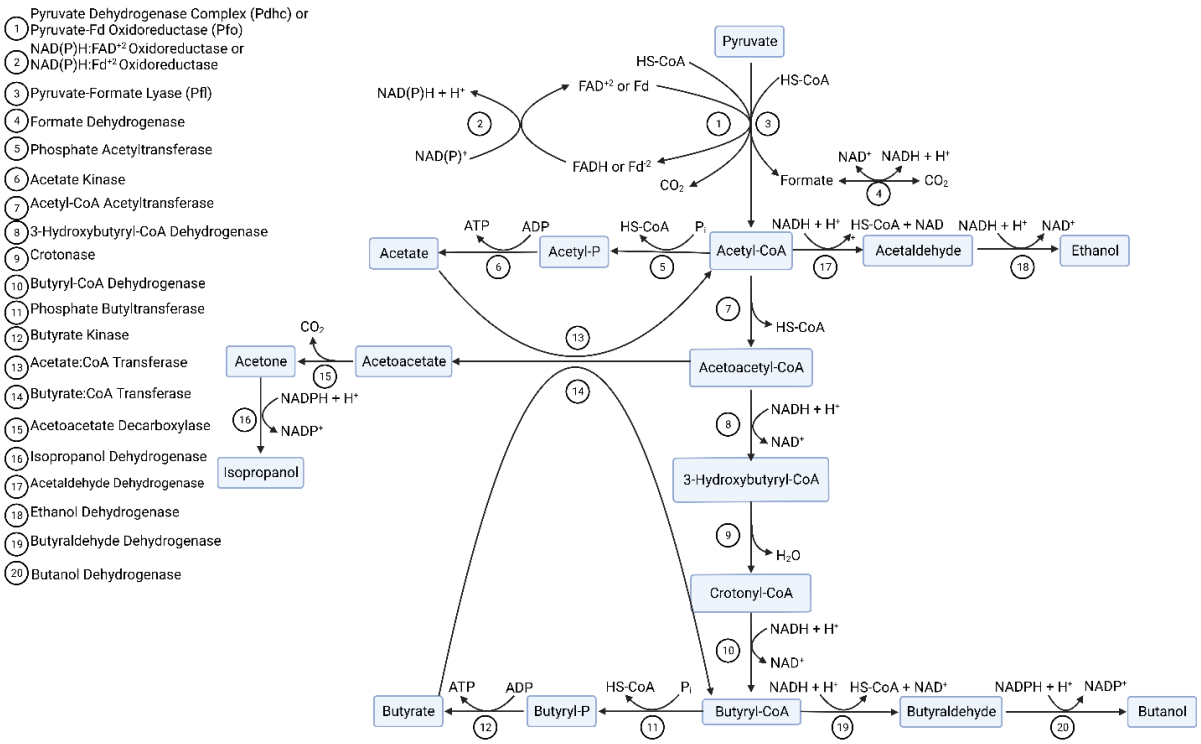

Figure 2: Metabolic Pathway Branches Concerning Acetyl-CoA Formation [30]

The metabolic pathways associated with acetate and butyrate formation are major source of ATP synthesis in mixed culture fermentation system and as such are responsible for about half the SLP in an EMP system [30]. The formation of acetate through pathway branching from acetyl-CoA begins with phosphorylation of the latter via phosphate acetyltransferase to yield acetyl-P. The acetyl-P is then converted in an ADP dependent reaction to form acetate and ATP. In comparison, butyrate formation proceeds through butyryl-CoA derived from acetyl-CoA through the intermediates β-hydroxybutyryl-CoA and crotonyl-CoA [35]. The butyryl-CoA thus formed can be converted to butyrate through one of two pathways which are: (1) production of butyrate phosphate via phosphotransbutyrylase which is subsequently converted to butyrate through butyrate kinase, and (2) via an interconversion reaction involving simultaneous conversion of butyryl-CoA to butyrate and acetate to acetyl-CoA through butyryl-CoA:acetate-CoA transferase [35]. In addition to butyrate, ethanol and butanol produced from pathway branching from acetyl-CoA also serve as electron sinks [30].

$$C_3H_3O_3^- + 2 \cdot H_2O \rightarrow CH_3COO^- + H_2 + HCO_3^- + H^+ \quad (5)$$

$$C_6H_{12}O_6 + 2 \cdot H_2O \rightarrow 2 \cdot CH_3COO^- + 2 \cdot H^+ + 4 \cdot H_2 + 2 \cdot CO_2 \quad (6)$$

$$2 \cdot CO_2 + 4 \cdot H_2 \rightarrow CH_3COO^- + H^+ + 2 \cdot H_2O \quad (7)$$

$$C_6H_{12}O_6 \rightarrow C_4H_7O_2^- + H^+ + 2\cdot H_2 + 2\cdot CO_2 \quad (8)$$

$$2\cdot C_3H_3O_3^- + 2\cdot H_2O \rightarrow C_4H_7O_2^- + 2\cdot HCO_3^- + H^+ \quad (9)$$

$$2\cdot C_3H_3O_3^- + H_2O + 2\cdot H_2 \rightarrow C_4H_9OH + 2\cdot HCO_3^- \quad (10)$$

$$2\cdot C_3H_3O_3^- + 3\cdot H_2O \rightarrow C_3H_7OH + H2 + 3\cdot HCO_3^- + H^+ \quad (11)$$

$$C_4H_7O_2^- + H^+ + 2\cdot H_2O \rightarrow 2\cdot CH_3COO^- + 2\cdot H^+ + 2\cdot H_2 \quad (12)$$

$$2\cdot C_3H_5O_3^- + H^+ \rightarrow C_4H_7O_2^- + CO_2 + H_2 \quad (13)$$

2.2.4 Lactate Branches

The lactate branches begin with reduction of pyruvate to lactate under conditions where oxidation of pyruvate and NADH is impeded and there is increased reliance on cytosolic ATP turnover [33]. The overall equilibrium of reaction under such conditions strongly favors lactate formation which is catalyzed by lactate dehydrogenase [32]. The reduction of pyruvate in this way consumes one proton from the cytosol [33]. Thus, the accumulation of lactate and propionate in anaerobic fermentation process is associated with shock load of organic feedstock and is viewed as evidence of inhibition [30].

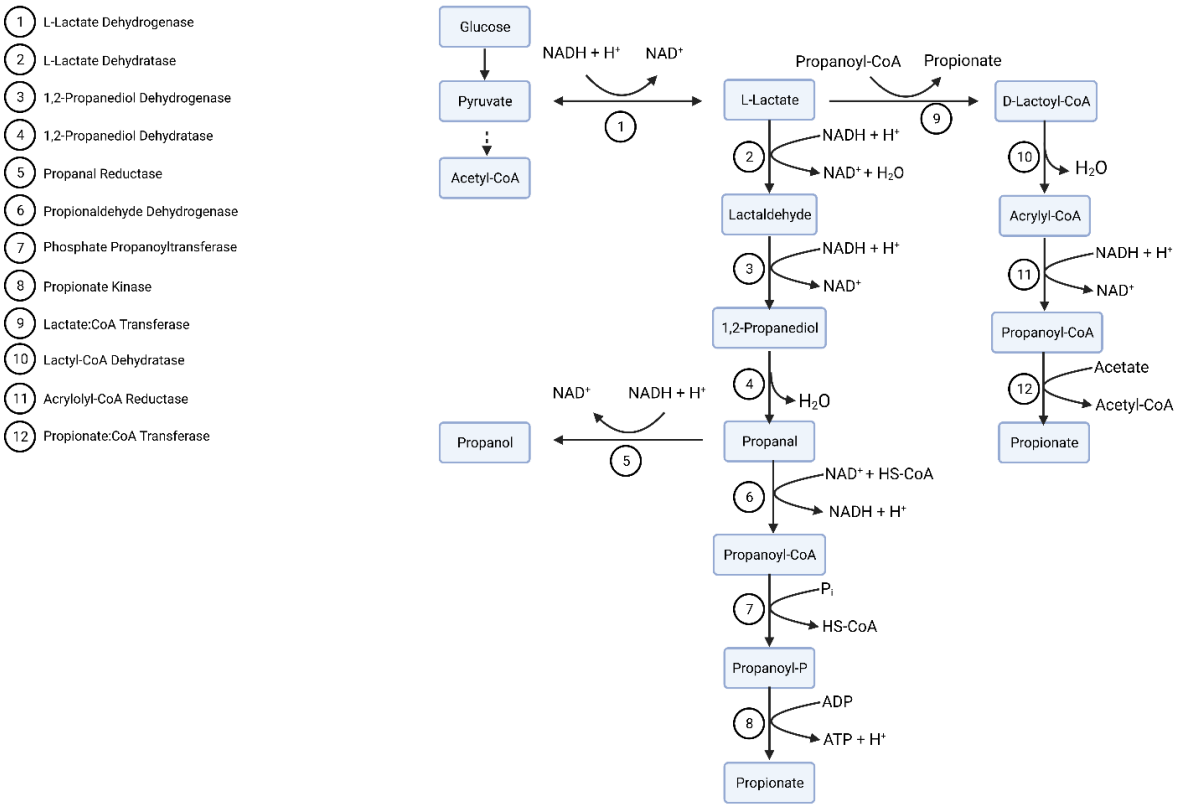

Figure 3: Metabolic Pathway Branches Concerning Lactate Formation [30]

The metabolic pathways associated with production of lactate and/or propionate can be categorized into: (1) 1,2-propanediol pathway, and (2) acrylate pathway [36]. The 1,2-propanediol pathway proceeds with reduction of L-lactate to lactaldehyde which is subsequently reduced to 1,2-propanediol (PDO) by NADH [36]. The PDO thus formed can be catabolized stoichiometrically through an energetically favorable reaction to propionate, yielding one ATP and one reduced cofactor [36]. Though this pathway increases the net ATP yield from glucose by one, there is lack of biochemical evidence to support its existence starting from L-lactate [36]. Unlike the PDO pathway, the acrylate pathway allows an ATP neutral conversion of L-lactate to propionate [36]. This pathway begins with conversion of L-lactate into D-lactoyl-CoA which is dehydrated in the next step to produce Acrylyl-CoA. Following reduction by NADH to yield propanoyl-CoA, propionate is formed after the transfer of the CoA from propanoyl-CoA to acetate [30]. While a variety of substrate can be catabolized to form propionate, in native producers glucose fermentation does not appear to primarily result in propionate production presumably due to its inability to trigger expression of lactate racemase required to initiate the cycle [36].

$$C_3H_3O_3^- + NADH + H^+ \rightarrow C_3H_5O_3^- + NAD^+ \quad (14)$$

$$C_3H_3O_3^- + 2 \cdot H_2 \rightarrow C_3H_5O_2^- + H_2O \quad (15)$$

$$C_6H_{12}O_6 + 2 \cdot H_2 \rightarrow 2 \cdot C_3H_5O_2^- + 2H^+ + 2H_2O \quad (16)$$

2.2.5 Alternative Pyruvate Branches

In addition to the other products, glucose can also be fermented to produce products such as ethanol, acetoin and 2,3-butanediol by decarboxylation of pyruvate as illustrated in Figure 4 [30]. Ethanol formation in this way is a two-step process which bypasses the formation of acetyl-CoA [30]. In the first step, pyruvate is decarboxylated by pyruvate decarboxylase in an irreversible reaction to produce acetaldehyde without oxidizing pyruvate [32]. In the second step, a polarized carbonyl oxygen of acetaldehyde allows its reduction through transfer of hydride ion from NADH [32]. Subsequently, the reduced intermediate forms ethanol by acquiring a proton from the medium [32]. The effect of ethanol on the electron balance is similar regardless of which pathway was utilized for its formation [30].

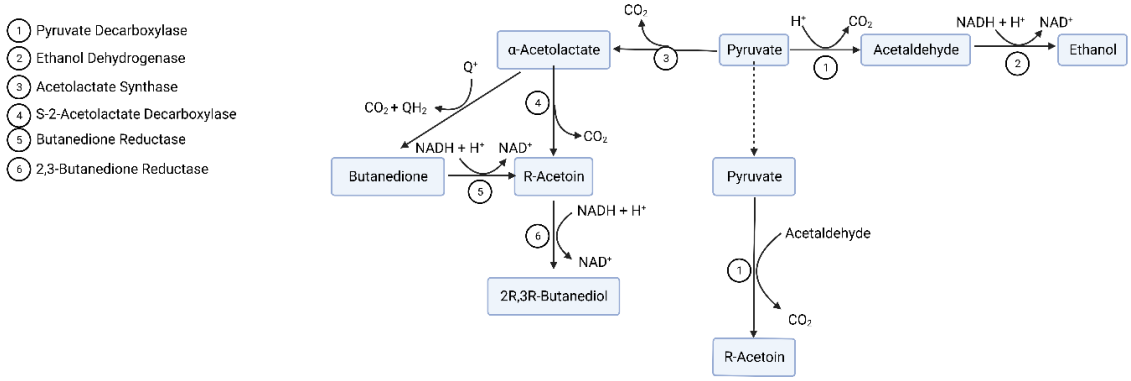

Figure 4: Alternative Metabolic Pathways Branching From Pyruvate [30]

$$C_3H_4O_3 + H_2 \rightarrow C_2H_5OH + CO_2 \quad (17)$$

$$2 \cdot C_3H_3O_3^- + H_2O + H_2 \rightarrow C_4H_{10}O_2 + 2 \cdot HCO_3^- \quad (18)$$

Akin to ethanol formation, the production of acetoin and 2,3-butanediol also proceeds with the initial decarboxylation of pyruvate, albeit to α-acetolactate instead of acetaldehyde [30]. These products are not associated with additional post-glycolysis synthesis of ATP [30]. However, both acetoin and 2,3-butanediol represent a net reduction of ECs when counting from glucose [30]. Therefore, while formation of acetoin as an electron sink is not useful for the cell, to maintain cellular redox balance 2,3-butanediol must be produced alongside other reduced cofactors [30].

2.2.6 Chain Elongation

Chain elongation (CE) refers to the biological process in which carboxylates of variable carbon-chain lengths are extended to corresponding carboxylates of higher chain-lengths using electron donors under anaerobic conditions [37]. Such cyclic interactions between the electron acceptors and electron donors can take place using one of the following mixed culture fermentation pathways[37]: (1) Fatty Acid Biosynthesis (FAB), and (2) Reverse Beta Oxidation (RBO) [37-40]. Both pathways will be discussed in this section.

2.2.6.1 Fatty Acid Biosynthesis: Type 2

FAB represents a central conserved process in all living organisms for production of acyl chains [32]. The FAB system can be distinguished into two major classes, type 1 and type 2 with the main difference between the two being the organization of genes and proteins required for FAB [32]. Whereas type 1 FAB system expresses large multienzyme complexes carrying all the proteins required for FAB, the type 2 FAB system is disassociated with each step in the synthesis being catalyzed by separate

and freely diffusible enzyme [32]. In this study, the focus would be on the type 2 FAB pathway system which is found mostly in bacteria and is illustrated in figure 5.

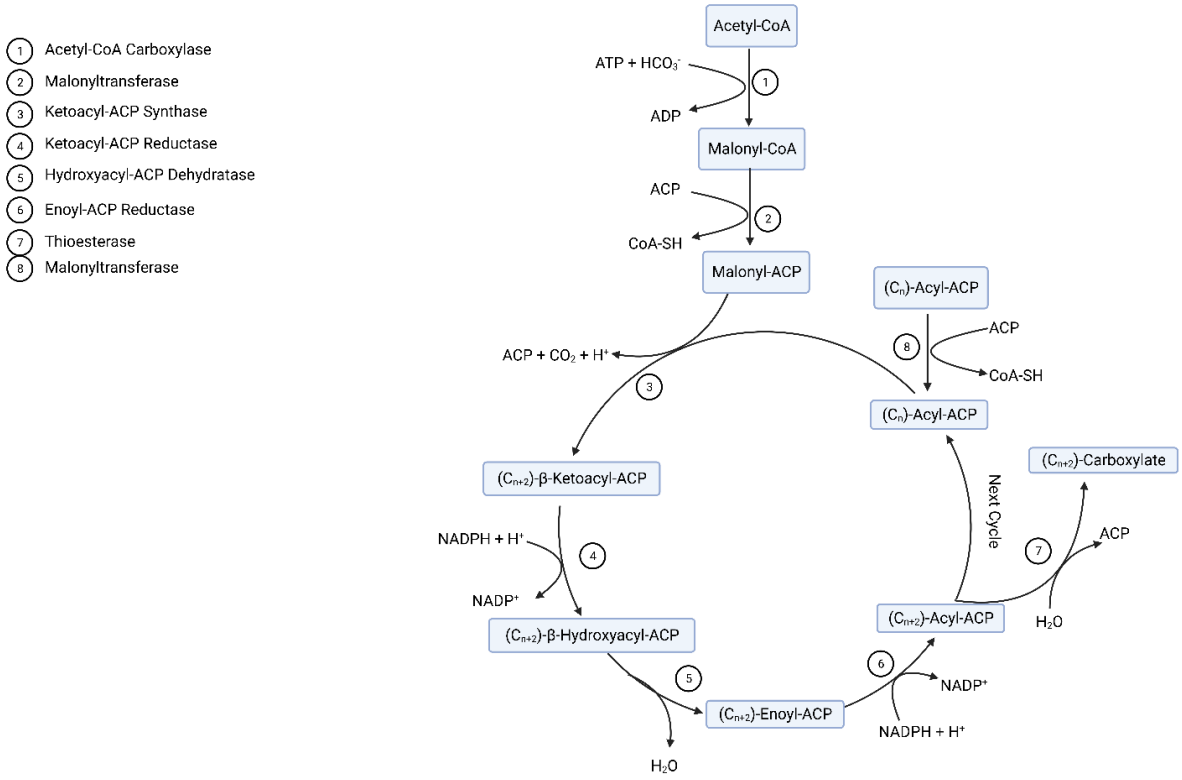

Figure 5: Type 2 Fatty Acid Biosynthesis Pathway [37, 41]

$$2\cdot Acetyl-CoA+ATP+2\cdot NADPH+2\cdot H^{+}\rightarrow Butyrate^{-}+2\cdot CoA+ADP+P_{i}+2\cdot NADP^{+}+H_{2}O \qquad (19)$$

Akin to other biosynthetic pathways, FAB reaction sequences are endergonic and reductive [32]. The preparatory stage of FAB pathway begins with an ATP dependent reaction in which a bicarbonate ion is activated by ATP to produce carboxyphosphate(s) [32]. The carboxyl group derived from the carboxyphosphate is transferred to a temporary CO_2 carrier, biotin, to form carboxybiotin [32]. Subsequently, biotin transfers the carboxyl group to acetyl-CoA in an irreversible process catalyzed by acetyl-CoA carboxylase to yield malonyl-CoA [32]. Additionally, before the condensation reaction that builds up the carbon chain can begin, the two thiol groups must be charged with appropriate acyl groups for chain-lengthening process [32]. This is accomplished through transfer of acetyl and malonyl group from acetyl-CoA and malonyl-CoA to the SH-group of the acyl carrier protein (ACP) [41].

In all organisms, the carbon chains of carboxylates are assembled through a repeating four-step sequence catalyzed by fatty acid synthase. First, in a formal Claisen condensation reaction, the malonyl-ACP combines with an acetyl-CoA to form β-oxobutyryl-ACP while simultaneously releasing a CO_2 molecule [32, 41]. Second, the β-oxobutyryl-ACP is reduced by the electron donor NADPH at C-3 to form β-hydroxyacyl-ACP [32]. Third, a water molecule is released upon dehydration of the C-2 and C-3 of the β-hydroxyacyl-ACP to yield a double bond in the product with trans-2-enoyl group [32]. Finally, the double bond of the product is reduced by the electron donor NADPH to yield butyryl-ACP which is extended by two carbon-units compared to the substrate in the first step [32]. Consequently, following the fourth step a new cycle can begin through condensation reaction of the acyl-ACP with another malonyl-ACP group produced as described before [32, 41].

2.2.6.2 Reverse Beta Oxidation

As implied by its name, the RBO pathway functions by reversing the direction of the β-oxidation cycle used by microorganisms for catabolism of chained-carboxylates of variable carbon length [39]. Such functional reversal occurs by circumventing the regulatory system of carboxylate degradation pathway and generating a thermodynamic pull in the direction of the CE [39]. This thermodynamic pull can be achieved through specific environmental conditions favorable towards the RBO such as: (1) availability of energy rich and reduced electron donors (ED) to provide acetyl-CoA; (2) appropriate pH_2 levels in the reactor to prevent oxidation of carboxylates, and (3) availability of appropriate carboxylate to serves as electron acceptor (EA) [40, 42].

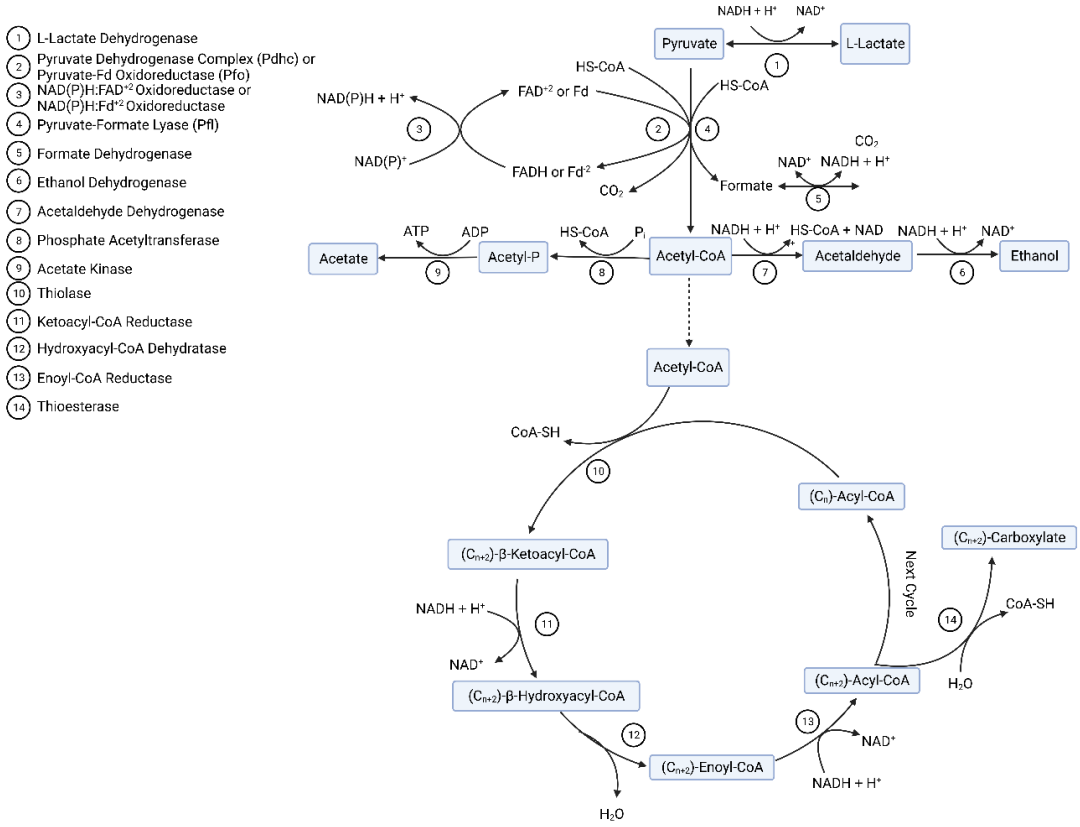

Figure 6: Reverse Beta Oxidation Metabolic Pathway

Much like FAB pathway, RBO is an endergonic and reductive cyclic process in which an acetyl-CoA derived from EDs such as ethanol or lactate is added to the EA carboxylate in every turn of cycle [30]. However, unlike FAB pathway the RBO pathway does not require ATP which contributes to its energy efficiency [39]. The energy in the RBO pathway is gained by coupling the oxidation of EDs and the reductive elongation of acetyl-CoA with EA carboxylate [38]. The reductive step in this process is required to make the production of ATP in the initial oxidative step thermodynamically favorable [38]. Additionally, RBO offers other advantages over FAB pathway such as: (1) it circumvents loss of carbon in form of CO_2 thus increasing carbon yield and efficiency; (2) RBO utilizes NADH as reducing equivalents instead of NADPH which alleviates the requirement of NADPH supple though PPP and, (3) RBO is not inherently limited in flux as acetyl-CoA is a common precursor which enable the use of diverse carbon sources [39]. Thus, the RBO pathway enables an iterative non-decarboxylative elongation of the EA with no ATP requirement and only four enzymes [39].

$$C_3H_5O_3^- + CH_3COO^- + H^+ \rightarrow C_4H_7O_2^- + CO_2 + H_2 \quad (20)$$

2.2.7 Dicarboxylate Cycle

The dicarboxylate cycle in mixed culture fermentation acts analogously to the tricarboxylic acid cycle (TCA) of aerobic metabolism albeit in reverse direction. This cyclic pathway has two distinct entry points, one from phosphoenolpyruvate (PEP) and one from pyruvate [30]. In either case, the pathway begins with activation of bicarbonate ion through consumption of one ATP to form carboxyphosphate which ultimately attaches to the biotin carrier [32]. This carboxylate group is then attached to either PEP or pyruvate to yield oxaloacetate [32]. Subsequently, the oxaloacetate is reduced to succinate through L-malate and fumarate. Conversion of pyruvate to succinate in such a way presents an alternative electron sink to ethanol [36]. Though an ATP is consumed in this cyclic pathway, it is partially compensated for by an anerobic electron chain consisting of fumarate reductase and NADH dehydrogenase [36].

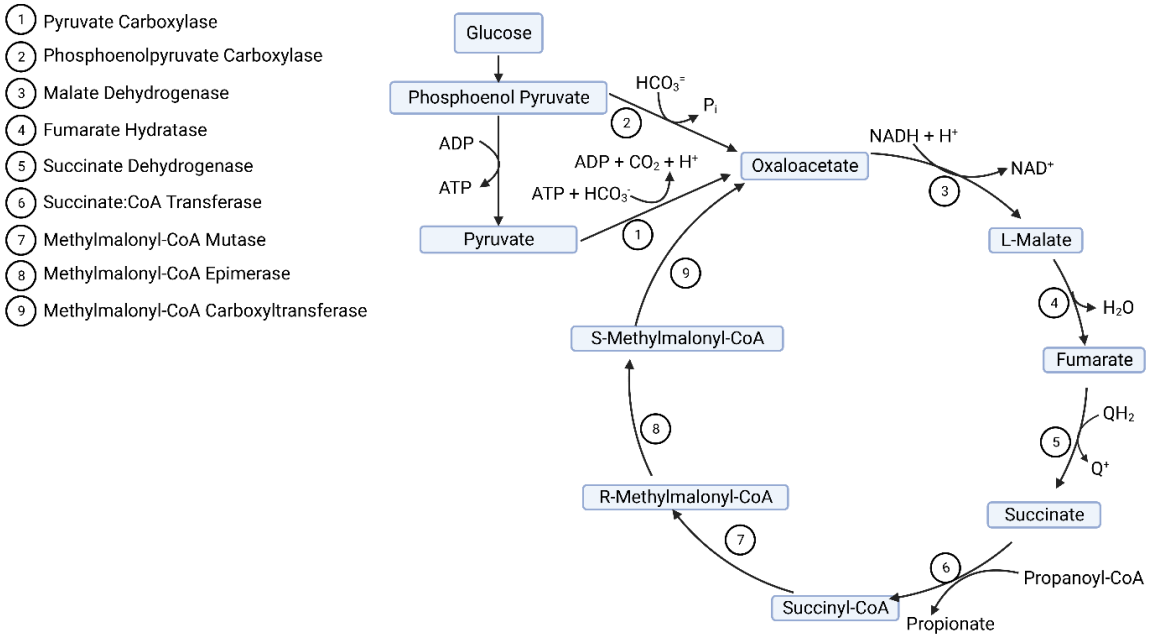

Figure 7: Dicarboxylate Cycle Metabolic Pathway [43]

Some microorganisms have adapted energy conservation strategies via the mixed carboxylate fermentation by further decarboxylation of succinate to propionate in the penultimate step following which the regeneration of oxaloacetate occurs via transfer of CoA to pyruvate thus producing Propanoyl-CoA [30]. Propionate formation in this manner is the result of CoA transfer to pyruvate while the formation of other dicarboxylates is result of PEP entering the dicarboxylate cycle [30]. Therefore, propionate formation in this pathway is associated with the final ATP yielding step of glycolysis while the other products are not [30]. Furthermore,

since each turn of the dicarboxylate cycle results in formation of one propionate, the oxidized electron carrier yield of each propionate is equivalent to full oxidized electron carrier yield of one turn of cycle [30].

$$C_3H_3O_3^- + 2 \cdot H_2 + HCO_3^- \rightarrow C_4H_5O_4^- + H_2O \quad (21)$$

2.3 Influence of Process Conditions on Product Formation

The interaction of process conditions with the microbial communities and cellular metabolism has been suggested to have a significant role in fermentative product formation [19]. Several lines of investigation suggests that that among the process conditions, pH, substrate concentration, gaseous partial pressure and temperature influence the thermodynamic feasibility of metabolic pathways along with the structure and diversity of microbial community structure in the anaerobic systems [19, 44-46]. Among these process parameters, the effects of pH, substrate concentration, and temperature on the product spectrum have been the subject of extensive research. However, there still exists uncertainty regarding the relation between process parameters type biochemical formed. For MCF to be cost-effective, mechanistic understanding of how process conditions can be manipulated to steer MCF towards specific biochemical formation is crucial.

2.3.1 Substrate Type

The fermentation of different types of substrates proceeds from different entry points in the metabolic pathway [30]. Consequently, different types of substrates would produce different sets of products. However, the relationship between the effect of substrate types and the specific product formation remains ambiguous, even for substrates which share a portion of metabolic pathways [19]. This ambiguity would be further discussed in this section through comparison of product spectrums obtained from fermentation of: (1) glucose and xylose, and (2) glucose and glycerol.

First, the comparison between glucose and xylose fermentation begins from their respective metabolic pathways. Whereas the glucose fermentation may proceed with either EMP or ED pathway, xylose would first have to be converted to D-xylulose-5-phosphate (X5P) before it can metabolized through either the PPP or the phophoketolase pathway (PKP) [31]. Regardless of the metabolic pathway followed by xylose, it would be eventually converted into G3P at which point its pathway would converge with that of glucose. Despite this convergence, the product spectrum from xylose fermentation reported in literature differs drastically from that of glucose. This comparison was best made in the study conducted by Temudo et al. where for the same substrate concentration, the xylose fermentation produced more

n-butyrate than glucose fermentation [31]. This contrast in the specific product formation from glucose and xylose fermentation in literature is surprising given their comparable oxidation states and similar thermodynamic feasibility of their reactions. Interestingly, a review of studies on xylose fermentation suggests that xylose predominantly produced more even-carbon products regardless of the process conditions it was subjected to [47-56]. Conversely, only a few publications have reported significant formation of odd-carbon products from xylose fermentation. In contrast, the description of odd or even-carbon products formed from glucose fermentation was found to be inconsistent.

Second, much like glucose and xylose, glycerol and glucose also share the metabolic pathway from G3P to pyruvate [31]. However, unlike glucose and xylose, fermentation of glycerol reportedly produces nearly negligible amounts of acetate and n-butyrate in a comparative research experiment conducted by Temudo et al. [31]. Instead, it was found that the glycerol fermentation produced 1,3-propanediol and ethanol as a major product. Furthermore, in their experiment to study the effects of substrate concentrations, the authors observed metabolic shifts for both glucose and glycerol. Whereas increase in glucose concentrations resulted in metabolic shift from n-butyrate to acetate and ethanol, higher concentrations of glycerol resulted in a metabolic shift from ethanol and formate to produce 1,3-propanediol and acetate instead. These findings were supported by the study conducted by Moscoviz et al. who also reported production of 1,3-propanediol from glycerol fermentation under various pH conditions in their experiments [57].

2.3.2 Substrate Concentration

The flux of substrate through a metabolic pathway directly influences the quantity of chemical energy that can be extracted by the heterotrophic cell to do work and transform the free energy into ATP(s) and other biochemicals. While at lower substrate concentrations cells prefer metabolic pathways with higher ATP yield, high initial substrate concentrations have been suggested to steer product formation towards metabolic pathways through which the NAD^+ required for glycolysis can be acquired. However, despite the abundance of publications, the description of the biochemical formation as a function of initial substrate concentration has been inconsistent.

While some authors have reported higher fraction of n-butyrate at high substrate concentration, others have documented the formation of lactic acid and propionate [24, 58-63]. In a comprehensive study on glucose fermentation, Hoelzle et al. investigated the effect of varying substrate concentration from 5 g/l to 20 g/l on product formation [59]. The authors observed that when the substrate concentration exceeded the cellular consumption capacity, the product spectrum shifted from acetate-n-butyrate type fermentation to lactate-propionate fermentation. This metabolic shift occurred at substrate concentration of around 15 g/L of glucose.

Based on metaproteomic and end-product analysis, the authors concluded that the metabolic shift at higher concentrations was associated with internal detoxification response of the microbial cell [59]. These observations were reinforced by findings of the research undertaken by Rafay et al. and Eng et al. on the fermentation of glucose and sucrose respectively [60, 61]. Whereas Rafay et al. observed this metabolic shift at 33 gCOD/L and 38 gCOD/L, Eng et al. reported increase in lactate formation at 12 gCOD/L for an HRTs ranging from 6-8 hours. Similar observations were also reported by Jiang et al. and Liang et al. who reported formation of valerate and lactate respectively from real waste streams albeit at different high substrate concentrations [58, 64, 65]. These observations suggest that lactate-propionate type fermentation is triggered at high substrate concentrations of glucose and its polymers. However, it is unclear what determines the concentration at which such fermentation would be triggered.

In contrast to formation of odd-carbon carboxylates, there exists a comparable body of literature advocating that increase in substrate concentration results in higher production of even-carbon carboxylates. However, such results should be inferred with caution because of the following reasons. First, for instance in a study conducted by Arslan et al. on the effect of headspace conditions on carboxylate formation, the magnitude of increase in n-butyrate yield was three orders of magnitude lower than the input substrate concentration [24]. Second, in the studies conducted by Wijekoon et al. and Jankowska et al. on the effects of increasing organic loading rate on product formations, an increase in n-butyrate concentration was also observed with increase in organic loading rate (OLR), however the range of concentration they explored in the experiments was narrow and in any case below the 15 g/L condition at which Hoelzle et al. observed lactate production [59, 62, 66]. Third, in the research conducted by Agler et al., an increase in n-butyrate was once against observed with increase in OLR [67, 68]. However, the corn fiber waste stream fermented by the authors also contained xylose which, as described in the previous section, predominantly produces even-carbon carboxylates.

2.3.3 pH

The effects of pH have been identified as a major contributing factor to the product formation in mixed culture fermentation due to its various roles in the fermentation system [19, 46]. For example, besides determining the degree of disassociation of the acidic species present in the fermentation system, pH is also known for its effects on enzyme(s) catalyzing metabolic reactions and on microbial community structures. Perhaps the best-known study illustrating this metabolic shift was carried out by Temudo who studied product formation by fermenting 4 g/L of glucose at various pH conditions using undefined mixed culture and made observations which would be discussed in this section [31].

First amongst the observations of interest is the metabolic shift between molecular hydrogen and formate. This metabolic shift was first described by Temudo who observed formation of molecular hydrogen at low pH during glucose fermentation and formation of formate at alkaline pH [31]. These findings were further reinforced by the study conducted by Zaki who also reported formation of molecular hydrogen at low pH and that of formate at alkaline pH during fermentation of 5 g/L of glucose during both, the progressive pH increase, and the pH reset experiments [69]. Moreover, according to Zaki, while formate and H2 formed a greater fraction of total products during experiments at high pH level. Temudo suggested that the production of CO_2 and H_2 over formate at low pH was related to the thermodynamics of formate dehydrogenation to CO_2 and H_2. However, it is unclear whether this metabolic shift occurred due to, (1) pH-dependent activity of Fdh with only Pfl expression or, (2) relative simultaneous activity of Pfl and Pdhc/Pfo or, (3) combination of both functions [30, 31].

Second amongst the observation of interest is the production butyrate or ethanol as a function of pH. In the study conducted by Temudo on glucose fermentation at different pH, the author observed acetate-butyrate type fermentation during experiments at acidic pH and acetate-ethanol type fermentation during experiments at alkaline pH [31]. Once again, similar observations were also made by Zaki in her experiments on glucose fermentation at various pH values [69]. However, whereas this transition to ethanol was gradual in the progressive pH increase experiments conducted by Zaki, the transition was found to be abrupt in her pH reset experiments for 6.5 pH where butyrate was completely replaced by ethanol formation. The author attributed this metabolic shift to change in dominant microbial species from Clostridium at acidic pH to Klebsiella at alkaline pH [69]. Temudo on the other hand insinuated that this metabolic shift was associated with the formate-molecular hydrogen metabolic shift discussed in the previous paragraph but was unable to substantiate this claim [31]. In contrast to the observations made by Temudo and Zaki, Ren et al. reported increase ethanol production from glucose fermentation when the pH was between 4.3 and 4.9 whereas Kim et. al reported increase n-butyrate fractions with increase in pH from 5 to 9 when fermenting food waste [70, 71]. Thus, from the literature reviewed in this section so far, it can be concluded that the reason for n-butyrate production at low pH in some cases and ethanol production at low pH in other cases is not fully understood.

2.3.4 Temperature

The temperature of an anaerobic fermentation system directly effects the activation energy of the metabolic reaction(s) and the thermodynamics of reaction equilibrium. Additionally, it is well documented that the temperature of the system provides selective pressure on the ecology of the mixed culture fermentation system.

However, due to lack of systematic investigation a clear link between the effects of temperature and product formation cannot be established.

For instance, whereas both Shin et al. and Jiang et al. reported an increase in n-butyrate production with concomitant increase in system temperature, Valdez-Vazuez et al. reported the opposite findings in their study on hydrogen production [58, 72-74]. It is unclear whether these contradictory findings were influenced by the differences in the substrate used in the respective studies or the methodology adopted in the studies. Therefore, a thorough and systematic research is required to clearly establish the effect of temperature on product formation.

2.3.5 Partial Pressure: Molecular Hydrogen

The existing research on microbial biochemical formation recognizes the controversial role of molecular hydrogen in anaerobic fermentation of organic feedstock. As such, the effects of hydrogen partial pressure (pH_2) on product formation and/or degradation has been subject of several studies. In general, these studies can be categorized into: (1) cases of metabolic inhibition induced by hydrogen gas; (2) cases of pH_2 influencing the product formation from fermentation of organic feedstock, and (3) reports of pH_2 having no inhibitory effects on substrate degradations.

First, of particular significance among the studies on metabolic inhibition induced by pH_2 is the detailed and seminal work of Mosey which pioneered the approach to examining the pH_2 parameter as a tool for monitoring anaerobic fermentation systems [75]. In this work, Mosey proposed a relationship between pH_2 and oxidation states of NAD^+:NADH EMC couple which allowed him to rewrite rate equations developed from ratios of EC couples in the form of pH_2 [75]. Consequently, the model of anaerobic digestion process thus formed described a proposed mechanism of SCC formation from glucose fermentation on basis of pH_2. Through this proposed influence of pH_2 over the cellular redox state, Mosey claimed that increasing the hydrogen gas concentration in anaerobic system: (1) reduces overall rates of organic acid formations, and (2) influences the product spectrum of carbohydrate fermentation by increasing the proportion of feedstock converted to propionate and butyrate while decreasing the proportion of feedstock converted to acetate [75]. This model of the anaerobic digestion process was later extended by Ruzicka, Costello et al., Keller et al., Rodriguez et al. and Kleerebezem et al. [76-81]. Whereas Ruzicka proposed the concept of nonequilibrium hydrogen effect on glucose fermentation and split the formation of SCCs into even-carbon and odd-carbon groups as directed by pH_2, other researchers introduced terms for product inhibition, pH inhibition, shock loadings and redox considerations with FAD, Fd and NAD into the anaerobic digestion model [76-81]. Much like the Mosey model, these extensions and models were also built on an assumed influence of pH_2 on the cellular redox state. However, this assumed relation was challenged by De Kok et al.

who experimentally tested the proposed relationship between pH_2 and NAD+:NADH ratio by subjecting an undefined mixed culture to various pH_2 conditions and found that pH_2 had no significant effect on the EMC couple ratios and by extension on the cellular redox state [82].

Second, in addition to invalidating the proposed relation between pH_2 and cellular redox state, De Kok et al. also reported no clear trend between the observed butyrate production and pH_2 [82]. Conversely, in their study on the effects of pH_2 on product spectrum obtained from mixed culture fermentation of carbohydrate-rich waste stream, Arslan et al. observed an increase in butyrate production relative to the product spectrum obtained for control condition [23, 24]. However, in contrast to expected results and claims made by Mosey, the authors reported cessation of propionate production upon application of 2 bars of pH_2. The authors suggested the possibility of adaption by microorganisms to conditions of pH_2 as a possible explanation, but no description or analysis of the microbial community was given to support this argument [23, 24]. Moreover, similar observations were made by the authors in a follow-up study where the authors subjected various concentrations of the carbohydrate-rich waste stream to 2 bars of pH_2 but did not observe any increase in propionate production. Based on these results, the authors concluded that contrary to the theory of product formation, propionate production does not always increase with a concomitant increase in pH_2 [23, 24]. These observations were further supported by the experiments conducted by Ren et al. and Inanc et al. who also reported no significant change in propionate production upon application of pH_2 [83, 84].

Third, the first observations of pH_2 having no inhibitory effects on substrate degradations was reported by Denac et al. who investigated the effects of pH_2 on degradation rates of organic acids produced from fermentation of molasses wastewater under constant pH [85]. In their study, the authors measured the uptake rate of organic acids after subjecting the undefined mixed culture to three conditions: (1) no gassing; (2) H_2 gassing, and (3) N_2 gassing. Subsequently, the according to the author's report they did not detect any inhibitory effect of pH_2 on the degradation rate of the organic acids produced from anaerobic digestion of the molasses wastewater. These results were contrary to what was expected based on the anaerobic digestion model proposed by Mosey [75]. Similar results were also reported by Steinbusch who subjected undefined mixed cultures to 1.5 bar of pH_2 and low pH but still observed oxidation of organic acids [86]. In her study, Steinbusch applied 1.5 bar of pH_2 to three batch reactors with acetate, or propionate, or butyrate as substrates respectively and observed their reduction to the corresponding alcohols with the conversion efficiency being higher for propionate and butyrate than for acetate [86]. However, despite the application of 1.5 bar pH_2, Steinbusch still reported methanogenesis for batch reactors containing propionate or butyrate as substrates, even though their oxidation to acetate were thermodynamically unfavorable [86]. The conversion efficiencies for the organic

acids were as follows: (1) 33.6 ± 9.6% for acetate; (2) 27.1 ± 7.1% for propionate, and (3) 36.6 ± 2.2% for butyrate [86].

2.3.6 Partial Pressure: Carbon Dioxide

Existing research microbial biochemical formation recognizes the emerging role of hydrogen partial pressure (pH_2) and carbon dioxide partial pressure (pCO_2) in specific product formation from MCF [19, 23, 24]. Owing to their association with various fermentation reactions, either as substrate, intermediate or co-product, pH2 and pCO_2 are vital in determining the thermodynamic feasibility of metabolic pathways involved in biochemical formation. Whereas a considerable amount of literature exists on the effect of pH_2 product formation, pCO_2 has been the subject of relatively fewer extensive studies.

In a seminal research on the effects of pH_2 and pCO_2, Arslan et al. reported that whereas fermentation under 2 bar pCO_2 selectively produced n-butyrate for carbohydrate-rich waste stream, the fermentation of protein and lipid rich-waste streams produced acetate instead [23, 24]. Furthermore, the authors also observed that elevated pCO_2 adversely effected hydrolysis of carbohydrates, while the hydrolysis of protein and lipid rich waste streams was found to be unaffected. [19, 23, 24] These observations are consistent with other investigations where elevated pCO_2 was found to have detrimental effect on microbial growth and glucosidase activity [87]. Surprisingly, despite the arrested hydrolysis the carbohydrate rich waste stream had higher total volatile fatty acids (tVFA) after thirty days compared to the low tVFAs observed from fermentation of protein and lipid rich-waste streams [23]. In fact, after thirty days carbohydrate-rich waste stream under 2 bar pCO_2 was found to have higher tVFA compared to control conditions even though their degree of hydrolysis were similar [23]. On the basis of these findings, the authors hypothesized that the final product type was not only determined by the effect of pCO_2 or the effect of substrate, but also by the combined effect of pCO_2 and substrate [23].

In a follow-up study, Arslan et al. investigated the combined effect of varying substrate concentrations and modified headspace on carboxylate production by fermenting a carbohydrate-rich waste stream under 2 bar pCO2 in acidic conditions at three substrate concentrations: 8 gCOD/L, 13.5 gCOD/L and 23 gCOD/L [24]. A detailed examination of their results showed that under 2 bar pCO2 substrate concentrations up to 13.5 gCOD/L had significantly higher n-butyrate selectivity compared to control conditions after one week of fermentation [24]. Remarkably, further increase in substrate concentration to 23 gCOD/L resulted in higher n-butyrate selectivity for control conditions compared to the conditions with 2 bar pCO_2 [24]. Consequently, the authors concluded that there are two methods for increasing n-butyrate selectivity: : i) application of CO_2 if the substrate concentration

is lower than 13.5 gCOD/l, and ii) increasing substrate concentrations without any headspace modification [24].

The results and conclusions of studies conducted by Arslan et al. suggests that optimization of pCO_2 could play an important role in obtaining the high selectivity and product concentration required to make MCF market-competitive [19, 23, 24]. However, these findings should be interpreted with caution as alternative explanation for their observations cannot be ruled out due to the methodological limitations of their investigations. For instance, Arslan et al. clearly mentioned the different compositions of the batch used for obtaining substrate concentration of 23 gCOD/L compared to the batches used for obtaining 8 gCOD/L and 13.5 gCOD/L may have had some influence on the experimental results [24]. Additionally, it should be emphasized that the dilutions applied by Arslan et al. for obtaining 23 gCOD/L substrate concentration inadvertently resulted in lower carbohydrate content compared to the batches for lower substrate concentrations [23, 24]. Together, these issues with methodology raise questions regarding the validity of results obtained by Arslan et al. for experiments with 23 gCOD/L [24].

Though the contradictory findings for product formation at high carbohydrate concentrations can be explained based on the methodological limitations of investigation by Arslan et al., the mechanism that underpins the conversion of carbohydrates into n-butyrate under 2 bar pCO_2 remains speculative [19, 23, 24]. These findings were surprising as elevated pCO_2 was expected to decrease the thermodynamic feasibility of n-butyrate formation in favor of propionate. It was suggested by Arslan et al. that the lactate present in their waste stream could have induced chain elongation which resulted in higher n-butyrate formation [19, 23, 24]. However, their proposed mechanism would contradict the findings of Lemmer et al. who observed higher propionate concentrations when a waste stream containing lactate was fermented under elevated pCO_2 [88]. Though some investigations have reported increased activity of lactate dehydrogenase (LDH) enzyme under elevated pCO_2, these findings further exacerbate the uncertainty regarding the role of lactate as it is well established that LDH catalyzes both the forward and backward reaction [89].

3. Methodology

3.1 Materials Used

3.1.1 Bioreactors

The designed experiments were conducted in two different experimental setups: atmospheric pressure bioreactors (APB), and elevated pressure bioreactors (EPB). The APBs were reserved for experimental conditions where the initial gas pressures would be below 2.0 bar. Conversely, the EPBs were reserved for experimental conditions where the initial gas pressure would exceed 2.0 bar.

3.1.1.1 Atmospheric Pressure Reactors

The experiments reserved for APBs were conducted in 1.0 L bioreactors fabricated using borosilicate glass. The APB openings would be plugged with butyl rubber septum with attached stopcocks for sampling. This would ensure air-tight anaerobic conditions and prevent accidental gas leakage due to the repeated piercing of the septum by needle during sampling process.

3.1.1.2 Elevated Pressure Reactors

The experiments reserved for EPBs would be conducted in 1.6 L bioreactors fabricated using stainless steels. The EPBs were composed of two parts: one 7 mm thick main cylindrical steel vessel (SS316) and one steel head plate lid (SS316; 12mm). To make the EPBs impermeable to air, the steel vessel and the lid were fixed with flanges (SS316; 12 mm) using PTFE gaskets and fastened with nuts and bolts (SS316 L; 8 No.).

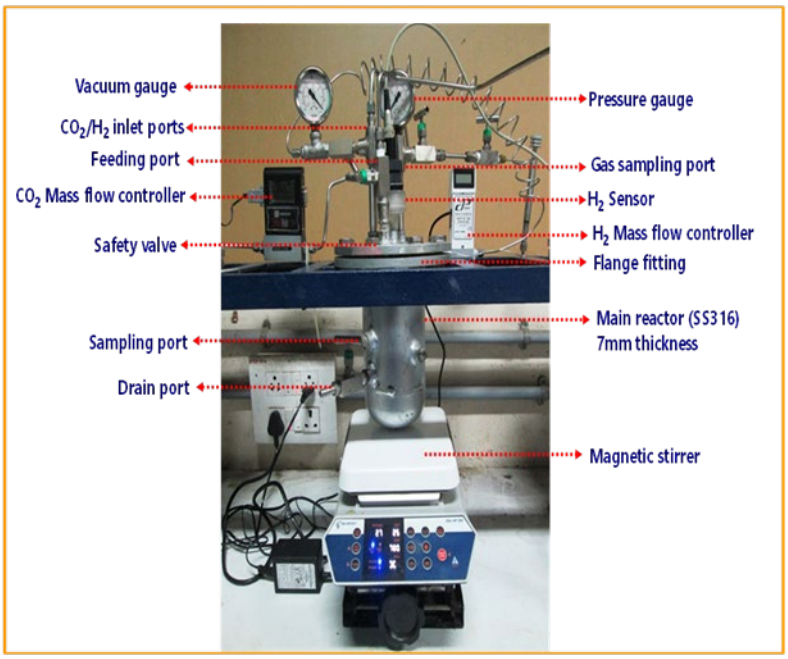

Figure 8: Elevated Pressure Reactor

The EPBs were custom designed to withstand gas pressure up to 15 bar with provisions for one safety valve (15 bar), one pressure release knob and one drain valve. Additionally, the EPBs were fabricated with two gas inlet ports, one sampling port and one feedstock inlet port. Each EPB was also equipped with one pressure gauge (WIKA; SS316; 40 bar).

3.2 Biomass & Culture

In this study, anaerobic sludge procured from the local wastewater treatment plant was used as parent inoculum for studying the effects of selected process parameters on microbial product formation.

3.3 Media

3.3.1 Substrate

For investigating the effects of selected experimental conditions on product formation, this study utilized glucose as the model carbohydrates. This synthetic feedstock was prepared by mixing the required concentration of glucose in separate Schott bottles.

After the addition of the feedstocks, the Schott bottles would be sealed by plugging butyl rubber septum into the openings. Then the Schott bottles would be sparged with 99% N_2 gas through the stopcock for ten minutes to ensure an anaerobic environment.

3.3.2 Nutrient Media

A mineral medium containing macronutrients and micronutrients was prepared without sulphates to prevent sulphate reduction during the experiments. The prepared nutrient medium was further fortified with BME-Vitamins 100x solution to promote growth of lactic acid bacteria in the bioreactors. Additionally, 100 mM of $NaHCO_3$ and 20 mM of 2-bromoethanesulfonate (BES) was dosed in the reactor to provide buffer and inhibit methanogenic activity respectively. The composition of the nutrient broth is presented in Tables 3-5.

3.3.3 Activation Media

Tryptone-Glucose-Yeast extract (TGY) medium was prepared in Schott bottles for the purpose of activating the anaerobic sludge prior to commencement of the experiments. Similar to the synthetic feedstock described in Section 3.1.3.1, the bottles containing the activation medium were first plugged with butyl rubber septum and then flushed with 99% N_2 gas to ensure an anaerobic environment. The composition of the nutrient broth is presented in Table 6.

3.4 Experimental Setup

To test the effects of initial substrate concentrations and pCO_2 conditions on fermentative product formation, the experiments were conducted for a fixed hydraulic retention time (HRT) of 8 days under constant mesophilic condition (35°C ± 1) and at an initial pH of 7.0 without mechanical mixing.

The experiments at atmospheric and elevated pCO_2 conditions were performed with an initial liquid to gas ratio of 5:3. For EPBs, this ratio would compartmentalize the total reactor volume of 1600 ml into liquid volume of 1000 ml and gas volume of 600 mL, whereas for APBs the 1000mL reactor volume were compartmentalized into liquid volume of 625 mL and gas volume of 375 mL. The liquid volume of all bioreactors was further compartmentalized into substrate volume and sludge volume of 800 mL and 200 mL.

Before experimentation, the reactor headspace was first flushed with N_2 (>99%) for five minutes to ensure anaerobic environment in the reactor. Subsequently, the EPBs were subjected to two sequential pressurization and depressurization cycles before a final pressurization step with 100% CO_2.

3.5 Biophysiochemical Analytical Methods

3.5.1 Liquid Composition

In order to answer the research questions formulated for this study, the reactants and products of glucose fermentation present in the broth must first be identified and then quantified. For this reason, 1 mL samples were regularly collected from the bioreactors for measuring the analytes in a High-Performance Liquid Chromatography (HPLC) system. In this study, a custom method was developed for analysis capable of resolving all analytes of interest without any overlaps. The scope of analysis was restricted to SCCAs, ethanol, lactic acid, succinic acid, and residual concentration of glucose. Primary inclusion criteria for the aforementioned analytes were their relatively higher production in existing literature on mixed culture fermentation.

Prior to the HPLC analysis, the cell mass present in the broth samples must be removed to mitigate its adverse effect on the sample quality and the HPLC machine. Therefore, the samples collected from bioreactors were first centrifuged for ten minutes at 10,000 x g to separate the liquid fraction from the cell mass. Subsequently, the supernatant was carefully extracted from the sample using a syringe and then gently passed through a 0.2 µm filter to remove the residual cell mass. The filtered supernatant thus obtained was aliquoted into HPLC vials using pipette. Furthermore, to correct for potential loss of analytes during the sample preparation and analysis process, 1g.L-1 crotonic acid was spiked in each vial as internal standard for carboxylic acids. Subsequently, degassed H_2SO_4 was dosed into each HPLC vial using a pipette to acidify the solutions. Finally, the obtained sample was thoroughly mixed using a vortex mixer.

In addition to the sample vials, the HPLC batch analysis set would contain calibration standards and blanks. Whereas the calibration standards was used to calibrate the HPLC system prior to sample analysis, the blanks was used as control run and for mitigating cross-contamination of samples due to carry-over of analytes.

The prepared set of HPLC vials were analyzed by injecting 60 µL of sample into the HPLC (Prominence Series, Shimadzu, Japan) equipped with Rheodyne injection port (7725i Series, Shimadzu, USA) with a loop volume of 20 µL. Such overfilling of the loop ensures more precision by injecting the similar volume of sample into the system with each manual injection. The HPLC was operated at a flow rate of 0.6 mL.min-1 in "binary gradient" mode using 0.03 N of H_2SO_4 as mobile phase which carried the injected sample from the injection port to the 300 x 7.8 mm RHM Monosaccharide column for analyte separation. Prior to usage, the mobile phase was thoroughly degassed using sonication to avoid formation of air bubbles. The column temperature was maintained at 40˚C using a column oven to prevent fluctuations during analysis and mitigate possible inversion of disaccharides and polysaccharides. Prior research on similar topic(s) were limited in their ability to separate key analytes using conventional chromatography approaches leading to uncertainty in their conclusions which is why in this study, a mixed-phase chromatography approach was used to separate the analytes as prior. As such, in

this study sugars were separated on basis of size-exclusion, the alcohols were separated on basis of reverse phase chromatography and the organic acids were separated on basis on ion-exclusion and normal phase chromatography.

For the purpose of analyte detection, the HPLC was equipped with an RID (RID-20A, Shimadzu, Japan) which was also maintained at 40˚C. Additionally, the sampling and base rate were set to 50 Hz whereas the response time was set to 0.05 sec. These settings enabled the detection of steep analyte peaks in the chromatograph. Finally, integration was done as per USP method.

3.5.2 Headspace Composition

The total consumption and production of gases during experimental runs was monitored by collecting gas samples from bioreactors to determine the headspace composition using Gas Chromatography with Thermal Conductivity Detector (GC-TCD).

Prior to sample collections, to prevent accidental injection of impurities into the bioreactors or their gas outlet lines the gas-tight syringes was first flushed with 99% N2 gas and then flushed once again with the headspace gas. Once the syringes were adequately flushed, 1 mL of samples was carefully withdrawn from the bioreactors to prevent accidental pressure loss due to gas leakage. To ensure that backflow from gas outlets do not skew gas measurements, the headspace sampling always preceded other samplings.

The collected gas samples were directly injected into a GC-TCD (Agilent Technologies: 7890) equipped with Heysep Q 80/100 column (2.0 m length, 1/8 inch OD, 2.0 mm ID, Agilent, CA, USA). Whereas the injection and detector temperatures would be maintained at 80 °C each, the oven would be operated at 100 °C. Nitrogen was used as the carrier gas for measuring the composition of headspace samples.

3.5.3 Chemical Oxygen Demand

In the pre-experiment and post-experiment phase, samples were collected from the parent inoculum and the fermentation broth for determining the total chemical oxygen demand (tCOD) and the soluble chemical oxygen demand using COD-closed refluxing-dichromate method as outlined in the standard methods [90]. The difference between the tCOD and sCOD measurements reflected the COD content of the inoculum.

In contrast to samples for tCOD measurements, the sCOD samples were subjected to an additional preparation step involving centrifugation and filtration to remove cell mass from the liquid fraction. This preparation step was similar to that undertaken for HPLC samples described in Section 3.5.1.

3.5.4 Characterization: Biomass & Broth

In the pre-experiment and post-experiment phase, samples were collected from the parent inoculum and the fermentation broth for three additional physiochemical analysis: bicarbonate content, and dry cell weight (DCW). Whereas the bicarbonate content and TVFA were determined using standard methods, the dry cell weight (DCW) would be determined from curve relating optical density at 660 nm (OD660) to the dry weight where an OD660 of 1.0 represent 420 mgDCW.L-1 [90].

3.6 Design of Experiments

Conventional research on mixed culture fermentation have relied upon 'One Factor at a Time' (OFAT) experimental design where only one factor or variable is changed at a time. However, there exists certain limitations associated with the use of such experimental design such as its inability to estimate interactions and its higher run requirement for estimating effects with same precision. For this reason, this research was designed as a mixed factorial between subject study capable of estimating interaction effects of the factors, initial substrate concentration and CO_2 partial pressure on product formation. The experimental conditions under which APBs and EPBs were operated in this study are presented in Table 1.

Table 1: Experimental Conditions Used in This Study

S. No	pCO_2	Substrate Concentration	S. No	pCO_2	Substrate Concentration
1.	0.3 bar	5 g/L	9.	3 bar	15 g/L
2.	2 bar	5 g/L	10.	4 bar	15 g/L
3.	4 bar	5 g/L	11.	0.3 bar	20 g/L
4.	0.3 bar	10 g/L	12.	2 bar	20 g/L
5.	2 bar	10 g/L	13.	3 bar	20 g/L
6.	3 bar	10 g/L	14.	0.3 bar	25 g/L
7.	0.3 bar	15 g/L	15.	2 bar	25 g/L
8.	2 bar	15 g/L	16.	4 bar	25 g/L

3.7 Calculations

3.7.1 Bioenergetic Calculations

The thermodynamic feasibility of pertinent biochemical reactions at the experimental conditions was assessed by calculating the actual Gibbs Free Energy for the reaction(s) using Equation 22.

$$\Delta G_R^1 = \Delta G_R^{01} + R \cdot T \sum_{i=1}^{n} Y_{Si}^R \cdot \ln\left(a_{Si}\right) \quad (22)$$

where:

ΔG_R^{01} *is the Gibbs free energy at pH 7 and* $308.15\ K$

R *is the gas constant* $\left(8.31\ J \cdot K^{-1} \cdot mol^{-1}\right)$

T *is the temperature in Kelvin*

Y_{Si}^R *is the stoichiometric coefficient for compound i*

a_{Si} *is the molar concentration of the compound i*

The Gibbs free energy at standard conditions were corrected for temperature using the Gibbs-Helmholtz equation according to the procedure of Lieng et. al where the magnitudes of ΔG_R^1 at standard conditions were taken from Heijnen and Kleerebezem [91].

3.7.2 Selectivity

To faithfully replicate the studies conducted by Arslan et al. the substrates and products detected through chromatographic methods were quantified on the basis of their selectivity as described in Equation 23 [23, 24]. However, to avoid bias towards the compounds with more mass, it was decided to calculate the selectivity as a mole fraction instead. The selectivity was then calculated for all individual compounds detected as well as the even-carbon or odd-carbon carboxylate group they belong to. It should be emphasized that formate was excluded from the calculation of selectivity of odd-carbon carboxylates as the central focus of this research is the comparison between lactate and acetyl-CoA derived carboxylates.

$$Selectivity\ (\%) = \left(\frac{Molarity_{\ substrate\ or\ product}}{Molarity_{\ substrate} + Molarity_{\ product}}\right) \times 100 \quad (23)$$

4. Results & Discussion

4.1 Results

The main objective of this research was to study the effects of initial substrate concentrations (g/L) and elevated pCO_2 conditions on the product spectrum of glucose fermentation under mesophilic conditions. To achieve this objective batch experiments were conducted as per the design of experiments to statistically gauge the main and interaction effects of initial substrate concentration and elevated pCO_2 conditions on product spectrum of glucose fermentation. The data acquired from these experiments was then used to achieve the sub-objective of this research which was to replicate the studies conducted by Arslan et al. on the effects of elevated pCO_2 levels on mixed culture fermentation and reassess the generalizability of their findings across experimental conditions which differ from those utilized in the original studies [23, 24].

4.1.1 Thermodynamic Analysis

As the thermodynamic feasibility of acetate, propionate and butyrate producing reactions was already calculated for experimental conditions up to 5 bar pCO_2 in the original studies conducted by Arslan et al., these calculations were not repeated in this study [24]. As illustrated in Figure 9, all the reactions producing acetate or propionate or butyrate from glucose were exergonic for all headspace conditions. Additionally, the effect of pCO_2 on the thermodynamic feasibility of these reactions was computed to be negligible. Among all the reaction, the production of propionate from glucose was computed to be the most favorable reaction.

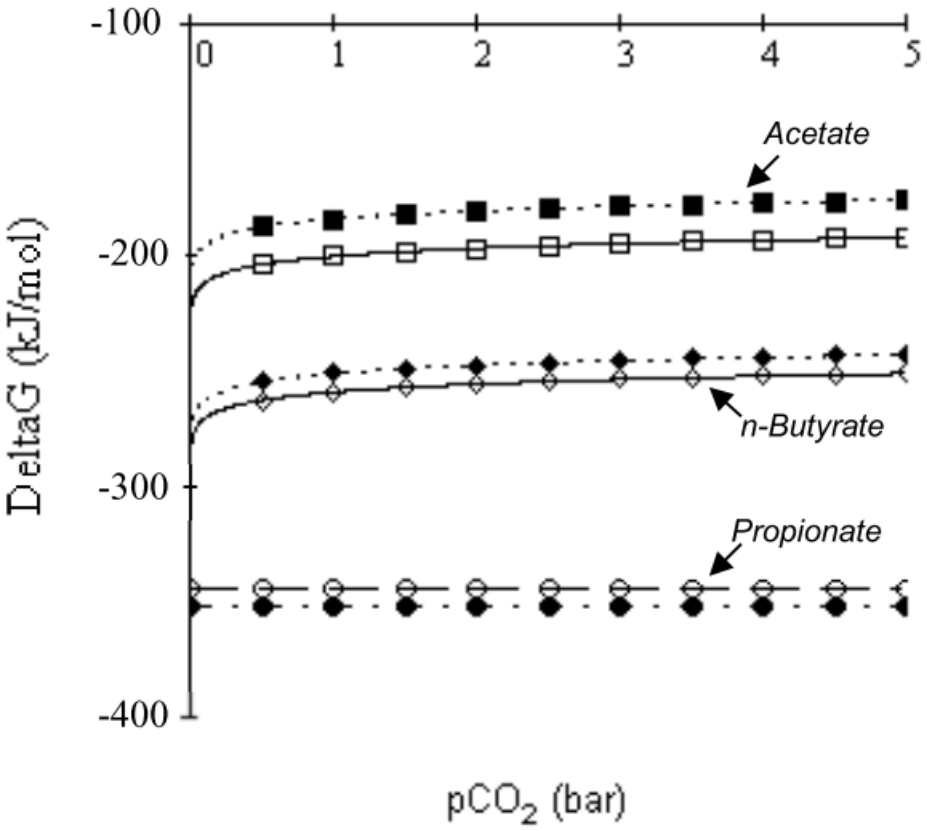

Figure 9: Thermodynamic feasibility of reaction producing acetate, propionate or butyrate from glucose under 1 and 5 bar pH_2 as a function of pCO_2. The hollowed markers represent 1 bar pH_2 and the filled out markers represent 5 bar pH_2.[24]

4.1.2 Chemical Oxygen Demand Balance

To investigate the main and interaction effects of initial substrate concentration and elevated pCO_2 conditions on the total carboxylate production, the data acquired from the experiments was used to check the COD balance by comparing the sum of COD for products and residual substrate with the COD of the substrate fed to the reactor. This comparison is presented in Table 2 and Figure 10.

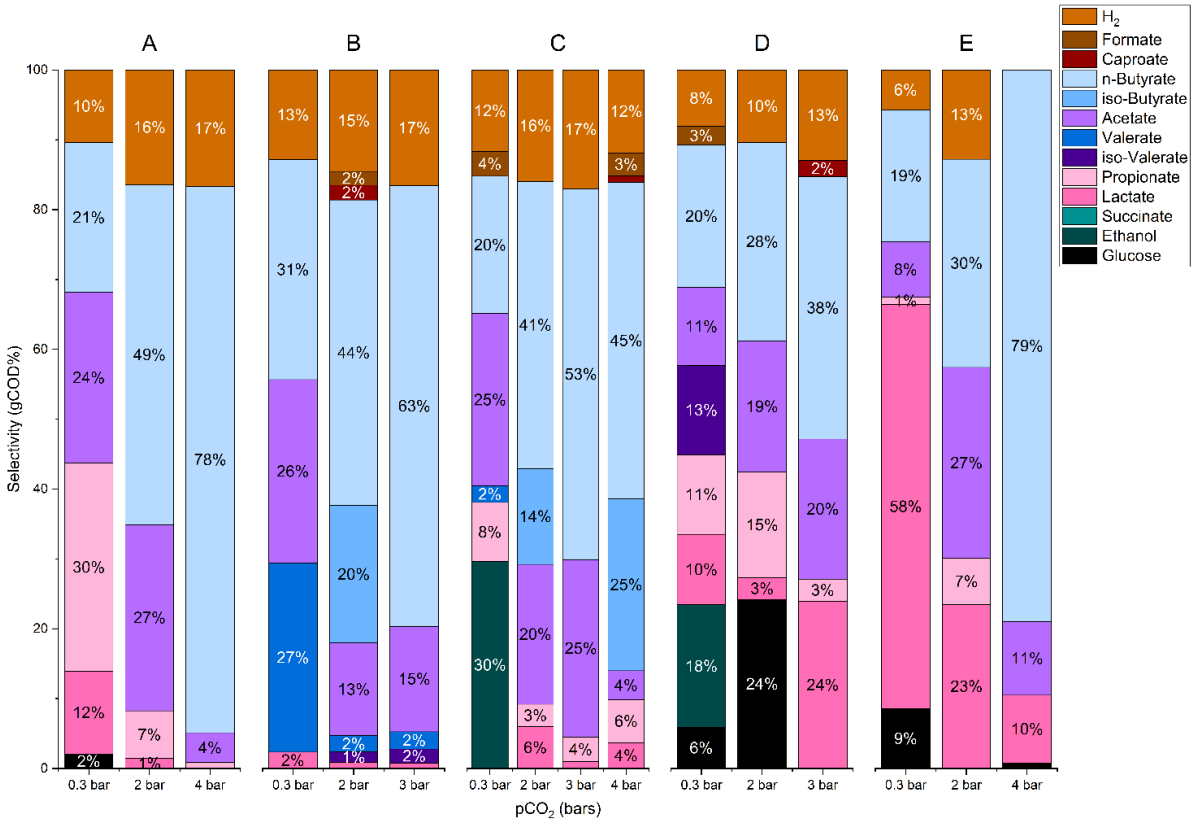

Figure 10: COD Balance of Experimental Conditions

In general, the summation of CODs for the experimental conditions were found to be closed between 83-133% recoveries. This indicates a closed electron balance and confirms that the major products in the experimental runs were identified. The computed variations in the COD balance may be attributed to either measurement errors, or CO_2 assimilation by homoacetogens in the reactors subjected to elevated pCO2 conditions.

Table 2: COD Balance for Experimental Conditions

pCO_2	Conc.	Glu	Eth	Lac	For	Ace	Pro	But	Pen	Hex	H_2	Total
(bar)	(g/L)	gCOD										
0.3	5	0.127	0.000	0.725	0.000	1.496	1.825	1.313	0.000	0.000	0.637	6.123
2		0.000	0.000	0.096	0.000	1.811	0.467	3.320	0.000	0.000	1.117	6.811
4		0.000	0.000	0.000	0.000	0.312	0.061	5.714	0.000	0.000	1.353	8.104
0.3	10	0.000	0.000	0.263	0.000	2.973	3.062	3.558	0.000	0.000	1.455	11.312
2		0.000	0.000	0.123	0.265	1.787	0.313	8.543	0.195	0.283	1.958	13.467
3		0.000	0.000	0.102	0.000	2.127	0.347	8.876	0.289	0.000	2.329	14.070
0.3	15	0.000	5.116	0.000	0.610	4.274	1.468	3.396	0.406	0.000	2.022	17.293
2		0.000	0.000	1.013	0.000	3.387	0.542	9.276	0.000	0.000	2.702	16.920
3		0.000	0.000	0.174	0.000	4.568	0.631	9.553	0.000	0.000	3.053	17.978
4		0.000	0.000	0.690	0.620	0.776	1.143	13.071	0.000	0.164	2.220	18.876
0.3	20	1.326	3.950	2.280	0.602	2.515	2.567	4.590	2.884	0.000	1.824	22.539
2		4.552	0.000	0.584	0.000	3.536	2.848	5.345	0.000	0.000	1.953	18.819
3		0.000	0.000	5.627	0.000	4.724	0.760	8.834	0.000	0.558	3.052	23.555
0.3	25	2.223	0.000	15.096	0.000	2.062	0.279	4.915	0.000	0.000	1.499	26.073
2		0.000	0.000	5.167	0.000	6.023	1.452	6.555	0.000	0.000	2.817	22.015
4		0.220	0.000	2.762	0.000	2.999	0.000	22.484	0.000	0.000	0.000	28.465

4.1.3 Product Analysis

The main research question in this study sought to determine the effect of the initial substrate concentrations and elevated pCO_2 conditions on selectivity of biomolecules produced from anaerobic conversion of glucose. To answer the research question the liquid and gas samples collected from the batch reactors were subjected to offline analysis. Subsequently, the results of the offline analysis were used in statistical analysis to test the hypothesis proposed in the introduction chapter. The results of these analysis are illustrated in figures 9-11 and the five themes inferred from these figures can be categorized as follows: (1) glucose uptake in ARBs and ERBs; (2) degradation of carboxylates; (3) metabolic shift between hydrogen and formate; (4) inhibition of certain minor products, and (5) metabolic shift between butyrate and lactate. These observations will be further elaborated upon in this section.

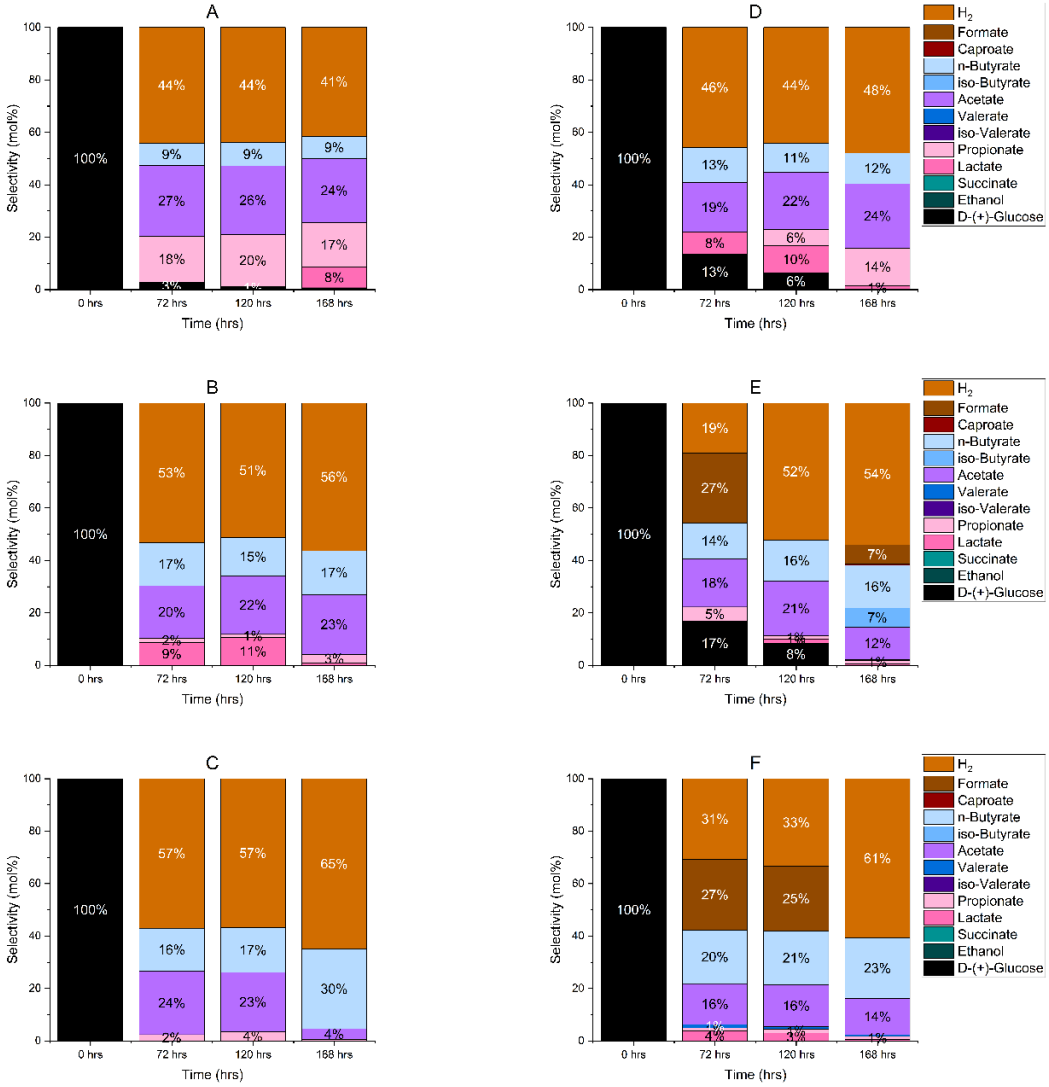

Figure 11:Product Selectivity Composition: A-5g/L & 0.3 bar; B-5g/L & 2 bar; C-5g/L & 4 bar;D-10g/L & 0.3 bar;E-10g/L & 2 bar; F--10g/L & 3 bar

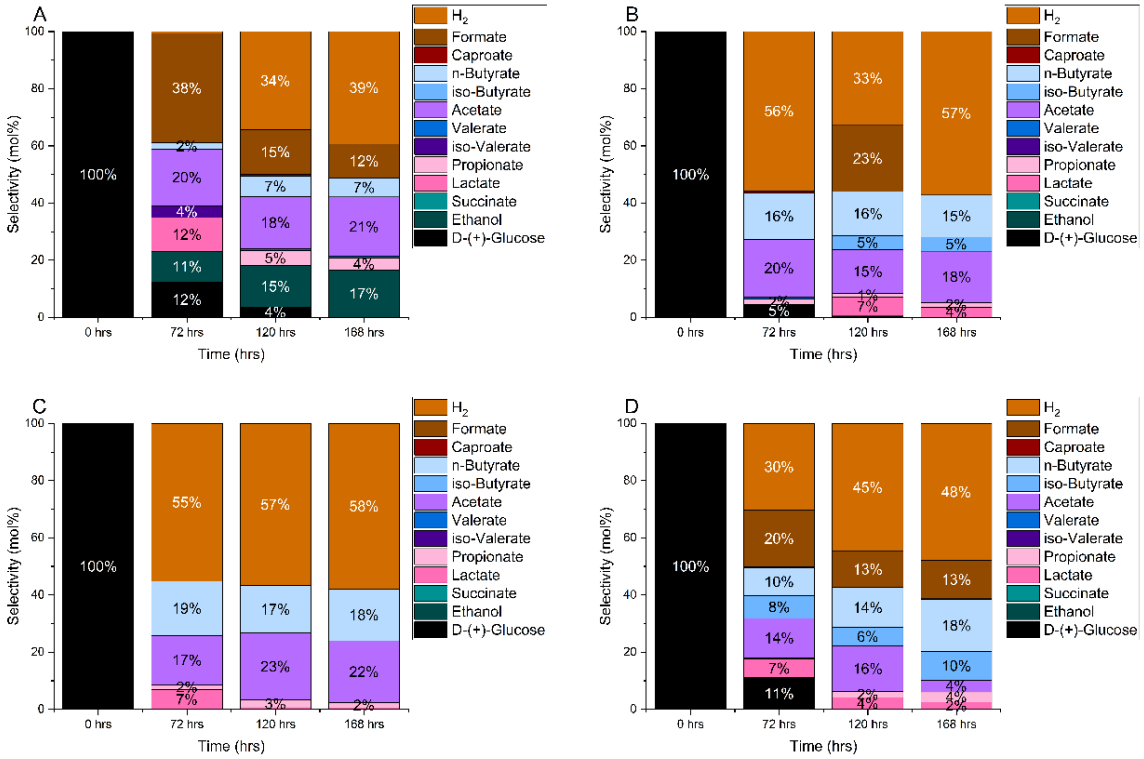

Figure 12: Product Selectivity Composition: A-15g/L & 0.3 bar; B-15g/L & 2 bar; C-15g/L & 3 bar; D-15g/L & 4 bar

First, as can be seen from the Figures 11-13, the glucose fed to the reactors was consumed within the first seventy-two hours for most of the experimental conditions. The exception to this observation were the following experimental conditions where the residual glucose was still detected by the HPLC system: (1) 10 g/L glucose and 0.3 bar pCO_2; (2) 10 g/L glucose and 2 bar pCO_2 ; (3) 15 g/L glucose and 0.3 bar pCO_2 ; (4) 20 g/L glucose and 0.3 bar pCO_2, and (5) 25 g/L glucose and 0.3 bar pCO_2. Additionally, it was observed that while the pressure in control reactors started increasing within the first twenty-four hours, the pressure in ERBs remained stable for the first forty-eight hours. It was found that during these forty-eight hours, the microbial activity in these ERBs were in lag phase and produced minimal amount of fermentative products (data not shown). However, after the first forty-eight hours, the pressures in the ERB were found to increase sharply in proportion to the increase in initial substrate concentration. While this pressure increase was minimal for ERBs operated with 5 g/L glucose concentration, the pressures for ERBS operating with glucose concentration higher than 10 g/L surpassed 8 bar during the experimental run. This marked increase in pressure indicated that the microbial activity in the reactor had entered the exponential phase where the glucose was being metabolized to produce acetyl-CoA and its derivatives. Surprisingly, this finding was contrary to the observations made by Arslan et al. in

their study on the effects of modified headspace on product selectivity where pressures were reported to be stable throughout the experimental run [23, 24]. This discrepancy in observed effect may have been the result of lower concentration of monosaccharides in the studies conducted by authors which consequently resulted lower flux of carbon through glycolysis yielding low concentrations of products and concomitantly lower production of gases [23, 24].

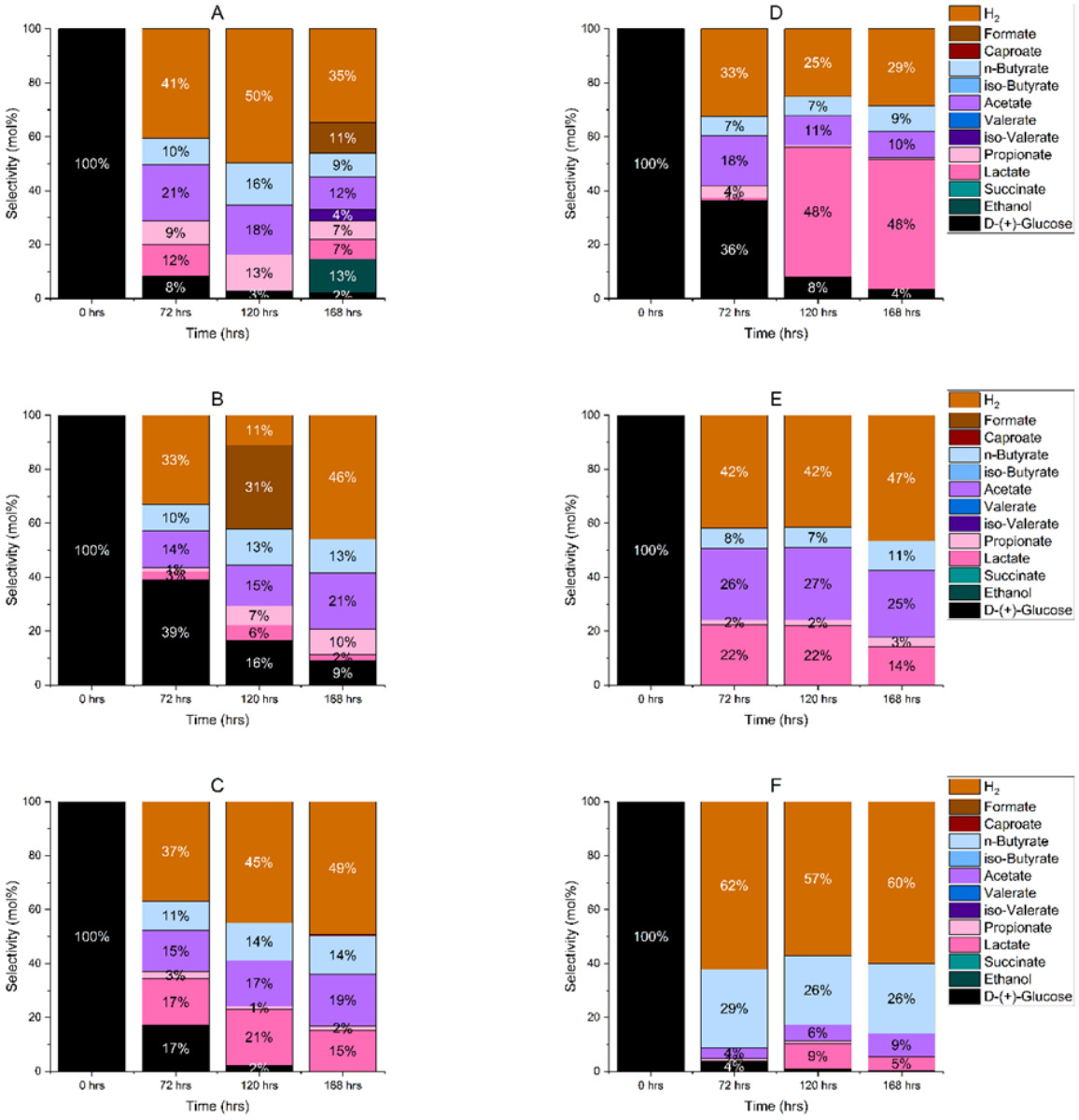

Figure 13: Product Selectivity Composition: A-20g/L & 0.3 bar; B-20g/L & 2 bar; C-20g/L & 3 bar; D-25g/L & 0.3 bar; E-25g/L & 2 bar; F--25g/L & 4 bar

Second, it can be inferred from Figures 11-13 that with successive increase in pCO_2 levels the degradation rate of higher carboxylates to acetate was found to decrease. These observations are in line with the results of studies on the effect of elevated CO_2 levels on anaerobic digestion of carboxylates where oxidation of carboxylates was found to be inhibited at higher pCO_2 levels. Kim et al. ascribed these observations to the inhibitory effect of CO_2 on acetogens which oxidize the carboxylates into acetate [92]. This suggests that the reactors in this study were operating in purely fermentation phase as glucose metabolism does not require hydrolysis and this study utilized BES to inhibit methanogens. Therefore, it can be assumed that the changes in the molecular hydrogen and carbon dioxide levels in this study were due to fermentation reactions.

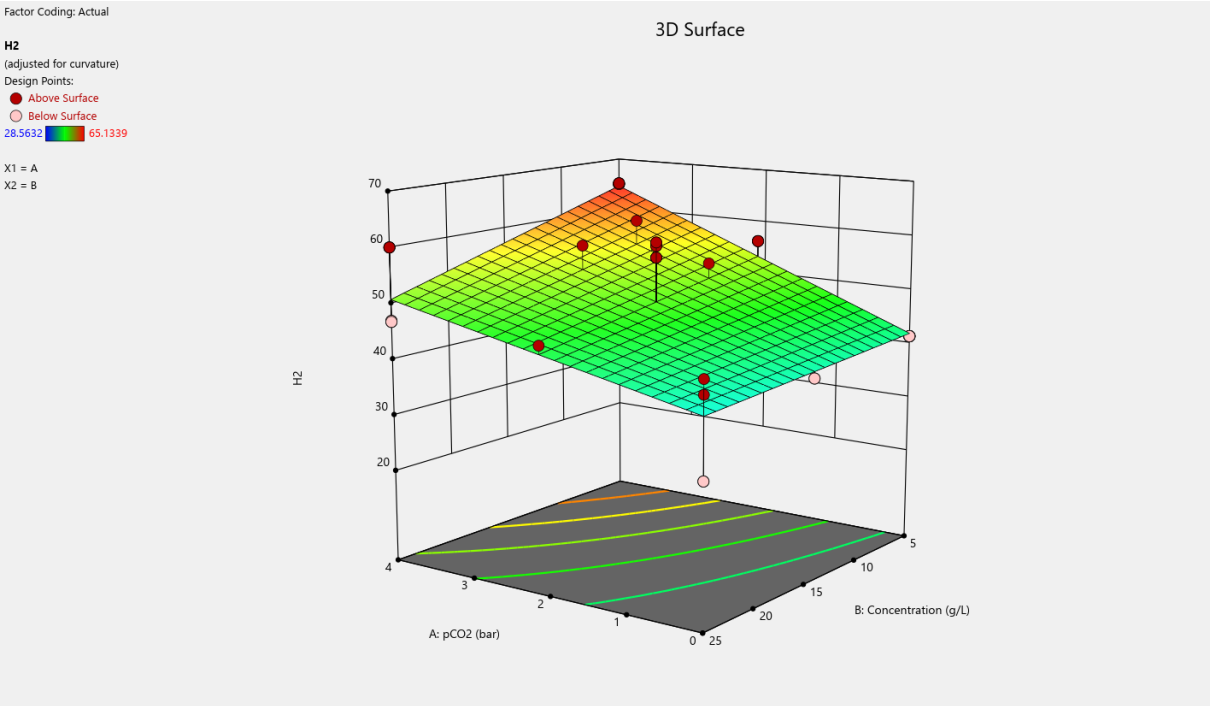

Figure 14: 3D Surface Depicting Hydrogen Formation Across All Experimental Conditions

Third, from the data illustrated in Figures 11-13, it is apparent that the formation of molecular hydrogen increased with concomitant increase in pCO_2 levels. Furthermore, it can be inferred from Figures 11-13 that the lowest H_2 level at 29% of total product concentration were measured for the control conditions with 25 g/L substrate concentration, the highest H_2 level at 65% of total product concentration were measured for 4 bar pCO_2 condition with 5 g/L substrate concentration. Once again, these findings are in line with those of Kim et al. who reported that sparging the reactor with external N_2 or CO_2 gases stimulated formation of molecular hydrogen [92]. Additionally, as no O_2 was detected in this study during the GC

analysis for any experimental condition, it is assumed that there were no major gas leakages from the reactors. Interestingly, a transient metabolic shift from hydrogen to formate was observed in this study for the ERBs. This was a rather unexpected result as the reactors of interest were operated at initial neutral pH whereas according to the studies cited in this research formate production predominantly occurred at alkaline pH for glucose fermentation [31].

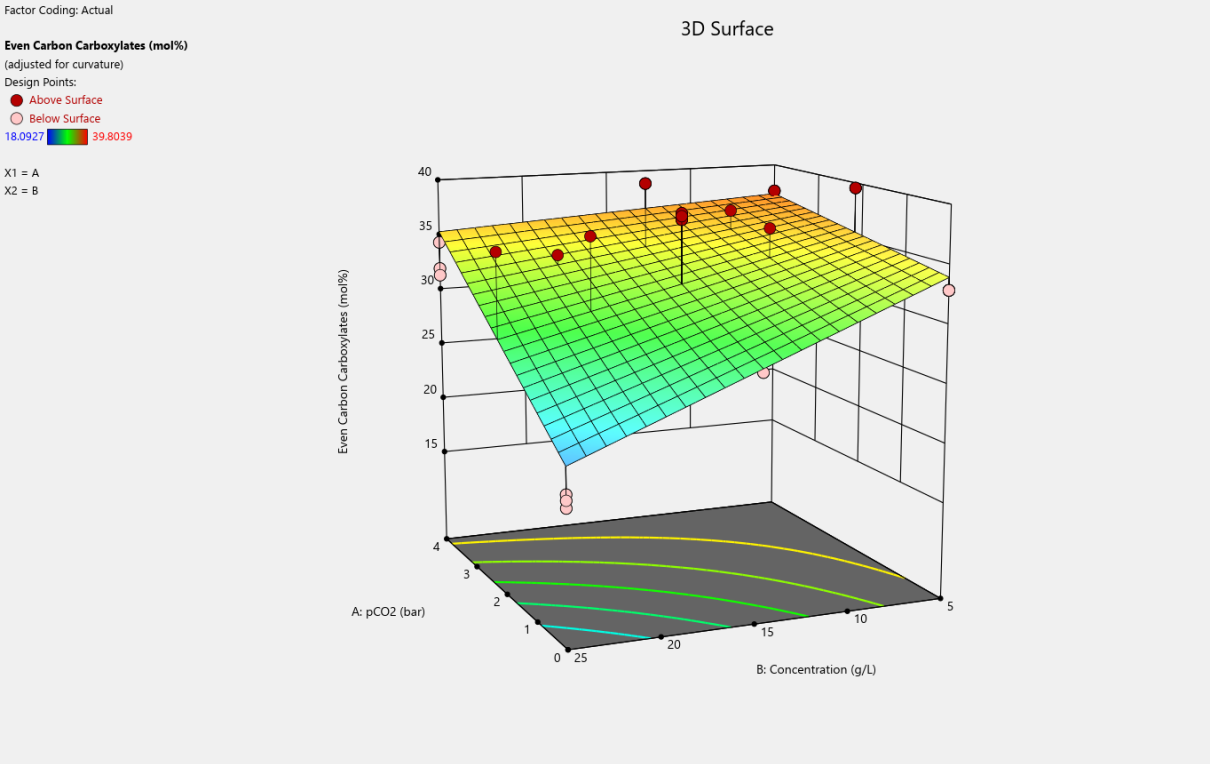

Figure 15: 3D Surface Depicting Even Carbon Carboxylate Formation Across All Experimental Conditions

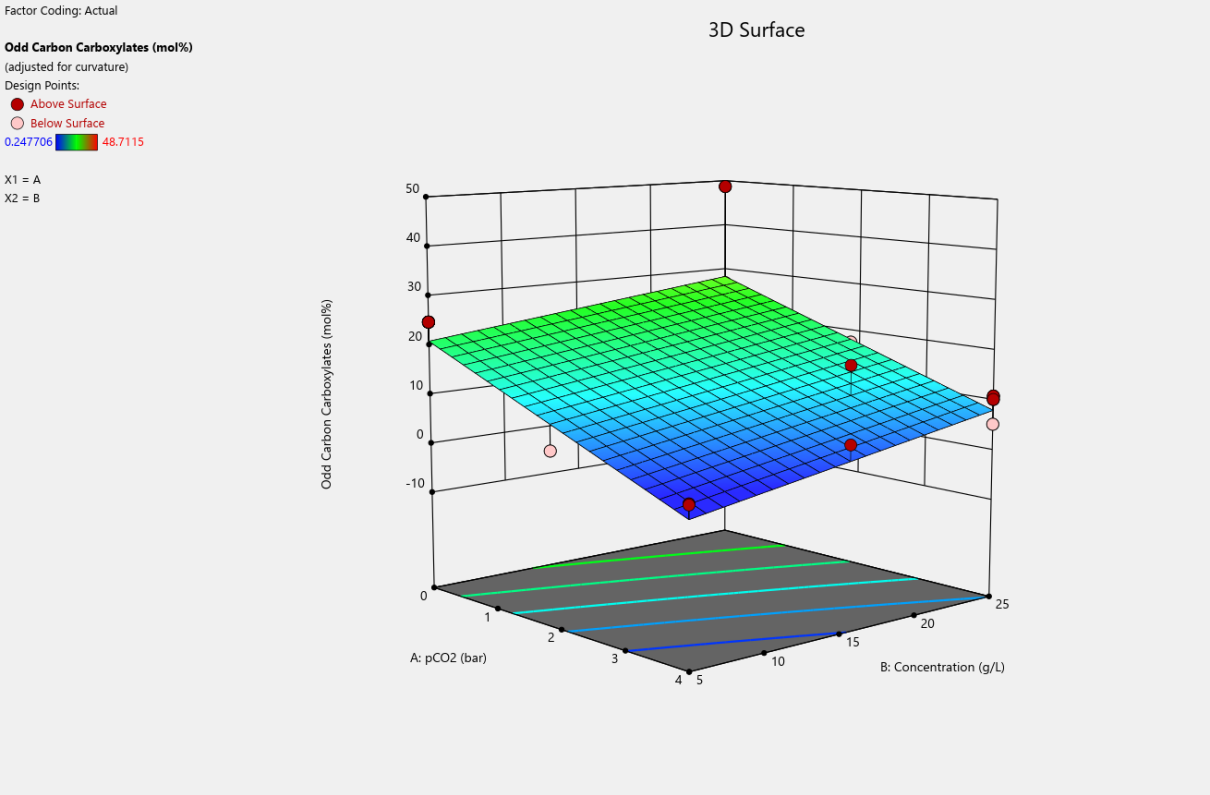

Figure 16: 3D Surface Depicting Odd Carbon Carboxylate Formation Across All Experimental Conditions

Fourth, alternative products of glucose fermentation such as oxaloacetate, malate, fumarate, and succinate were not detected by the HPLC analysis for any experimental conditions. This outcome was rather remarkable as according to the literature elevated pCO_2 levels were expected to stimulate the carboxylation reaction which results in the formation of these products. Other possible products of glucose fermentation such as glycerol, acetone, and isopropanol were also not detected for any experimental conditions. Furthermore, as can be seen from Figure 11-13, ethanol formation was only observed in control reactors irrespective of the substrate concentration. This observation is in line with the findings of Vezzu et al. who observed decreasing ethanol weight with increasing pCO_2 conditions and concomitant decrease in substrate concentration [93]. Additionally, it should be mentioned that minute peaks for sec-butanol were detected for multiple experimental conditions in this study. However, these peaks were assessed to be insignificant and thus were not included in the experimental results.

Fifth, a comparison of the product distribution data for ARBs and ERBs in Figures 11-13 reveals a clear metabolic shift from acetate and butyrate formation for elevated pCO_2 conditions compared to the control reactors where the dominant products were either acetate and lactate or acetate and ethanol. From Figure 11-13, it can be inferred that the highest concentration of lactate formation at 48% of total product

concentration occurred in control reactors with 25 g/L substrate concentration whereas the lowest lactate levels at 0-5% of total product concentration were observed for experimental conditions with 4 bar pCO_2 for substrate concentrations ranging from 5 g/L to 25 g/L. Similarly, the highest butyrate levels at 30% of total product concentration were measured in samples from experimental condition with 4 bar pCO_2 and 5 g/L substrate concentration. These observations are consistent with the conclusions reached by Hoelzle et al. who found the metabolic shift from butyrate to lactate and propionate to be a function of increasing substrate concentrations with butyrate being the dominant product in experimental conditions with low substrate concentrations and lactate being the dominant product in experimental conditions with high substrate concentrations [59]. Surprisingly, in this study lactate was found to accumulate in the liquid broth for all conditions where substrate concentration was increased from 5 g/L to 25 g/L regardless of the CO_2 levels.

4.1.4 Statistical Analysis

In order to test the hypothesis proposed in this study, statistical analysis was conducted using the substrate concentrations and pCO_2 levels as the factors of interest and the molar concentrations of reactants and products, as measured on the final day of experimental runs, as the response.

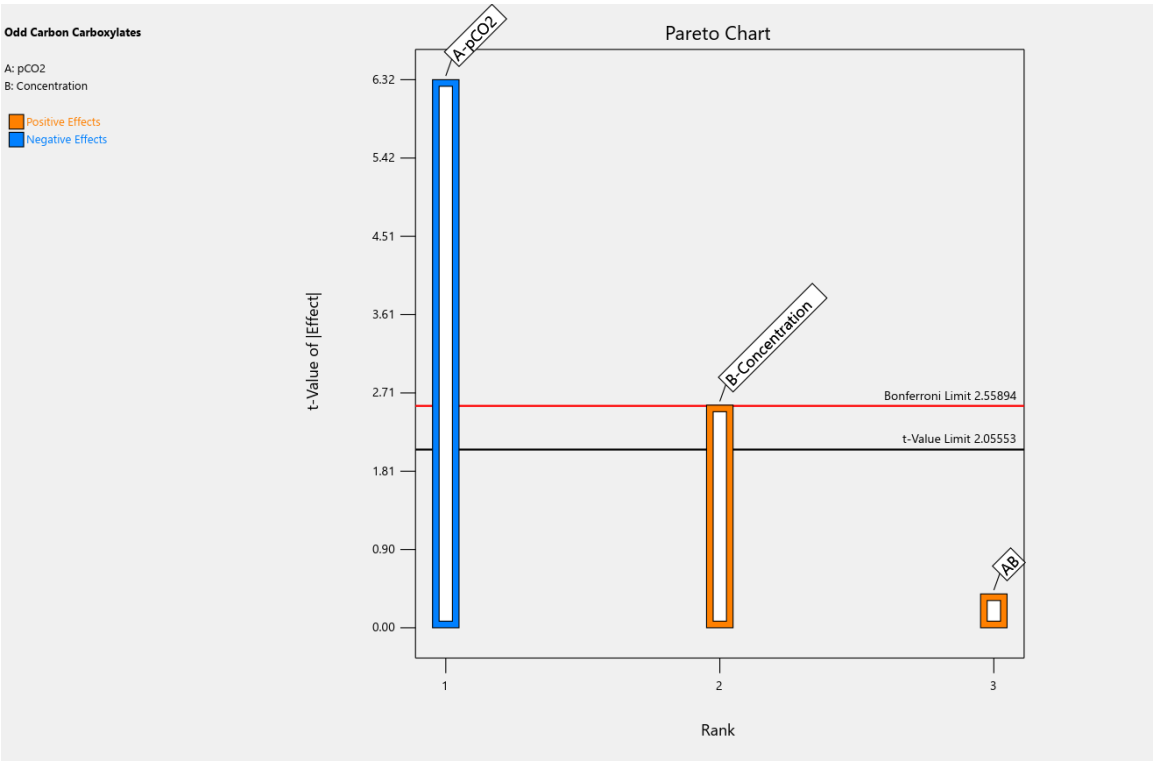

Figure 17: Pareto Chart Depicting Main & Interaction Effects on Odd Carbon Carboxylate Selectivity

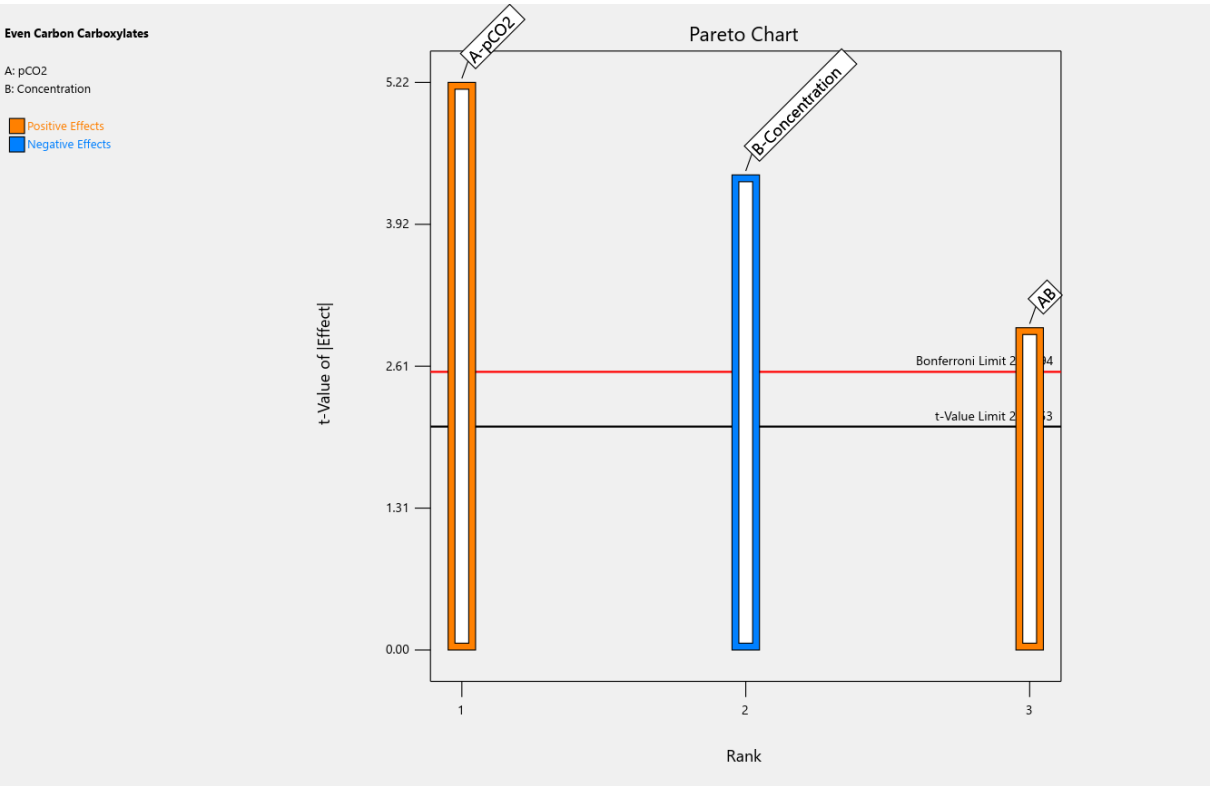

Figure 18: Pareto Chart Depicting Main & Interaction Effects on Even Carbon Carboxylate Selectivity

In the first step, t-test and ANOVA was used to determine the relationship between these factors and the response variables. The results of the t-test for odd-carbon carboxylates (OCC) and even-carbon carboxylates (ECC) are illustrated in Figures 17-18. It can be seen from the pareto chart illustrated in Figure 17 that the factor pCO_2 had a strong negative effect on the OCC concentration in the reactors whereas the effects of substrate concentration and its interaction with pCO_2 (AB) were weakly positive instead. However, only the effect of pCO_2 was found to be clearly significant ($p<0.0001$) as it was higher than the t-value limit and the Bonferroni limit. In comparison, the effect of substrate concentration may be significant ($p=0.0176$) as it was higher than the t-value limit but not the Bonferroni limit while the effect of AB on OCC concentration was clearly insignificant ($p=0.70$) as it was lower than the t-value limit.

In contrast to the effects on OCC, all the three terms were found to have significant effect on ECC as they were higher than the t-value limit, and the Bonferroni limit. This is apparent from Figure 18 which also indicates a negative effect of substrate concentration ($p=0.0002$) on ECC concentration along with the positive effects of the pCO_2 ($p<0.0001$) and AB ($p=0.0072$) terms on ECC. Additionally, a curvature check for OCC and ECC concentrations reveal significant curvatures caused by unknown source(s) for both the response variables.

Collectively, the results of t-test and ANOVA indicate that pCO_2 had a statistically significant negative effect on the OCC concentrations, t (1) = 6.32, $p < 0.0001$ and F = 39.94. Conversely, pCO_2 was found to have a statistically significant positive effect on ECC concentrations, t (1) = 5.22, $p < 0.0001$ and, F = 27.26. Thus, on basis of these statistical results the null hypotheses of no effects are refuted in favor of the alternative hypothesis H_1 (1.1-a) and H1 (1.1-b).

In the second step, the statistical analysis was repeated for the dominant fermentation product(s) belonging to the OCC and ECC categories. For ECC, the chosen dominant products were acetate and n-butyrate while for OCC, the chosen dominant product was lactate. The results of these analysis reveal that:

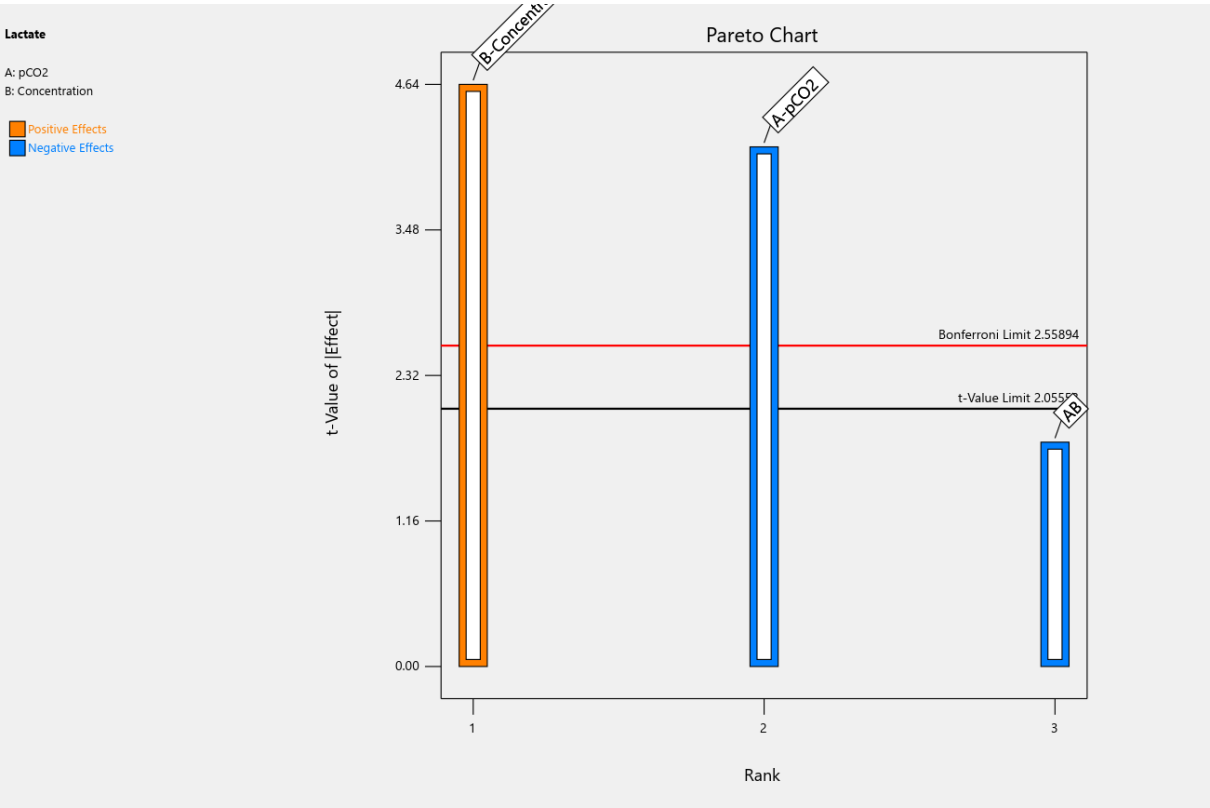

Figure 19: Pareto Chart Depicting Main & Interaction Effects on Lactate Selectivity

(1) While substrate concentration had a statistically significant positive effect ($p<0.0001$) on lactate concentration, the effect of pCO_2, while still statistically significant, was negative instead ($p=0.0004$).

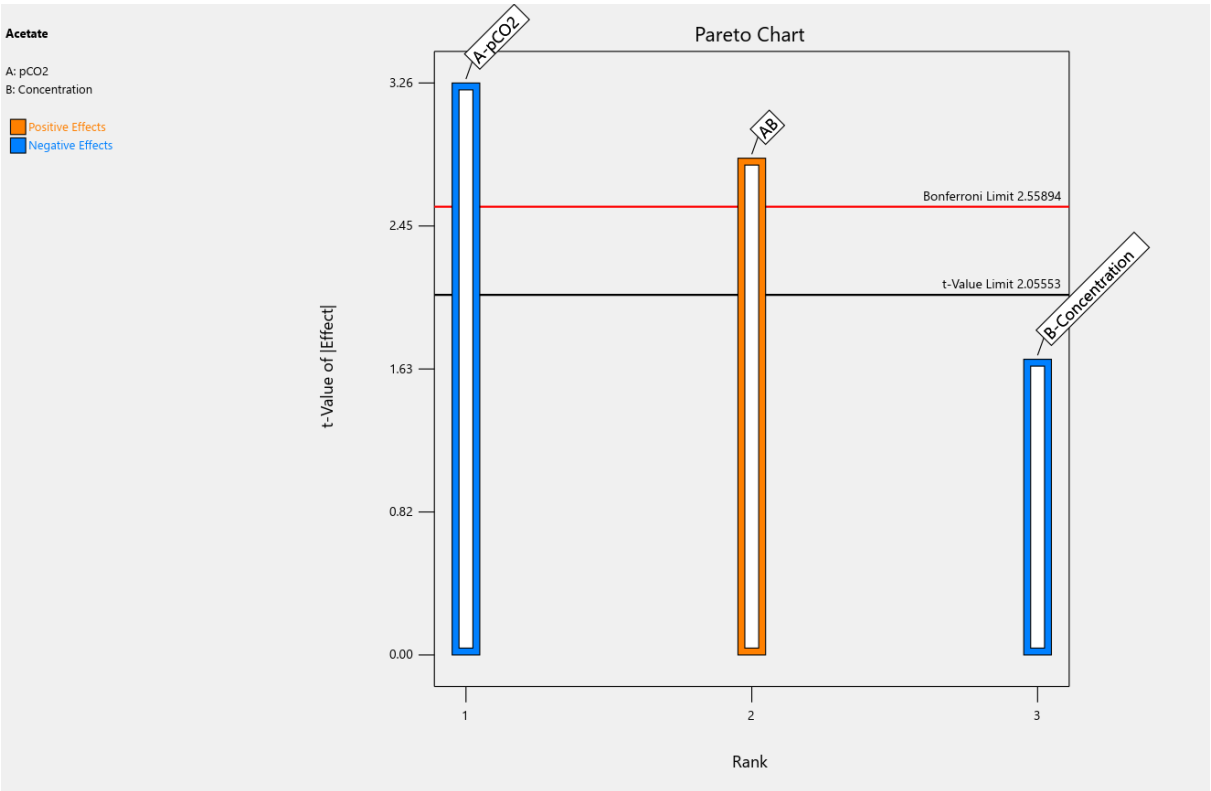

Figure 20: Pareto Chart Depicting Main & Interaction Effects on Acetate Selectivity

(2) Surprisingly, pCO_2 had a statistically significant negative effect on acetate concentrations in the reactors. This outcome was unexpected as the pCO_2 effect was found to be strongly positives for the ECC category to which acetate belongs.

(3) Only pCO_2 levels had statistically significant effect on n-butyrate concentrations in the reactors, and this effect was strongly positive. This outcome contradicts the conclusions reached by Arslan et al. according to which n-butyrate selectivity could be increase by both higher substrate concentrations as well as application of CO_2 in reactor headspace [23, 24].

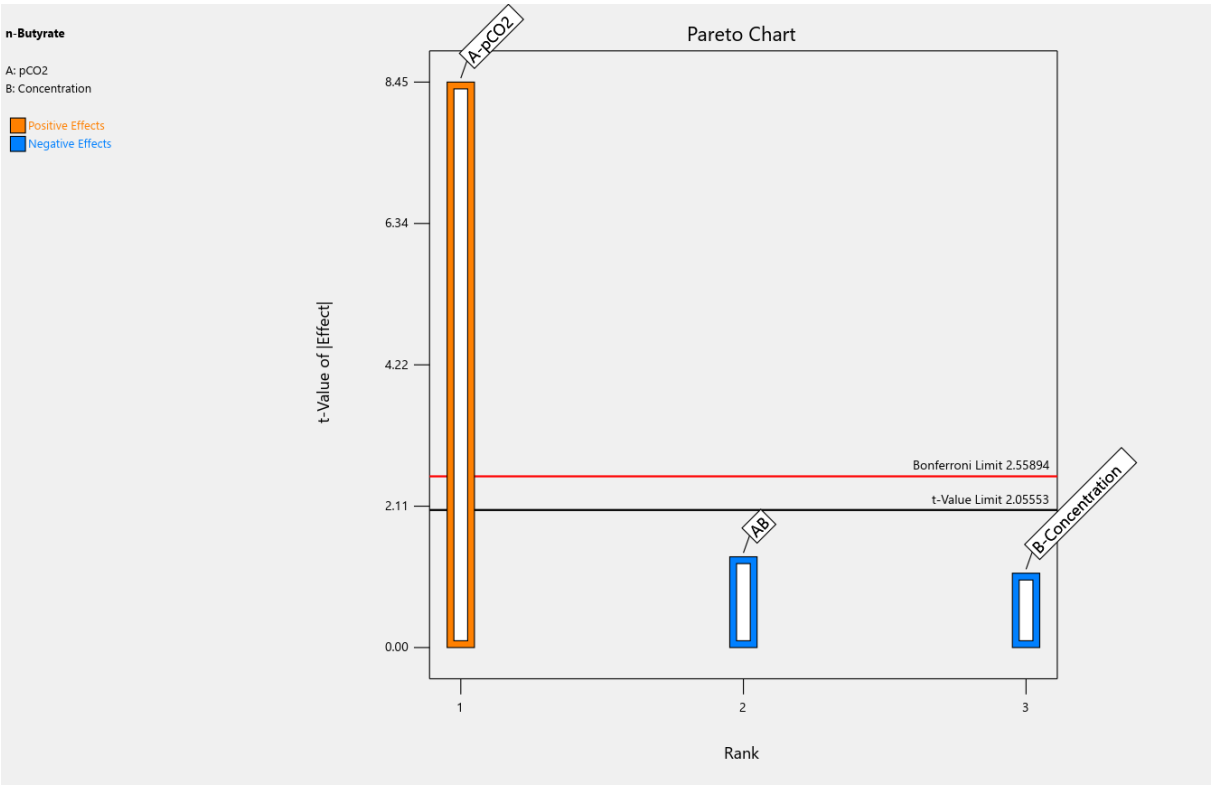

Figure 21: Pareto Chart Depicting Main & Interaction Effects on Butyrate Selectivity

4.2 Discussion

4.2.1 Effect of pCO_2 on total carboxylate concentration

The first finding of interest to this research was the effect of pCO_2 on total carboxylate production as described by Arslan et al. in their studies [23, 24]. According to the authors, after one week of incubation the total carboxylate production in the reactors subjected to 2 bar pCO_2 (EPB) was lower compared to the control reactor (APB). Whereas this concentration was 1.6 mgCOD/L for the reactor subjected to 2 bar pCO_2, in the control reactor the concentration reached 3.4 mgCOD/L [23, 24]. It should however be emphasized that the initial substrate concentration for both reactors was 8 gCOD/L which suggests that the carboxylate concentrations in both reactors were practically insignificant as they were three orders of magnitude less than the input concentration [23, 24]. Nevertheless, the authors ascribed the lower carboxylate concentration to the apparent inhibitory effect of pCO_2 on hydrolysis of complex substrate.

To test the hypothesis proposed by the authors, the effects of pCO_2 on total carboxylate production must be isolated from the effects of pCO_2 on hydrolysis of complex substrates. Therefore, the methodology of this study was designed to use a pure simple monosaccharide, glucose, as the substrate of choice while the hydraulic retention time was restricted to one week.

In general, the COD balance for the experimental conditions were found to be closed between 83-133% recoveries. This indicates a closed electron balance and confirms that the major products in the experimental runs were identified. The computed variations in the COD balance may be attributed to either measurement errors, or CO_2 assimilation by homoacetogens in the reactors subjected to elevated pCO_2 conditions. Furthermore, as can be seen in the data presented in Table 2, the differences in total carboxylate production between the control reactors and the reactors subjected to elevated pCO_2 levels were practically insignificant. This indicates that that the effect of pCO_2 on total carboxylates produced from fermentation of simple monosaccharides is insignificant. These results are in stark contrast with the outcomes reported earlier for the studies conducted by Arslan et al. [23, 24]. This suggests that the lower total carboxylate production reported by Arslan et al. may have been a result of adverse effects of pCO_2 on hydrolysis rather than the effects of pCO_2 on fermentation since a lower degree of hydrolysis would inevitably lead to lower product formation [23, 24].

4.2.2 Effect of pCO_2 on product formation

The second finding of interest to this research was the effect of pCO_2 on product formation described by Arsan et al. [23, 24]. In their study, the authors described the effects of pCO_2 on two carboxylates: butyrate and propionate [23, 24]. Whereas the authors reported that the application of pCO_2 in reactor favored the formation of butyrate, the production of propionate was found to be inhibited by the application of pCO_2 [23, 24]. Based on these results, the authors concluded that there are two ways for steering product formation from mixed culture fermentation of carbohydrates towards butyrate: (1) by increasing substrate concentration to sufficient level without modifying the headspace, and (2) by applying carbon dioxide in the headspace if the substrate concentration is below 13.5 gCOD/L) [24]. Therefore, to test the validity of these claims, a between-subject mixed factorial design study was developed to gauge both, the main and the interaction effects of substrate concentration and pCO_2 on product formation. It was anticipated that increase in pCO_2 levels would steer product formation towards butyrate formation while the increase in substrate concentration level would direct the product formation towards lactate and/or propionate production instead.

4.2.2.1 Effect of pCO_2 on even-carbon carboxylates

For testing whether increasing the substrate concentration results in higher butyrate production, the data acquired only from APB experiments were used for comparative analysis. The results of these experiments revealed that, contrary to the claim made by Arslan et al., increasing the substrate concentration steers the product formation towards lactate and propionate instead of butyrate [23, 24]. This is

supported by the fact that both, the selectivity and the concentration of lactate and propionate were found to increase with increase in glucose concentration in control reactors whereas the selectivity and concentration of butyrate were found to be comparable for all substrate concentration levels. Furthermore, the statistical analysis also indicates that the factor substrate concentration had insignificant effect on butyrate formation while its effect on formation of OCC were found to be significant instead. Consequently, this implies that the butyrate formation in this study could not have occurred only through the conversion of glucose to butyrate as described in Reactions 8 and 9 because in that case the effect of substrate concentration on butyrate formation would have been statistically significant.

As mentioned in the literature review, there are four other metabolic pathways which can lead to butyrate formation during mixed culture fermentation of glucose: (1) oxidation of lactate to butyrate; (2) interconversion of acetate and butyrate; (3) reverse beta oxidation, and (4) fatty acid biosynthesis []. However, the results of this study do not completely match the stoichiometry of the first three reactions as the amount of butyrate produced exceeds the amount of lactate, acetate, H_2 and CO_2 consumed. Thus, the high levels of butyrate formed cannot be justified only on basis of one of these reactions. Therefore, it is less plausible that these metabolic routes were utilized to produce n-butyrate.

For testing whether the application of carbon dioxide in the headspace could steer product formation towards butyrate formation, the data acquired from all the experiments were used for comparative and statistical analysis. The results of these analysis suggest that the claim made by Arslan et al. underemphasized the role of carbon dioxide in butyrate formation as the application of carbon dioxide in headspace was found to increase the butyrate formation not only for experimental conditions with substrate concentration below 13.5 gCOD/L but also for the experimental conditions with higher substrate concentration [23, 24]. This is supported by the fact that the selectivity and concentration of butyrate were always found to be higher in EPBs compared to the control reactors (APBs). Furthermore, the statistical analysis of these results revealed that pCO_2 is a statistically significant contributory factor to butyrate formation. Consequently, this indicates that the pCO_2 may be at least partially responsible for the high levels of butyrate selectivity and concentration observed in this study due to its stimulating effect on the FAB Type 2 pathway [32]. Such a causality would be in line with the observation made by Arslan et al. regarding CO_2 effecting the butyrate formation mechanism in some way without being consumed [23, 24].

4.2.2.2 Effect of pCO_2 on odd-carbon carboxylates

In contrast to the stimulatory effect of pCO_2 on butyrate formation, there is much less information about the inhibitory effects of pCO_2 on lactate and propionate formation. Therefore, in view of this paucity of information in prior studies, this

research would proffer a possible theory regarding the mechanism through which pCO_2 could potentially inhibit the formation of lactate and propionate. However, before such a theory concerning the effects of pCO_2 on OCC formation can be presented, it is imperative to first understand how OCC such as lactate and propionate are formed. The empirical and theoretical evidence central to the formation of OCC are as follows:

1. **Carbohydrate metabolism can only yield Propanoyl-CoA through the acrylate pathway.** As mentioned in the literature review, there are three metabolic pathways through which carbohydrate metabolism can produce propionate: (1) acrylate pathway; (2) 1,2-propanediol pathway and, (3) dicarboxylate pathway. Despite the diversity of pathways available for propionate formation, the final reaction leading to its production always involves an interconversion reaction in which the -CoA from propanoyl-CoA is transferred to another carboxylate such as succinate, lactate, or acetate [36]. It should be emphasized that while propionate formation can occur through the 1,2-propanediol pathway, there is a lack of biochemical evidence to support the existence of this pathway. This implies that the acrylate pathway is the only known source of propanoyl-CoA formation. Consequently, this means that propionate formation through any pathway is conditional upon the formation of lactate from pyruvate reduction.

2. **Carbohydrate metabolism through EMP produces carboxylates and not carboxylic acids.** During anaerobic fermentation, the formation of "carboxylic acids" begins with the phosphoglycerate kinase reaction involving transfer of phosphoryl-group from C-1 of 1,3-biphosphoglycerate to ADP to yield 3-Phosphoglycerate and ATP [32-34]. The removal of this phosphoryl-group leaves an ionized carboxylic acid functional group which remains the same for 2-phosphoglycerate, phosphoenolpyruvate, pyruvate and finally lactate. This implies that during the course of carbohydrate fermentation, there is never a production of 3-phosphoglyceric acid or 2-phosphoglyceric acid or phosphoenolpyruvic acid or pyruvic acid or lactic acid as there is never a proton to be dissociated from glycolytic acid intermediates [32, 34, 94].

3. **Lactate fermentation consumes proton(s) and not molecular hydrogen.** In his seminal papers, Robergs et al. proved that the biological production of lactic acid is not possible and that the in the cells exposed to systemic physiological range of pH, the glycolytic metabolites function as bases and not acids [34, 94]. Additionally, the authors also elaborated upon the biochemistry of lactate formation through the lactate dehydrogenase (LDH) reaction to prove that lactate formation consumes proton(s) released during glycolysis [33, 34]. This implies that the conventional explanation for lactate formation in the field of AD and MCF is incorrect as it is grounded on the belief that such formation requires consumption of H_2 which is not the case [23, 24, 31, 95]. Indeed, if this were the

case then lactate formation in non-mitochondrial cells such as red blood cells and erythrocytes would never occur as the only source for molecular hydrogen in humans are the microbial communities in the gut [32-34]. Nevertheless, this is not the case as lactate is in fact produced in these non-mitochondrial cells despite the absence of molecular hydrogen (H_2) . This indicates that presence of hydrogen is not necessary for the LDH reaction to take place as it is the proton and/or hydride ions and not H_2 which are the true substrates for lactate formation [32-34]. This would also explain Consequently, this suggests that the chemical reactions predominantly used in MCF literature to present propionate production from glucose or pyruvate are also incorrect as, much like lactate production reaction, the propionate formation reaction consumes proton and/or hydride ions instead of molecular hydrogen. If this is indeed true, then the correct reactions for production of lactate and propionate from pyruvate would be as follows:

$$C_3H_3O_3^- + NADH + H^+ \rightarrow C_3H_5O_3^- + NAD^+$$

$$C_3H_3O_3^- + 2 \cdot NADH + 2 \cdot H^+ \rightarrow C_3H_5O_2^- + H_2O + 2 \cdot NAD^+$$

Additionally, if these reactions indeed consume proton and not H_2 then it would explain why elevated molecular hydrogen levels (pH_2) did not result in increased propionate formation in the studies conducted by Ren et al. and Arslan et al [23, 24, 83].

From the statistical and product distribution analysis conducted in this research it can be inferred that administration of elevated pCO_2 in reactor headspace stimulated the butyrate formation. As mentioned earlier, the production of butyrate could have occurred through five different reactions: (1) fermentation of glucose into butyrate; (2) oxidation of lactate to butyrate; (3) interconversion of acetate and butyrate; (4) reverse beta oxidation, and (5) type 2 fatty acid biosynthesis reaction. However, it is unlikely that the observed increase in butyrate formation could be contributed to the glucose fermentation reaction as the factor substrate concentration was found to have a statistically significant strong negative effect on butyrate selectivity while increasing the substrate concentration was found to steer product formation towards lactate and propionate instead of butyrate. Additionally, the results of this study do not completely match the stoichiometry of second, third and fourth butyrate forming reactions as the amount of butyrate produced exceeds the amount of lactate, acetate, H_2 and CO_2 consumed. Thus, it is less plausible that these metabolic routes were utilized to produce n-butyrate as the high levels of butyrate formed cannot be justified only on basis of one of these reactions. This suggests that the observed increase in butyrate formation may have been caused, at least partially, by the FAB type 2 pathway. This inference is supported by the fact that the elevated pCO_2 conditions would have inevitably led to increase in bicarbonate

buffer pool size inside the cell which is required by FAB type 2 pathway to proceed. If we entertain the possibility that the administration of elevated pCO_2 in the reactor headspace did in fact result in increased butyrate formation through FAB type 2 pathway, then it follows that the increased bicarbonate buffer system thus formed would have adverse effects of lactate and propionate formation due to competitive inhibition for consumption of H^+ which is a common substrate for both carbonic anhydrase and LDH reaction. Consequently, this would explain why pCO_2 has a statistically significant negative effect on OCC and lactate formation and why the lactate and propionate selectivity was found to decrease following the application of pCO_2 in the reactor headspace. Indeed, such a reciprocal relation between bicarbonate ion and lactate formation has been well documented in the medical research where the bicarbonate ion concentration has been found to decrease with a concomitant increase in lactate formation [96-99]. Since the metabolic reactions from glycolysis to LDH reaction are essentially the same in all living cells, it can be assumed that the microbial community in mixed culture fermentation studies would behave similarly the cells in the aforementioned medical research following the administration of elevated pCO_2 in the reactor headspace.

5. Conclusion

5.1 Conclusions

The key objectives of this research were: (1) to investigate the effect of initial substrate concentrations (g/L) and elevated pCO_2 conditions on the product spectrum of glucose fermentation under mesophilic conditions, and (2) to reevaluate the findings of Arslan et al. by replicating their studies across experimental conditions differing from those utilized in the original studies [24]. The conclusions reached through the analysis of data acquired from the statistically designed experiments are as follows:

- **Elevated pCO_2 levels stimulates butyrate formation from fermentation of glucose.** This study confirms that application of carbon dioxide in the reactor headspace has statistically significant effect on butyrate formation $t(1) = 8.45$, $p < 0.0001$ and, $F = 74.42$. In the reactors with elevated levels of CO_2 the selectivity reached up to 30.41% when substrate concentrations were between 5-15 g/L whereas in control reactors with same substrate concentration range the highest butyrate selectivity was measured to be 11.72%. Similarly, for EPBs with substrate concentrations between 15-25 g/L, the butyrate selectivity reached up to 25.77% whereas for the control reactors with same substrate concentration range the highest butyrate selectivity was only 9.37%.

- **Elevated pCO_2 levels inhibit production of lactate from glucose fermentation.** One of the more significant findings to emerge from this study is the inhibition of lactate formation from glucose fermentation due to elevated levels of pCO_2 in batch reactors. Whereas for APBs the highest lactate selectivity was measured to be 47.95% for substrate concentration of 25 g/L, for EPBs the highest selectivity was measured to be only 15.13% when 20 g/L of glucose was fermented under 3 bar pCO_2. Furthermore, the effect of pCO_2 was found to have a strong negative effect of lactate and this effect was statistically significant, $p < 0.0004$ and, $F = 17.16$.

- **Increasing the initial substrate concentration in control reactors results in increased production of lactate.** Contrary to the findings reported by Arslan et al. the findings of this study clearly indicate that increasing the initial substrate concentration in batch reactors without modifying the reactor headspace results in increased formation of lactate and not butyrate [24]. Whereas for APBs the highest lactate selectivity was measured to be 47.95% for substrate concentration of 25 g/L, for EPBs the highest selectivity was measured to be 15.13% when the substrate concentration was 20 g/L and pCO_2 level was 3 bars. Furthermore, the effect of substrate concentration on

lactate selectivity was found to be statistically significant, $t(1) = 4.64$, $p < 0.0001$ and, $F = 21.54$, whereas its effect on butyrate selectivity was found to be insignificant.

- **There is no evidence that elevated pCO_2 conditions adversely effects the total carboxylate production from mixed culture fermentation of carbohydrates.** This study has shown that, contrary to the findings of Arslan et al., the application of carbon dioxide in the reactor headspace did not result in lower total carboxylate formation from fermentation of glucose [23, 24]. In fact, after seven days of fermentation the total carboxylate concentration in EPBs were found to be comparable to those in control reactors.

- **Application of CO_2 in reactor headspace stimulated the formation of molecular hydrogen.** This study has shown that compared to control reactors there is concomitant increase in H_2 formation with increase in CO_2 levels in the reactor. Following seven days of glucose fermentation, the highest selectivity of H2 at 64.97% was observed in EPBs for conditions with 4 bar pCO_2 and 5 g/L substrate concentration. In comparison, the highest selectivity of H2 at 47.93% in control reactors was observed for the atmospheric condition with substrate concentration of 10 g/L. This effect of pCO_2 was also found to be statistically significant $t(1) = 7.61$, $p < 0.0001$ and, $F = 57.89$.

5.2 Limitations & Recommendations

- **Confirm the role of FAB Type 2 pathway in butyrate formation through metagenomic analysis.** In this study the contribution of FAB type 2 pathway towards butyrate formation in EPBs was deduced on the basis of statistical and stoichiometric analysis. As such the biophysiochemical analysis utilized in this study were limited to liquid and gas chromatography. Therefore, a natural progression of this work would be to investigate the effect of pCO_2 on expression of gene associated with FAB type 2 pathway using metagenomic analysis in randomized controlled trials. It is expected that in comparison with the control reactors, the genes expressing FAB type 2 pathway would be more active in reactors subjected to elevated CO_2 levels and consequently the butyrate yield in these reactors would be higher.

- **Asses the main and interaction effect of pH, pCO_2 and substrate concentration on product formation using a central composite experimental design**. In the current study a two-factor statistical experiment design was utilized to gauge the main and interaction effect of initial substrate

concentration and pCO_2 on product formation. However, this experimental design is inadequate for gauging the effect of tertium quid such as the pH which is widely accepted to have an effect on product formation. Therefore, it is recommended that further studies utilize a three-factor central composite experimental design to study the main and interaction effects of pH, initial substrate concentration and pCO_2. Such an undertaking would require considerably more work, but nevertheless it would help ascertain the degree to which the pH and its interactions with substrate concentration and pCO_2 influence product formation from fermentation of glucose.

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7. Appendix A

Table 3: Composition of Macronutrients Solution Used in this Study

Macronutrients	Concentration
NaCl	0.5 g/L
$MgCl_2.6H_2O$	0.5 g/L
$K_2HPO_4.3H_2O$	0.5 g/L
$Na_2S.9H_2O$	0.5 g/L
NH_4Cl	5 g/L

Table 4: Composition of Micronutrients Solution Used in this Study

Micronutrients	Concentration
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.0005 g/L
$CoCl_2.6H_2O$	0.0005 g/L
$CaCl_2.2H_2O$	0.006 g/L
$FeCl_2.4H_2O$	0.01 g/L
$MnCl_2.4H_2O$	0.0005 g/L
$NiCl_2.6H_2O$	0.0005 g/L
$Na_2SeO_3.5H_2O$	0.0005 g/L
$Na_2WO_4.2H_2O$	0.0005 g/L
H_3BO_3	0.0005 g/L
$CuCl_2.2H_2O$	0.0005 g/L
$ZnCl_2$	0.0005 g/L
$AlCl_3$	0.0005 g/L
EDTA	2.5 g/L

Table 5: Composition of Vitamin Solution Used in this Study

Vitamins	Concentration
D-Biotin	0.0005 g/L
Choline Chloride	0.0005 g/L
Folic Acid	0.0005 g/L
myo-Inositol	0.001 g/L
Niacinamide	0.0005 g/L
p-Amino Benzoic Acid	-
D-Pantothenic Acid•½Ca	0.0005 g/L
"Pyridoxal.HCl	-
Pyridoxine•HCl"	0.0005 g/L
Riboflavin	0.00005 g/L
Thiamine•HCl	0.0005 g/L
Vitamin B-12	-
NaCl	0.0425 g/L

Table 6: Composition of Anaerobic Basal Broth Used in this Study

Activation Media	Concentration
Peptone	0.8 g/L
Yeast Extract	0.35 g/L
Sodium chloride	0.25 g/L
Starch	0.05 g/L
Dextrose	0.05 g/L
Sodium pyruvate	0.05 g/L

Arginine	0.05 g/L
Sodium succinate	0.025 g/L
Sodium bicarbonate	0.02 g/L
L-Cysteine HCl	0.025 g/L
Ferric pyrophosphate	0.025 g/L
Hemin	0.00025 g/L
Vitamin K	0.000025 g/L
Dithiothreitol	0.05 g/L
Sodium thioglycollate	0.025 g/L