



Application of a fermentation strategy to produce succinic acid using *Actinobacillus succinogenes*

Alaa Salma

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Par

« **Alaa SALMA** »

« **Application of a fermentation strategy to produce succinic acid using
*Actinobacillus succinogenes*** »

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Details create the big picture.

Before my PhD, I used to admire people who knew exactly what they wanted in life, people who not only have the conviction but also the persistence and dedication to focus all their energy on one specific goal. The older I get the more such people made me feel anxious about my own future and life decisions. Like many others, I started to compare myself to those people and thought I felt short in every aspect of life. At some point I tried to convince myself that by traveling and working around the world, me and my life's purpose would be bound to eventually cross paths, telling myself when it would I'd be surely become as dedicated as them.

During my PhD, every time I found particular interest in something new, I asked myself if this is the one thing I could be great at, the one thing I just might pursue for the rest of my life. Considering that I am a person of many interests it is needless to say that none of these passionate episodes lasted all too long. Which in turn made me feel like I was wasting my time and that I was giving up on everything that I had started.

After my PhD, I realized that for the entire time that I was searching for the one big piece to fill the void, all I had to do was take a step back and see all the little pieces, the ones that I had given up on, becoming one. This PhD taught me that filling my void is a process of growth. What I previously viewed as mostly failures were actually valuable pieces of lessons learned knowledge as well as understanding only gained by experience. Those experiences may have been positive or negative, but either way it always resulted in new skills and knowledge, shaping me into who I am.

Dedication

Most of all, thanks to God the Divine who continues to make the impossible possible.

There are a number of people without whom this thesis might not have been written, and to whom I am greatly indebted.

To the soul of my grandparents...

To my dear Dad, Muhamed, who represented to me 'living proof' of women ability to redefine and recreate our lives and who always picked me up on time and encouraged me to go on every adventure, especially this one!

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General introduction

General introduction

Over the years, coal, gas and oil have been used in the chemical industry to produce a wide majority of industrial products [1]. However, with diminishing supplies of these resources and the looming dangers of excessive carbon dioxide (CO₂) emissions, there is a dire need to replace and to develop a sustainable and renewable based economy that allows to produce the same products in a safe and cost effective way [2–4]. Green technology using renewable resource is therefore becoming a driving force in the chemical industry [5,6]. Biomass as a renewable resource (e.g., wood, lignocellulosics crop, whey, corn, sugarcane, etc..) has proved its economic and sustainable potential for replacing a large petroleum-based bulk chemical market [2,6]. Several companies have evaluated the potential of both bio-based chemicals and polymers to identify lucrative products including glycerol, dicarboxylic acid, fatty acids and others [7,8]. One of the most promising contenders is succinic acid [9], which is a great platform chemical because of its ability to convert into several important products and the relative ease of its bio-based production [10,11].

Succinic acid (SA) is a substance used in many industries such as: plastics, textiles, pharmaceuticals, solvents, and as a food additive [12]. Today, SA is predominately produced from a four-carbon hydrocarbon via maleic anhydride, utilizing the C₄-fraction of naphtha at about a total weight of 15,000 tons per year [13]. For this reason, it may be appropriate for companies to develop and to invest in an alternative process as the production of bio-based succinic acid [14]. Succinic acid is an intermediate of the citric acid cycle and is one of the end-products of anaerobic metabolism during fermentation of a number of microorganisms [12]. Bio-based succinic acid production avoids the use of this route and instead takes advantage of a fermentation process [15]. Fermentation is an energy-deriving reactions occurring in eukaryotic and prokaryotic organisms, which converts a simple carbon source into various volatile fatty, acids and gas [7,16]. This reaction is more specifically carried out by bacterial or fungal cells that possess specific genes encoding enzymes necessary for their pathways; each pathway contains its own distinctive intermediates, co-factors, and enzymes [17]. One of the best features is that CO₂ is needed as a second substrate by the microorganisms for succinic acid production [18–20]. Therefore, in addition to producing succinic acid, this process uses up CO₂ [21,22]. Such a process was described using ruminal bacteria [9,21]. Despite the huge number of research studies concerning the amelioration of microorganisms through genetic engineering techniques, at present only a few genetically modified microorganisms (GMM) strains are commercially used or pending approval [23–25]. This is mainly due to the reluctance

of the public regarding this subject and to legal restraints [26]. In the process thesis under review, succinic acid is produced by native microorganisms using glucose and/or fructose as fermentation feedstock.

As described in detail later in this thesis, many improvements have been made to this process, which entails the addition of one of tricarboxylic cycle acid (TCA) components to these microorganisms to increase both the rate of production and the overall yield of succinic acid. A number of experiments will be discussed regarding changes of medium, types of substrates, metabolic pathways, partnership with another microorganisms and electro-fermentation, etc. These novel ideas serve to improve the efficiency and quantity of succinic acid produced, with a concomitant decrease in the production of by-products. This dissertation examines the major technical aspects of bio-based succinic acid, with descriptions and discussion of two modes of culture: Pure and Co-culture technologies and fermentation /electro-fermentation.

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Objectives of the thesis

This work focuses on the development of a bio-based process for the production of succinic acid in three different targets. The main target was to optimize the production of succinic acid as well as to develop new process toward the succinic acid production. The second was to combine an electrochemical cell within the fermentation process in a new concept to produce succinic acid to improve the process efficiency in addition to the application of scale-up studies. The third was to apply a co-culture system using mixed inocula containing both *Actinobacillus succinogenes* and *Saccharomyces cerevisiae*, which can be an attractive biotechnological solution that combines the advantages of succinic acid production without the need for a costly mineral carbon source as well as gives idea about how microorganisms interact to each other.

Thesis Layout

This thesis develops an innovative method to enhance the bioproduction of the succinic acid through novel technology of co-culture and bioelectrochemical process. The manuscript is divided into four main parts: a literature review (Chapter 1), Material and methods (Chapter 2), Results and discussion (Chapters 3-5) followed by Conclusions and perspectives. The work completed in this thesis meets these objectives in the following ways:

The current state of the art research in the platform molecule field as well as the global bio-based demand and supply of current and future resources are introduced in chapter 1. From this chapter, it becomes apparent that existing succinic acid industries must undertake many critical changes towards sustainable development. The fermentation which investigates the catalytic and the biological conversion of glucose, fructose, sucrose, galactose and xylose to value added chemicals are also emphasized.

In Chapter 2, the succinic acid fermentation from substrate using pure and co-culture microorganisms is introduced. The methodology that is used, in this study, to implement the biological experiments is developed and a detailed description considering the analytical instrumentation that was employed is also given.

In Chapter 3, fermentation runs with optimization studies for maximizing yield, concentration and productivity of succinic acid in batch systems are conducted. The predictions of Tricarboxylic cycle acid key elements (Fumarate C4-dicarboxylic) additions to improve succinic acid are validated with experimental runs in conditions designed by optimization studies.

In Chapter 4, scale up studies are executed at laboratory scale in the optimal conditions for succinic acid production to generate data that can be used to anticipate and design large scale

industrial fermenters with great confidence that they will run as expected. In addition, the electro-fermentation tools, as well as the parameters of the procedure, are described and preliminary experimental data are given to compare with the experimental results, obtained in Chapter 3.

In Chapter 5, the use of co-culture technology for the efficient production of chemicals is emphasized. An overview of existing experimental and theoretical co-culture set-ups in synthetic biology and adjacent fields is given here, and challenges and opportunities involved in such experiments are discussed. Here, we build up a partnership between *A. succinogenes* and *S. cerevisiae* to construct a co-culture in order to produce succinic acid by efficient co-utilization of sugar mixtures. The concept centers on the fact that during fermentation, the *S. cerevisiae* yeast strain consume the oxygen present in the media and produce carbon dioxide; this later could be used as mineral carbon source by the *A. succinogenes* strain and allowing that way succinic acid production.

The overall conclusions of this study are extracted and presented with many recommendations. Moreover, implications of research for future work indicating the directions modern biorefineries should follow towards sustainable development are also included.

Chapter I. Platform molecule from sustainable raw materials; case study succinic acid

Preamble

Facing the shortage of crude oil supply and demand of sustainable development, biological production of platform molecules from renewable resources has become a topic of worldwide interest. Succinic acid is a four-carbon dicarboxylic acid, which has attracted much interest due to its abroad usage as a precursor of many industrially important chemicals in the food, chemicals, and pharmaceutical industries.

In recent decades, robust producing strain selection, metabolic engineering of model strains, and process optimization for succinic acid production have been developed. This review provides an overview of platform molecules production technology; highlight some of the successful metabolic engineering approaches.

Platform molecule from sustainable raw materials; case study succinic acid

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Abstract

Platform molecules were defined by the US Department of Energy, as bio-based or bio-derived chemical where their constituting elements originated wholly from biomass and that can be utilized as building blocks for production of commodity and fine chemicals. These chemicals can subsequently be converted into a number of high-value bio-based chemicals or materials. Nowadays, there is a growing concern towards discovering a more economically and environmentally cleaner way for production of platform molecules from renewable substrate as a carbon source. Succinic acid (SA) is considered as key platform chemical since it is used as precursor for other valuable chemicals and has drawn worldwide interests for its wide applications. This review aims to highlight the currently available information about mechanisms involved platform molecule production, especially the SA production. In this review, the processing technologies used in platform molecules production are described, in addition to the information regarding key parameters optimisation, genetic engineering mechanisms, redox potential and purification processes. These lasts, known as alternative cost-competitive providers for fossil fuels.

Keywords: platform molecules; biomass; succinic acid production; fermentation; downstream separation methods; processing technology; redox potential.

I. Background

Early in the 20th century, non- renewable raw materials satisfy our energy needs and provide a broad spectrum of chemicals that enrich our daily live [1]. By 2035 the International Energy Agency (IEA) predicts a significant drop of about 75% of fossil raw materials. However, the depletion of fossil feedstocks followed by vigorous consumers demand for eco-friendly energy sources has made the governments as well as the industry interested in the production of chemicals from bio-based resources, to form a sustainable global economy, unlimited and ubiquitous alternative to chemical industry, which should replace the current petroleum based methods to produce energy and chemicals [2]. The United State Department of energy (US DOE) compiled a list of above 300 possible chemicals as building block from a wide assortment of raw materials in an accessible database, according to the US DOE, the National Renewable Energy Laboratory reports (NREL) and the academic patents available in literature. This database comprises the chemical name designation as a commodity, structure, feedstock source, current potential production processes and its specialty for the consumer goods. A screening criterion was set using feedstock, estimated cost of process, actual market volumes and prices. In the petrochemical industry all products derived from 7 or 8 foundation chemicals (Table1). A different approach was developed from the initial list of 300 chemicals; 30 building blocks chemicals were considered as analogues to the petrochemical industry and they were selected first using the petrochemical criteria considered as down selection number 1. Later the biorefinery strategic fit criteria considered as down selection number 2 (direct product substitution that competes directly against existing products and chemicals derived from petroleum, novel products that possesses new and improved properties for replacement of existing functionality or new applications, and potential utility of these chemicals as building block intermediate that provide basis of a diverse portfolio of chemicals from a single intermediate). These products and materials were mostly produced by petrochemical industry. Twenty years ago, the researchers have striven to prove that bio-economy could provide similar products, by set of inexpensive, simple and eco-friendly chemical building block from biomass, also known as a platform molecule. In the late twentieth century, a new concept of bio-derived platform molecules was envisaged; as they are small simple molecules basically biomass derived and might be utilized as building blocks for high-value added chemicals and materials. In 2004, focused and intensive research was oriented towards product derived from biomass and a report by the US DOE on Top Value Added Chemicals from Biomass was published [3]. It has been widely claimed that biomass feedstock can replace fossil fuels and implies a

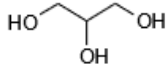
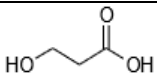
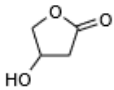
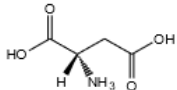
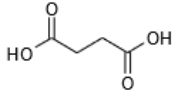
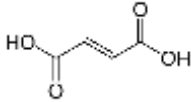
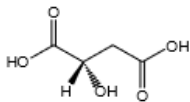
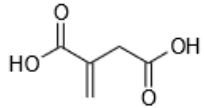
successful execution of integrated biorefineries competitively against petroleum refineries, in order of manufacturing fuels, energy, chemical and material products. A critical assessment was set by the US DOE towards the progression and the acceleration of a bio-based economy and the implementation of biorefineries technics, by generating a large list of biomass-derivable chemicals, the most top promising candidates, and thus emphasis efforts in universal research. Biorefinery products, which represent annually $\leq 10\%$ of fossil resources extract, would be a primary source of dividends in many operations and often petrochemicals way profits a higher sideline than energy and fossil fuels. However, the report made by the US DOE in 2004 interestingly debated the analogy to base chemicals fossil-derived from sugars, lignin, syngas and triglycerides, but did not perform a reference with regard to 'platform molecule' term [4]. The aim of US DOE report was to list on a significant scale, the molecules produced which have a set of options to be transformed to a higher-value added chemicals and materials [5]. The down selection round 2 determined 12 building blocks sugar-derived from the top 30 building block candidates, and their structure are given in table 2.

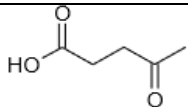
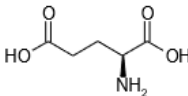
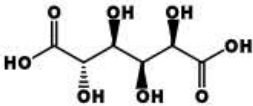
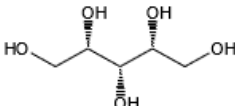
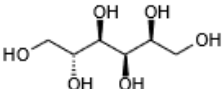

This review highlights the power and the potential of platform molecules to be used as biochemical replacing chemicals from fossil resources. Additionally, it serves the purpose of reviewing the pertinent literature, and therefore, the main advances put forward in the last years, so as to create the necessary base for a constructive and supported review. However, in order to eventually replace fossil resources that are depleted, the bio-economy require ways to produce the range of familiar chemicals that are crucial in our live, mainly synthetic textiles, surfactants, pharmaceuticals, plastics, pigments, dyes and agrochemicals, also home and personal care products. In this review the status of base chemicals fossil derived industry is shortly described as building blocks chemicals and equivalent bio-based chemicals is debated. Actual notion of platform molecules is under discussion, leading to a label definition of those small bio derived chemicals. The composition of biomass feedstock in its constituent parts with their derivable chemicals from each composite are featured, regarding to molecules obtainable from it. A vast list of platform molecules is given and their processing technologies from fossil and biomass feedstocks are described.

Table 1. Base chemicals fossil-derived

Feedstocks	Base chemicals	Commodity chemical	Intermediates Chemicals	Consumer goods	References
Naphtha	Ethene	Ethylene oxide	-Polyethylene oxide -Antifreeze -Polyesters	Transportation	[6]
		1,2-dichloroethan		Housing	[7]
		Polyethylene			[8]
		Vinyl chloride	-Polyvinyl chloride	Health, hygiene	[9]
		Ethylbenzene	-Solvents	Housing, safe food supply	[10]
		Styrene	-Styrene butadiene rubber	Housing	[11]
	Propene	Propene oxide	-Propandiol	Recreation	[12]
		Propene	-Polypropene		
	Butadiene	Propan-2-ol	-Solvents	Housing	[13]
			Polybutadiene rubber	Recreation, transportation	
	Benzene	Phenol	-Polycarbonates -Bisphenol A	Safe food supply	[14]
		Cyclohexane	-Nylon	Textiles	[15]
		Aniline	-Dyes		[16]
	Toluene	Toluene diisocyanate	-Polyurethanes		[17]
	Xylenes	Terephthalic acid	-Polyethene terephthalate	Housing, transportation	[18]
		Isophthalic acid	-Polyethene terephthalate -Adhesives		
Syngas	Methanol	Acetic acid		Health, hygiene	[20]
		Formaldehyde	-Resins	Communications, textiles	[21]
		Methyl methacrylate	-Latex -Paints		

Table 2. 12 sugars-derived building blocks.

Carbon number	Building blocks chemicals	Structure	Derivatives	Applications and consumer's goods	Ref
C3	Glycerol		Epichlorohydrin, 1,2-propanediol, 1,3-Propanediol, Mono-, di- and tri-esters, Glyceric acid, Acrolein	Triacetin, food additive, Pharmaceuticals, Personal Care Polyols, Alkyd resins, Tobacco, Explosives, Detergents, Cellophane	[23]
	3-hydroxy propionic acid		1,3-Propanediol, Malonic, Acrylic acid, Acrylamide, Methyl acrylate	Acrylic polymers, Speciality polyesters	[24]
C4	3-hydroxy butyrolactone		Pyrrolidones, γ -butenyl-lactone, acrylatelactone	Intermediate for high value pharma compounds	[25]
	Aspartic acid		Amino- γ -butyrolactone, Aspartic anhydride, 3Aminotetrahydrofuran, 2-Amino-1,4,-butanediol, Polyaspartic acid	Production of sweetener: aspartame	[26]
	1,4 diacids (Succinic acid)		1,4-Butanediol, γ -Butyrolactone, Tetrahydrofuran, N-Methyl-2-pyrrolidone Di-esters, Polyamides, Polyesters	Sweetener in food and beverages	[27]
	Fumaric acid		1,4-Butanediol, γ -Butyrolactone, Tetrahydrofuran, Unsaturated polyesters, L-Aspartic acid, L-Alanine, Succinic acid	Unsaturated polyester resins, Food additive, Animal feed, Dye mordant, Polyhydric alcohols	[28]
	Malic acid		Chlorosuccinic acid	Artificial Sweetener, food additives	[29]
C5	Itaconic acid		Methyl-1,4-BDO, 3-Methyl THF, 3-&4-GBL, 2-Methyl-1,4-butanediamine, Pyrrolidones	Copolymer in styrene butadiene polymers (provides dye receptivity for fibers); Nitrile latex	[30]

	Levulinic acid		Methyltetrahydrofuran, Esters, γ -Valerolactone, 5-Methyl-2-pyrrolidone, Diphenolic acid	Potential replacement for Bisphenol-A	[31]
	Glutamic acid		Glutaminol, 5-amino-1-butanol, 1,5-pentanediol, Norvoline	Monomers for polyesters and polyamide	[32]
	Glucaric acid		Polyglucaric esters and amides	Solvents	[32]
	Xylitol/Arabinitol		Ethylene and propylene glycol, Polyesters, Xylaric, Xylonic, Arabonic, Arabinoic acid	Sweetener, Deicer, Automotive antifreeze	[33]
C6	Sorbitol		1,4-Sorbitan, Isosorbide, Polyetherpolyols, Ascorbic acid	Sweetener, Humectant, Thickener, Cryo-stabiliser, Amateur rocket fuel	[34]
	2,5-furan dicarboxylic Acid		2,5-Dihydroxymethylfuran, 2,5-DihydroxyMethyl tetrahydrofuran, 2,5-Bis(aminomethyl) tetrahydrofuran	Potential as monomer to replace terephthalic acid	[35]

II. Platform molecule definition

The US DOE reports are responsible of releasing the platform molecules research articles knowing that no accurate definition has been set to describe this term due to weakness of consensus in the knowledge of a platform molecule. Therefore, many terms have been published in articles, reports, patents and books referring chemicals building-block manufactured from biomass, meant by bio-based platform molecules. Many nomination has been settled for these kind of molecules such as, bio-platform molecules [36], platform molecules [37], biomass-based platform chemicals [38], biomass-derived platform molecules [39], base chemicals from biomass [40], building-block chemicals from biomass [41] and platforms (syngas and triglyceride platform). Some authors define ‘platforms’ as molecules with multiple functional groups owing to the potential to be converted into new families of useful molecules [42]. An extensive range of chemical products result when numerous building-block chemicals are grouped together. Molecules produced from chemical and thermal processing applied during the biological production of platform molecules can be also considered as bio-derived or bio- based platform molecules. Same terms have been applied newly by European and international projects, as well as by governmental, academic and industrial bodies when products are based from biomass, also called bio-derived products. Nowadays, according to the European Commission definition there is no necessity that product or article to be bio-based, on the contrary to the US Farm Bill that emphasis that whole or significant extent of product should be formed from bio-based materials. Bio-based products are defined as: commercial or industrial products (non-food or non-feed) that are constituted in significant part or in whole, of renewable biological products, agricultural materials (e.g., plant, animal, and forestry materials), or marine materials according to US 2002 Farm Bill. In 2007, the European Commission claimed that bio-based matrix are considered as product derived from biomass other than food or feed (plants, crops, trees, algae, biological waste from households, animals and marine organisms) [43]. Bio-based products are products of interest and considered as high added value fine chemicals in many fields such as, cosmetics, food additives, pharmaceuticals, and many others.

The European Committee Standardized (CEN) updated the definition of biomass which clarifies that fossil fuels are initially coming from the transformation of biological material undergone many natural processes. Geological and/or fossilized formations are excluded from biomass or from biological material origin. All cited definitions between bio-derived and bio-

based are authorized for the application of 'bio-based' taking in consideration bio-based platform molecule term, referring to author's opinion that it could interchange between both. Considering the cited previously terms, bio-based platform molecule would be defined simply as: chemical compound whose constituent elements originate wholly from biomass (material of biological origin, excluding fossil carbon sources), and could be used as a building block for the production of another high value added chemicals. Some cases exist, where various molecules in the reaction pathway can be seen as platform molecules, complicating further, a correct assignment of real platform molecule. Taking example, fructose as feedstock if isolated can be converted to hydroxymethylfurfural (HMF), but also, it can be directly converted without isolation to levulinic acid and γ -valerolactone [44], making therefore these two compounds as bio-derived platform molecules, as directly produced from biomass, depending on their production route.

III. Platform chemicals root

1. Fossil materials as feedstocks

Previously, a wide range spectrum of chemicals was provided by fossil raw feedstocks which comply with our energy needs and reinforce our lives. Fossil feedstocks depletion crisis entail severe impacts in both economic and social side, in addition their utilization is rightly perceived as a major source of pollution. The most of all products building from the chemical industry using fossil raw materials as feedstocks are generally coming from seven simple building-block molecules (overall natural gas and crude oil) by cracking, reforming fractionation and distillation, called 'base chemicals fossil derived', view table 1 [45]. The global annual scale of base chemicals produced exceeds 10 million tons, and exceeded more than 360 million tons in 2010 [46].

2. From low cost sustainable materials into platform chemical

Many researchers published in their articles some alternative renewable feedstocks for replacing non-renewable raw materials mitigating that way the dwindling of fossil resources such as solar resources; wind, hydropower, thermal, photovoltaic [47], biomass; energy and chemicals [48], planet movements; ocean tides, waves, geothermal; energy, nuclear energy; fusion and fission, carbon dioxide; hydrogenation to hydrocarbons and oxygenates [49]. Therefore, the most common feedstocks utilized in biorefinery such as saccharides, lignin, protein, extracts or any combination of these raw materials as biomass take more advantages

(see fig 1). According to author's knowledge, platform molecule definition was refined to those available molecules at low cost and on a large scale. Current platform molecules are listed and provided in table 3, grouped together relying on their processing technology to generate these molecules from biomass. A wide list has been compiled in table 3 according to the definitions described previously, performing the diversity and given the broadest vision considered for any process or bio-derived product. Since the area of bio-based platform molecules developed, it is normal that certain chemicals become more widespread than others. Given that, every natural amino acid is derived from fermentation or protein depolymerisation of biomass; however, only glutamic and aspartic acid are mentioned below. A study done by Lammens et al., [50] figured out a broad range of amino acid sources from biomass fraction followed by protein hydrolysis, concluding that these two amino acids were the most substantial residues to become key platform molecules probably.

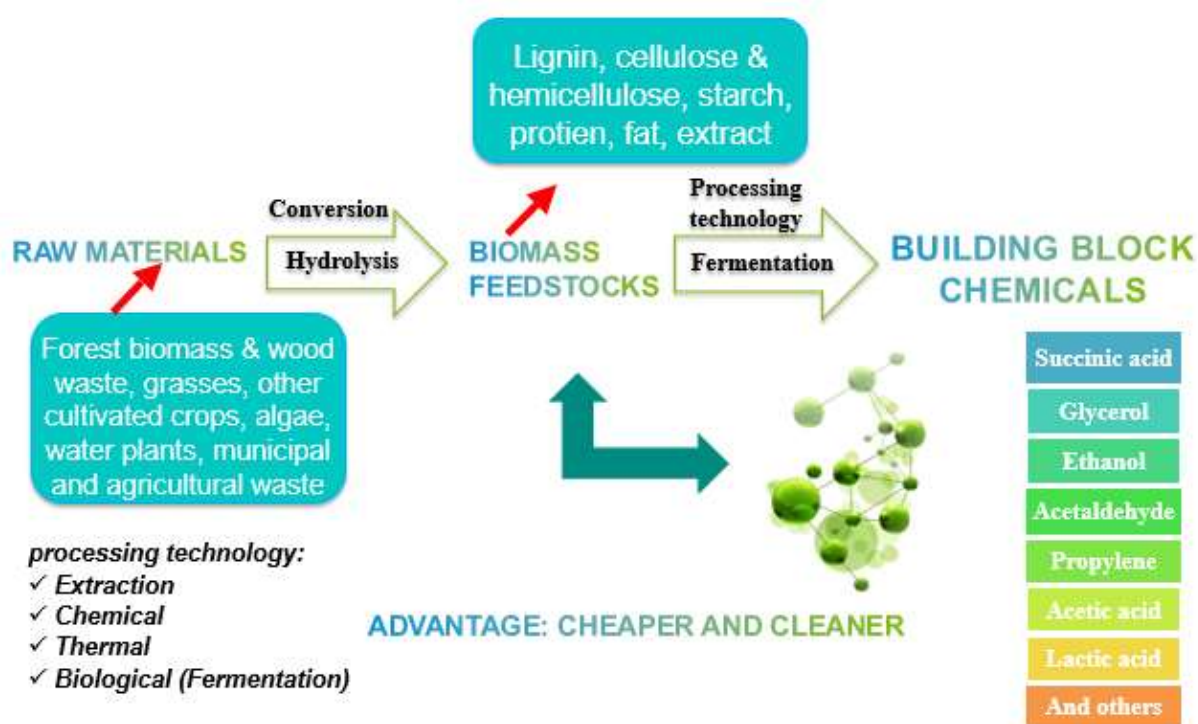


Figure 1. From raw materials to chemicals.

Table 3. List of platform molecule biomass derived

Platform molecule	Processing technology	Derived from	Ref
Fatty acid alkyl esters	Extraction, chemical-catalytic	Triglyceride	[51],[52]
Triglyceride	Extraction	Oil containing biomass	
Fatty acids (e.g. oleic acid)	Extraction, chemical-catalytic	Triglyceride	[53]
Mannitol		Sugars	
Glycerol		Triglyceride	
Citric acid Actonic acid Itaconic acid Succinic acid Maleic acid	Biological	Sugars	[55–57]
Amino acid (glutamic, Aspartic acid)		Sugars or protein	[58]
Ethanol			[59–61]
Butanol			[62]
Lactic acid		Sugars	[63–65]
Fumaric acid			[28, 66, 67]
3-hydroxy propionic acid			[68–70]
Xylitol	Chemical catalytic	Hemicellulose or xylose	[3, 33, 71, 72]
Sorbitol		Cellulose or sugars	[73–75]
Glucosamine	Chemical catalytic, Thermal	Chitin	[76–78]
Oxalic acid		Cellulose or sugars	[79–82]
Furfural	Chemical catalytic	hemicellulose or sugars	[83]
5- Hydroxy methyl furfural		Cellulose or sugars	[84, 85]
Levulinic acid			[3, 86–89]
Syringol	Thermal	Lignin	[90, 91]
Eugenol			[3, 92–94]

A comparison of bio-platform molecule to fossil-derived base chemicals is needed, since bio-economy matures, it is obvious that bio-based chemicals are unlikely fossil-derived base chemical in volumes they are manufactured and used in (table 4).

Table 4. Comparison between base chemical from fossil feedstocks and biomass derived (tons)

Feedstocks	Base chemicals	Annual production from fossils	Ref	Annual production from biomass	Ref
Oil, gas	Ethene	≈ 123 300 000	[46, 95]	≈ 200000	[96]
	Propene	≈ 74900000		Pilot scale	[97]
	Butadiene	≈ 10200000		Pilot scale	[98]
Oil	Benzene	≈ 40200000		≥100	[99]
	Toluene	≈ 19800000		≥100	
	Xylenes	≈ 42500000		≥ 100	
Syngas	Methanol	≈ 49100000		≈ 340000	[100]

3. Pathways to the 12 building blocks from sugars

The 4-carbon diacids family can be combined together (succinic, fumaric and malic acids), as they are produced through similar biochemical pathways. Four carbon dicarboxylic acids produced from the Krebs cycle pathway from both biotransformation or fermentation are regarded as key building blocks for manufacturing of both commodity and specialty chemicals [101]. A critical technical challenge is the improvement of microbial biocatalyst to minify coproducts such as acetic acid; increase yields and productivities of these chemicals, recovery process at low costs are needed to remove unwanted salts and system integration scale-up. These building blocks are transformed into three primary derivatives family by three different process: reduction, reductive amination and direct polymerization [102].

A C3 chemical, 3-Hydroxypropionic acid (3- HPA) is considered as key building block, owing to the potential to derive fine chemicals. However, one of major technical challenges to develop 3-HPA as a building block is the development of fermentation process at less expensive cost [103]. Major technical considerations are the development and scale-up of system integration of fermentation process at low cost, with high yields and productivities by an appropriate organism with the improvement of microbial biocatalyst to reduce by-products formation, with an efficient low cost recovery process to reduce undesirable salts [104].

Glycerol holds the potential of being an extremely diverse building block within the biorefinery. Currently, glycerol is a well-recognized item of commerce with worldly annual production of $600 - 750 \times 10^3$ tons and industry expects its consumption of above 4 million tons by 2024 [105]. Many chemical industries firms such as Uniqema, Procter & Gamble and Stepan produce glycerol and its numerous derivatives (such as glycerol stearate, glycerol oleate and glycerol triacetate). Glycerol is manufactured as natural glycerol by-products, from biodiesel and

oleochemical industries, and as synthetic glycerol, from propylene. USA ranks the number 1 worldwide to be a provider and a consumer of glycerol. About 75% of glycerol supply in U.S are coming from natural sources, and the residual 25% are synthetic and refined before its many eventual use in markets reflecting glycerol's comparatively expensive price of \$0.60 – 0.90 per pound. However, increasing the use of biodiesel market led to much availability of low cost glycerol since it is a key byproduct in biodiesel manufacturing [106]. Glycerol prices could fall to \$0.20/lb which is the industrial average cost today for refined and unrefined glycerol; however, glycerin, could be present at lower cost. Glycerol can become a key building block in biorefinery if its prices drop into \$0.20 - \$0.50/lb range. The increasing in fatty acid consumption for fuels and products may significantly raise up the world glycerol production. If the US displaced about 2% of the on-road diesel by biodiesel in the coming years, almost 2 billion pounds of new glycerol would be produced [107].

Aspartic acid is a 4-carbon amino acid considered as a fundamental part of metabolism for protein production. Different arrangements of aspartic acid are present. However, L-aspartic which could be produced in four primary way, chemical synthesis, protein extraction, fermentation and enzymatic conversion, is the most common one used to produce a synthetic sweetener, aspartame [108]. Enzymatic conversion consists in reacting fumaric acid with ammonia, catalyzed by a lyase enzyme considered as the preferred method currently, since it provides higher yields and productivities, few byproducts and easy recovery process (crystallization). The main technical barriers are the development of cost-competitive fermentation process with the existing process (using low cost sugar as substrate), metabolic engineering of biocatalytic pathway to overexpress oxaloacetate without affecting viability in cells, managing operating for environment, oxaloacetate enzymatic oxidation, high cost recovery [109]. Direct fermentation methods are not cost competitive yet, but the utilization of biotechnological routes holds promising to surmount this obstacle. Another strategy for reducing aspartic acid cost is to make improvements to the current process. The primary focus of this effort would be to reduce fumaric acid cost, which is currently used as feedstock for aspartic acid production [110].

3-Hydroxybutyrolactone (3-HBL), a cyclic chemical with four carbons, is currently used in pharmaceutical production and produced through chemical transformations also via fermentation. The chemical synthesis of this building block is considered as “messy” because it requires multiple steps [111]. One possible way to form 3-HBL begin from malic acid, which could be cyclized to give hydroxysuccinic anhydride, then can be reduced to form hydroxybutyrolactone. Thus, the primary technical obstacles present in the development of this

building block involve the development of inexpensive fermentation process using wide range of lignocellulosics biomass as feedstocks that are able to tolerate its inhibitory components and averting odd oxidants [112].

Methyl-SA is a dicarboxylic acid with C5, also known as itaconic acid, considered as a key building block for its potential to derive fine chemicals. Itaconic acid basic chemistry is analogous to maleic acid/anhydride that is derived from petrochemicals. Itaconic acid used as specialty monomer is produced through aerobic fungal fermentation [113]. The primarily defy is related to the reduction of fermentation costs process to below \$0.25/pound to compete with petrochemical-derived products. However, many technical barriers are present in the development of a process to produce this building block compounds, including the evolving and scale-up of low costs fermentation system with an organism using low cost sugars (C5 and C6) and the recovery process with high rates, yields and productivities, reducing by-products formation by improving microbial biocatalyst to better control the operating environment [114]. Levulinic acid (LA) is one of the most and largest recognized building block family of potential derivatives, formed by the treatment of both 5 and 6 carbon sugar from starch, hemicelluloses (xylose, arabinose) or lignocellulosics with acid. LA is often used as starting material to produce a large amount of chemicals since it serves as a precious building block available from polysaccharides in biorefinery [31].

Glutamic acid (GA) is a C5 amino acid building block owing the potential with its derivatives to build five carbon polymers with new functionality when compared to petrochemicals derivatives from maleic anhydride [115]. The development of very low cost fermentation ways is considered as the main technical obstacle to the development of GA as a building block. One of the major challenges is primarily associated with the development of less expensive fermentation process by a strain that can produce GA as free acid. To compete petrochemicals derived products, productivity and final fermentation titer should be improved, and fermentation cost needs to be equal or less than \$0.25/pound [116].

One of glucose hydrogenation product is sorbitol, which is produced by several companies and its current volume is in the order of 200 million pounds per year. To ensure complete conversion of glucose, continuous process instead of a batch technology using catalyst such as Raney nickel is used in all commercial processes for reducing sugars amount in sorbitol production, and considered as very strict requirements because sorbitol major applications are in the food industry with no technical development for its use in derivatives, making sorbitol a very inexpensive feedstock [34]. The use of sorbitol as a building block essentially for derivatives does not require any technical development. The sole change was glucose conversion into

sorbitol in a continuous process instead of batch process. This would make sorbitol an inexpensive feedstock for producing derivatives [117].

Glucaric acid is an organic compound and member in large family of materials representing a significant market opportunity known as oxidized sugars which can be obtained by oxidation of glucose with nitric acid and considered as building blocks. The oxidation of glucose to glucaric acid in high yield occurs using chemical or biochemical catalysis [32]. However, in biorefinery the value of glucose conversion into glucaric acid arises from two proprieties: the first one is that a broad range of products are produced in high scale markets using glucaric acid as a starting point, and the second one is development of efficient and robust processes for glucaric acid production applicable for oxidation of other inexpensive sugars discussed in this review, such as xylose or arabinose [3].

Xylose and arabinose can be used to produce the corresponding sugars alcohols, xylitol and arabinitol via hydrogenation. At present, commercially, xylitol which is known as non-nutritive sweetener is produced in very limited quantity, whereas arabinitol is not commercially produced, even though there is no hurdles in its production [118]. The conversion model of xylose and arabinose into xylitol and arabinitol is based on conversion model of glucose to sorbitol with 99% yield range. Nickel, ruthenium and rhodium, hydrogenation catalysts, are used in hydrogenation of C5 sugars into sugar alcohols. No technical development requirements in xylitol production used as a building block for derivatives. The production of xylitol could be done at very low cost if the xylose feedstock is inexpensive which is not the case [115].

The oxidative dehydration of glucose using oxygen, or electrochemistry leads to FDCA formation (2,5-Furandicarboxylic acid), a member of the furan family. It can also be converted by oxidation of 5-hydroxymethylfurfural, an intermediate in LA production, one of the top 12 platform molecules from 6-carbon sugars [35]. The necessity of developing an effective and specified dehydration process create a challenge in the production and use of FDCA. The lack of knowledge in sugar dehydration control limits the innovation of powerful technology, including the production of new building blocks at a minimum cost. The production of new polymers from FDCA is also a challenge while the developing and controlling of esterification reactions and FDCA reactivity will be of great matter [119].

There are chemical compounds with low molecular weight characterized by the presence of at least one carboxylic functional group ($-\text{COOH}$) [120] also called organic acids. Organic acids were discovered by Scheele in 1784, and humans have used them since early ages. Organic acids are broadly produced in nature by all organisms such as micro-organisms, plants, animals and humans. The top important commercially organic acids are lactic acid, citric acid, gluconic

acid and itaconic acid. They are used in various applications as flavor enhancers in food, as ingredients in pharmaceuticals, cosmetics and beverages, also for the production of biopolymers, coatings, adhesives and others in chemical industry [121]. Global market of organic acids was estimated at approximately \$12 billion in 2014 and is expected to reach over \$18 billion by 2023 [122]. The vision is that these products can replace fossil fuel which is itself a societal goal.

As mentioned above, various organic acids can serve as platform chemicals for polymers, food, coatings, lubricating oils, pharmaceuticals, cosmetics, solvents and other materials production [123, 124]. They are products of interest due to their price and highly competitive existing market for large quantities for use in foods, beverages and chemicals. Currently, the highest volume products are acetic, citric, formic, lactic, propionic, and fumaric acid. Organic acids were identified as attractive target molecules derived from sugars or cellulose decomposition. It is difficult for the renewable materials prepared using extraction and/or advanced fermentation technology to compete given the current costs of petroleum derived starting materials [123]. A significant portion of multifunctional organics acids are already produced from renewable sources. Renewable materials can help to replace entirely petroleum derived organic acids, indicating a good management if costs could be lowered and integrated large scale processes are developed [55]. Significant advances in technology are needed to make renewable organic acids and derived products cost competitive with their petroleum analogs [125]. Significant investment in developing new strains and enzymes that produce these acids at higher yield should be continued. Of particular interest, using microorganisms that are resisting poisoning by higher concentrations of acid [126].

IV. Succinic acid : case study

As mentioned before, 12 building-block or platform chemicals were identified as the most promising candidates from a pool of 300 chemicals. A healthy shift in their production strategies using renewable feedstocks (eco-friendly), may then compete with energy-intensive processes using petroleum based [127, 128]. In the meantime, SA is at the top of the list and holds the title of being the only special product with an annual market demand of about 710 kilo tons with a net value of \$115.2 million, and expected to exceed \$1.1 billion by 2020 [129]. SA (butanedioic acid) is a dicarboxylic acid that occurs naturally in plant and animal tissues as well as microorganisms. This chemical is also known as “Spirit of Amber.” When it was first

discovered, it was extracted from amber by pulverizing and distilling it using a sand bath. It was primarily used externally for rheumatic aches and pains.

SA is a dicarboxylic acid comprised of four carbon atoms. In the bio-economy, this four carbon dicarboxylic acid (SA) progresses to a platform intermediate, as a substitute of maleic anhydride, to produce diverse number of chemicals considered as bulk/intermediate chemicals, among of them; maleic acid, adipic acid, 1,4- butanediol, γ -butyrolactone, N-methyl-2-pyrrolidone, tetrahydrofuran, 2-pyrrolidone, succinic esters and succinimide, which are used in green chemistry and in a wide number of industries including polymers (clothing fibres), food, surfactants and detergents, flavors and fragrances and in the pharmaceutical industry [28, 130]. SA conventional applications in industry involve about 9% polybutylene succinate-terephthalate and polybutylene succinate and 6.2% polyester polyols, 15.1% in pharmaceutical industry, 12.6% of SA is utilized in food industry, as acidulant, flavorant and sweetener, and 19.3% in resins, coatings and pigments production [131].

1. Production methods : an overview

Chemical synthesis is the construction of complex chemical compounds from simpler ones by which many bulk substances important to daily life are obtained. It is applied to all types of chemical compounds, but most syntheses concern organic molecules, allowing their availability in large quantity [132]. In the petrochemical process, SA is mostly produced from n-butane via hydrogenation of maleic anhydride pursued by hydration [133]. Other chemical methods have been studied but none have so far been able to compete effectively with the production of organic acid by fermentation [30].

Industrial production of organic acids began with the production of citric acid from lemons and the production of malic acid from apples. At the end of the 19th century, studies on microorganisms showed that they were capable of producing organic acids. From this discovery, researchers and industrialists have turned to the use of microorganisms for the production of platform molecules and became an important field of study for their various applications (Ke-Ke Cheng et al., 2012). Over 2 trillion \$ per year is the estimated number of chemical industry growth, if bio-based products replace petroleum-based resources, providing an additional income with an amount of over \$500 billion a year according to the U.S. Bio-based Products.

Bio-based feedstocks became less expensive during the past few decades, and it will remain an added value over the petroleum/gas feedstocks in the future. According to Huw Kidwell [135],

SA consumption was of about 25,000 to 30,000 tons annually in worldwide with approximately 10% raise up a year. SA total capacity is estimated to be more than 45–50 million lb/year. The World demand for SA was 51,000 tons in 2013 and is expected to increase to 593,400 tons by 2020 [129]. It is expected in the next few years that these figures will raise up in parallel with the fast demand rising and the cost-competitiveness of biosuccinim. Many companies are active in development of bio-based SA at commercial scale and three of them intend to bring more than 140,000 tons per year of capacity online by 2016 (Table 5) [132, 136].

Table 5. World-wide investments in the bio-based chemical SA by the producer's companies

Company	Raw material	Fermentation/ Microorganisms	Start-up	Investment made in	Capacity in thousands per year
BioAmber (DNP/ard)	Wheat glucose Corn glucose	<i>Candida Krusei</i>	2010	Joint venture between Canada and Pomacle, France	30
Reverdia (Roquette & DSM)	Starch/sugars	<i>Saccharomyces cerevisiae</i>	2012	Cassano Spinola, Italy	10
Myriant	Corn glucose	<i>Escherichia coli</i>	2013	Louisiana, USA	14
Succinity (BASF & Corbion – purac)	Glycerol	<i>Basfia succiniciproducens</i>	2013	Montmelo, Spain	10

The metabolism towards SA production is controlled by environment which can be either aerobic or anaerobic, SA is produced by numerous aerobic, facultative, and obligate anaerobic microorganisms using C6 sugars such as glucose and fructose metabolized by the Embden Meyerhof-Parnas pathway (glycolysis), providing per mole of sugar, two moles of pyruvate, ATP (energy), and NADH (reducing power) [137].

2. SA producer's microorganisms

A tremendous research is made to develop a biological process for SA production as a metabolite under aerobic, micro-aerobic and/or anaerobic conditions by employing microorganisms such as *Aspergillus niger*, *Aspergillus fumigatus*, *Byssoschlamys nivea*, *Lentinus degener*, *Paecilomyces varioti* and *Penicillium viniferum* [136, 138]. Recently, the topology of central carbon metabolism of *A. niger* and the metabolic network was deciphered by integrating genomic, biochemical and physiological information. Based on these information, a stoichiometric model was constructed suggesting that this organism can produce 1.5 mol/mol (mol of SA produced per mol of glucose consumed) under microaerobic conditions

[139]. However, the use of fungus was limited to food and beverages manufacture due to the low productivities as well as the difficulties in fermentation, separation and purification methods [140]. *Saccharomyces cerevisiae* has been studied to reach a higher concentration of SA in wine manufacturing [141], a series of its mutant strains were designed by the overexpression or inactivation of some genes, showing an increased levels of SA when compared to wild type strains, from 0.96 g L⁻¹ to 2.25 g L⁻¹ [142, 143]. SA is not accumulated intracellularly which makes it suitable and promising strain for the biotechnological production of SA production on an industrial scale [144]. Few Gram-positive bacteria such as *Corynebacterium glutamicum* and *Enterococcus faecalis* have been utilized for SA production. Several engineered *C. glutamicum* strains were designed by gene modifications, and their optimal culture conditions were developed [145]. However, SA rate was increased seven times, while glucose consumption rate was increased five times under deprived oxygen condition. SA metabolic pathways synthesis are diverse; however, in bacteria, the main one is the phosphoenolpyruvate (PEP) carboxylation reaction, while others microorganisms use multiple pathways. *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, *Anaerobiospirillum succiniciproducens* and recombinant *Escherichia coli*, are gram-negative bacteria which produce SA in various forms [146]. Table 6 represents the best candidates as they produce SA as a major fermentation product. A wild type of *E. coli* ferments primarily glucose into ethanol, formic, acetic and lactic acids with small detectable amounts of SA under anaerobic condition as stated by Clark et al [147]; the yield of SA on glucose obtainable is no more than 0.13 g/g. It is known that the metabolic pathway used by *E. coli* to form SA differs from that of the three bacteria mentioned above; the PEP carboxykinase plays a minor role. *E. coli* produce succinate naturally via TCA cycle reductive branch; however, the quantity is limited and this route is not applicable for large scale commercial production. Therefore, to achieve high SA production yields and to inhibit any by-product formation, there has been considerable interest in engineering *E. coli* by several gene disruptions [148]. The enzymatic pathway technology used to produce succinate, (SA ionized form) is explained by [149].

In his paper, the metabolic pathways of wild-type *E. coli* with gene alterations (or gene editing) are studied. To enhance and improve succinate overall yield, a number of genes were knocked out in the pathway. In the first step, *PTC* gene deletion leads to an abolishing of metabolic pathway, which converts phosphoenolpyruvate (PEP) to pyruvate, ensuring the inhibition of PEP and hence all the reaction is fed into the TCA cycle to produce succinate without any by-product formation. Gene deletions in the next set are lactose-dehydrogenase (*LDH*) and pyruvate formate lyase (*PFL*), preventing pyruvate conversion into lactate and acetyl-CoA, as

by-products; also pyruvate is recycled into TCA cycle, which improve succinate overall formation [150].

Table 6. SA producer's bacteria.

Microorganisms	Fermentation strategy	Substrate	Concentration (g L ⁻¹)	Yield (g SA/g Sugars)	Ref
<i>A. succinogenes</i>	Batch	Glucose	10	0.56 g/g	[151]
		Xylose	10	0.42 g/g	
		Mannose	10	0.38 g/g	
		Arabinose	10	0.44 g/g	
<i>A. succinogenes</i>	Continuous	glucose	26.5	0.74 g/g	[152]
<i>A. succinogenes</i> 130Z	Batch	xylose	80	0.74 g/g	[153]
		glucose	5.4	0.80 g/g	[154]
		mannose	5.7	0.85 g/g	
		arabinose	5.0	0.96 g/g	
		xylose	5.2	0.84 g/g	
<i>A. succinogenes</i> NJ113	Batch	Cellobiose	50	0.67 g/g	[155]
<i>A. succinogenes</i> CGMCC159	Batch	Straw hydrolaste	58	0.80 g/g	[156]
<i>M. succiniciproducens</i> LPK7	Fed batch	Glucose	20	0.76 g/g	[157]
	Continuous	Glucose	18	0.25 g/g	[158]
Recombinant <i>E. coli</i> AFPP11	Dual phase Fed batch	Glucose	40	1.1 g/g	[159]
Recombinant <i>E. coli</i> HL27659k	Fed batch	Glucose	Not reported	0.61 g/g	[160]

Table 6 presents data with regard to the conditions and fermentation efficiency, reported for different microbial strains that produce succinic acid. The production of SA by fermentative way has been achieved by nature and mutant strains. According to the American type culture collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), three facultative anaerobes bacteria isolated from the rumen, *Actinobacillus succinogenes*, *Basfia succiniciproducens* and *Mannheimia succiniciproducens* are classified as biosafety level 1 microorganisms and considered as the top promising wild -type bacterial stem due to their potential of consuming various sources of carbon and therefore achieving high fermentation efficiency. Better concentration of succinate (105.8 g L⁻¹) was achieved when *A. succinogenes* FZ53 mutant used glucose as carbon source leading to a yield and productivity of 0.82 g SA/g glucose and 1.36 g L⁻¹ h⁻¹, respectively [161]. *B. succiniciproducens* metabolic fluxes were investigated and two engineered strains were evolved but unfortunately its use for SA production was not investigated to a great extent [162].

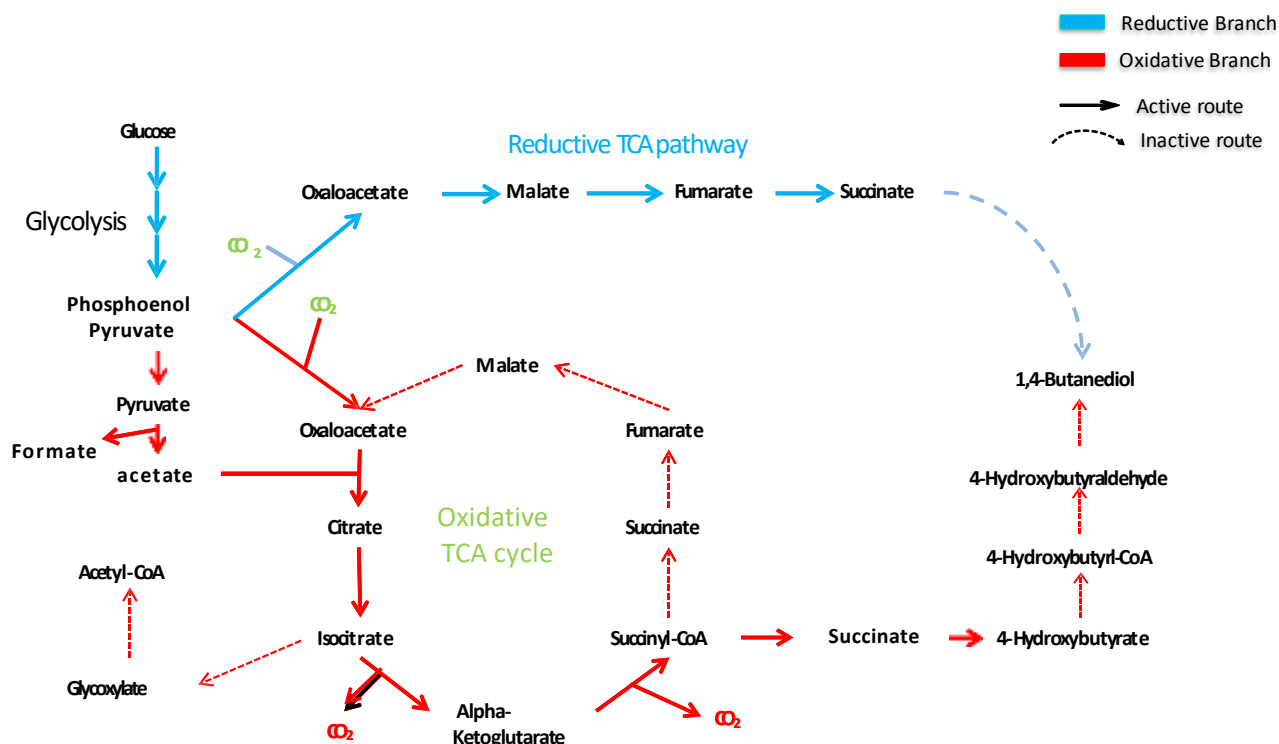
A genetically modified *E. coli* strain was built to produce highly efficiently succinic acid, such as in dual phase fermentation; the strain, *E. coli* AFP111/pTrc99A–pyc, gave a concentration of 99.2 g L⁻¹ of SA, with a top yield of 1.1 g of SA/g of glucose and a productivity of 1.3 g L⁻¹ h⁻¹. [163]. *Corynebacterium glutamicum* ΔldhA pCRA717 could also be a promising candidate to produce SA referred to its high volumetric productivity (3.17 g L⁻¹ h⁻¹) and yield (0.92 g SA/g glucose), achieved with a final concentration of (146 g L⁻¹) [164]. Research emphasized on the evolving of some modified yeast strains which could be considered to produce SA at low pH, which should be helpful in view of the reduction of the costs of operations downstream (purification and separation) of SA [132, 165].

The main features to exploit *A. succinogenes* to produce SA are the utilization of a wide variety of feedstocks containing carbon sources, suitable tolerance to inhibitors, sufficient as well as efficient fermentative pathway even with sustainable raw materials [166] ; while the principal downsides are the demanding nature of this strain towards nitrogen sources (corn steep liquor, yeast extract or NH₄Cl), vitamins such as biotin, the optimal pH demanded (nearly neutral), and furthermore the restricted tools of genetic modification handling [167]. SA bio-production using *A. succinogenes* is implemented in biorefineries employing cheap and complex renewable raw materials, which enrich fermentation medium at less expensive cost compared to commercial nutrient sources. pH neutralizers utilization in fermentation broth which result from the decrease of pH during *A. succinogenes* cultivations affect upstream and downstream separation costs [168]. The low pH values attained in fermentation broth led to minimizing the cost of downstream separation, as it affects SA dissociation level (pKa₁ = 4.16 and pKa₂ = 5.6) [132].

3. Metabolic network towards SA production in *A. succinogenes*

A. succinogenes has been isolated from bovine rumen. It is a fermentative Gram-negative, facultative anaerobic, non-motile, non-spore forming, capnophilic, pleomorphic rod and taxonomically referred to the Pasteurellaceae family, as follows: Bacteria; Proteobacteria; Gammaproteobacteria; Pasteurellales; Pasteurellaceae; *A. succinogenes*, based on the 16S rRNA amplification [169, 170]. *A. succinogenes* is mesophilic and grows well at 30-37 °C in chemically defined media, capable of consuming a broad range of C5 and C6 sugars as well as various disaccharides and other carbon sources, such as glucose, xylose, arabinose, mannose, galactose, fructose, sucrose, lactose, cellobiose, mannitol, maltose and glycerol [156, 171, 172]. Higher SA yields were achieved when reduced carbon resources were used as feedstocks, such as glycerol, sorbitol and mannitol, while C5 sugars utilization, such as arabinose and xylose,

led to lower succinate yields [173]. Normally, glucose is transported through the cell membrane into cell by means of permease action; hence, glucose phosphorylation occurs via phosphoenolpyruvate dependent phosphotransferase system (PTS) [174], and also through the activity of hexokinase to form glucose-6-phosphate, which is converted to phosphoenolpyruvate (PEP) via glycolytic pathway. The interference of the oxidative pentose phosphate pathway (OPPP) is limited contribute in glucose metabolism. Thus, NADH is generated through the activity of pyruvate and formate dehydrogenase, while NADPH is result through transhydrogenase and/or by combination of two enzyme activity of NADH-oxidizing malate dehydrogenase and NADP-reducing malic [174]. Additionally, flux distribution between C3 and C4 pathways is affected by the NADPH requirements, mainly refer to pyruvate and formate dehydrogenase activities (figure 2). Both, glyoxylate and Entner-Doudoroff pathways are not present in *A. succinogenes* fermentative metabolism. Four nodes are major in flux distribution to produce succinate, which are phosphoenolpyruvate (PEP), oxaloacetate (OAA), malate and pyruvate [175, 176]. Metabolic flux analysis has shown that SA main pathway is from PEP towards oxaloacetate, malate, fumarate ending into succinate catalyzed by key enzymes, which are PEP carboxykinase, malate dehydrogenase, fumarase and fumarate reductase [174, 177]. Nevertheless, via the C3 pathway, PEP could be transformed into formate, acetate and ethanol, and via the C4 network, it is converted to succinate by malic and OAA decarboxylase enzyme which hold the role of pyruvate bio-conversion, making therefore a reversible shunt between the C3 and C4 pathways [178].

Figure 2. Metabolic pathway occurs in *Actinobacillus succinogenes*

4. Key factors requirement

pH regulators

Oftentimes, pH is considered as a major key factor in fermentation processes; for optimal performances, almost microbial cultures require pH values near neutral. SA fermentation also led to organic acids production (formic and acetic acids) which provokes the acidification of the medium, necessitating therefore the addition of neutralizing agents. During fermentation, when pH values are near neutral, these by-products are in their dissociated shape leading to salts formation by neutralization. A study done by Zhu et al [170] reviewed that the highest SA production occurs in fermentation of *A. succinogenes* ATCC 55618 at optimum pH value of 7.5, while the addition of individual neutralizing agents, such as NaOH or KOH, results to fall down SA production, which may be related to acute cell flocculation. When ammonium hydroxide NH₄OH or calcium hydroxides are used as neutralizing agents, growth inhibition is observed. To prevent cell flocculation, 5 M NaOH and 0.47 M of MgCO₃ were combined, achieving 28 % higher SA concentration (59.2 g L⁻¹) in fermentation than when NaOH was

used as only neutralizing agent [179]. A stable production of SA occurs at a pH range of 6 to 7.2 as reported in another paper [180], while magnesium carbonate (MgCO_3) was considered as the most promising neutralizing agent when compared to Na_2CO_3 , NaOH , CaCO_3 and NH_4OH in *A. succinogenes* CGMCC1593 fermentation, using glucose as the sole carbon source. When NaOH or Na_2CO_3 were used, cell flocculation occurs at 12 hours, whereas cell flocculation was not observed when MgCO_3 was used. Li et al., [167] demonstrated that among many neutralizer agents, such as CaCO_3 , NaHCO_3 , NaOH , Na_2CO_3 , $\text{Ca}(\text{OH})_2$, $\text{Mg}(\text{OH})_2$, and $\text{NH}_3\cdot\text{H}_2\text{O}$, MgCO_3 was the most efficient regarding SA production, glucose utilization and growth of *A. succinogenes* NJ113, reaching a conversion yield of 0.73 g/g of glucose into SA and decreasing fermentation cost by 55.9%. The utilization of CaCO_3 , $\text{Ca}(\text{OH})_2$ and $\text{NH}_3\cdot\text{H}_2\text{O}$ as neutralizers, results in decreasing cell growth, and thus low production of SA; while the supply of mixed neutralizers of $\text{Mg}(\text{OH})_2$ and NaOH at a ratio of 1:1 led to the same efficiency for SA production as using only MgCO_3 as neutralizer. This could be referred to NaOH solubility and strong alkalinity, and the lack of Mg^{2+} as cofactor for PEP carboxykinase [180]. In the studies mentioned above [180], MgCO_3 was considered as the most efficient regulator of the pH, providing both CO_2 and Mg^{2+} ions serving as cofactors in the TCA cycle reductive branch for its first enzyme, PEP carboxykinase.

CO_2 Feed in

In fermentation, while gaseous CO_2 is sparged, the dissolved CO_2 concentration relies on fermentation broth components, temperature, agitation, CO_2 flow rate and partial pressure. During *A. succinogenes* fermentation, when carbonate (e.g. MgCO_3) or bicarbonate salts are added, higher dissolved CO_2 concentrations can be reached, regardless gaseous CO_2 supply. During fermentation, CO_2 availability, results in carbonates and bicarbonates ions conversion into HCO_3^- , and diffusion through cell membranes, which demands ATP consumption so as to maintain reaction equilibrium. A study by Zou et al [181] on the SA production by *A. succinogenes* ATCC 55618 explored the action of dissolved concentration of CO_2 , partial pressure of gaseous CO_2 as well as the MgCO_3 supply on SA production. The CO_2 dissolved concentration was not sufficient, when only gaseous CO_2 was sparged, so as to maximize SA production; consequently 0.47 M of MgCO_3 and CO_2 partial pressure of 101.33 kPa (100% CO_2 supplying gas) was supplied resulting in higher: concentration (60.4 g L^{-1}), productivity ($0.84 \text{ g L}^{-1} \text{ h}^{-1}$) and yield (0.58 g of SA/g of glucose) of succinic acid. Xi y et al [182] investigated the effect of agitation and other parameters on SA production. Maximum SA

production was performed when *A. succinogenes* NJ113 was grown in the following conditions, 200 rpm stirring speed, pH 6.8, 0.1 MPa CO₂ partial pressure, 37°C and 150 mM NaHCO₃, leading to CO₂ optimal fixation rate of 0.57 g L⁻¹ h⁻¹, with a concentration of 51.6 g L⁻¹ and 0.76 g g⁻¹ yield.

The CO₂ availability could raise owing to its market price of around 60 \$/t with an annual assessed carbon capture and storage of 90% [183]. An analogue solution could be the MgCO₃ production by precipitating CO₂ and Mg²⁺ ions that could be used in SA fermentation.

Redox potential

Redox state of the cells is known to affect metabolites profiles; more and more microbes are figured out with a process having the capacity of using extracellular electron transfer, in which they use either electron donors or acceptors for metabolic reactions [184]. This feature can be utilized to overcome cellular redox limitations and hence optimizing microbial production. Redox potential, also known as oxido-reduction potential (ORP), reflects overall electron transfer and redox balance involved in metabolic activity of microorganisms. ORP is identified by a ratio of oxidative to reductive redox couple in fermentation operation [185]. The majority of fermentation products produced under microaerobic or anaerobic conditions are considered as bulk commodities. Although many fermentation processes have been well developed with long-term operability, cost saving is an endless effort, particularly for biofuels and bio-based chemicals production at bulk quantity such as ethanol and butanol to address global concern on sustainable development [186]. Since redox reactions and homeostasis are basis for intracellular metabolism, ORP status controls metabolites profiles, which subsequently offers a strategy to direct the pathway for efficient production of more and more materials and energy [187]. With advanced technologies that can detect ORP levels and its intracellular influence on gene expression, protein biosynthesis and metabolites caused by ORP shifts, mechanism underlying this phenomenon can be elucidated. More robust strains and optimized processes can be developed by redox potential which can be controlled by metabolic modifications and bioprocess engineering levels, which have been highlighted in ethanol and butanol fermentations under microaerobic and anaerobic conditions [187]. Figure 3 shows the standard ORP of some redox pairs in intracellular metabolism and it is affected by temperature, aerobic or anaerobic conditions; therefore, strategies have been implemented to regulate redox potential, including gene modification, energy input, reagents supplementation and sparging gases. A study done by Zeikus et al [188] showed the impact of redox potential regulation on

succinate formation which is enhanced by 20% when using *A. succinogenes* in bioelectrical reactors (described elsewhere in this review), and its efficiency depends on the interactions between microorganisms and the working electrode. In batch fermentation, optical cell density and metabolites formation were shifted with respect to redox potential levels in the range of -100 to -450 mV, recording an optimal ORP level of -350 mV, which result in higher SA yield (1.28 mol/mol), productivity (1.18 g/ L/h) and higher mole ratio of SA to acetic acid (2.02) as reported in literature [184]. Another study revealed that the use of redox mediators has the potential to improve fermentation end-products, such as the case of *Corynebacterium crenatum*, and more reduced environment benefited SA production when using reagent supplements such as $K_3Fe(CN)_6$, Na_2S [189].

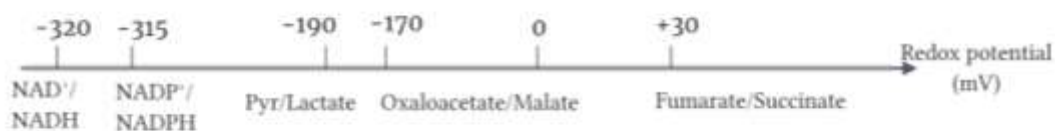


Figure 3. Standard potential redox E° (mv) at 25°C of some redox pair involved in intracellular metabolism [190]. This photo was done by the authors themselves.

V. Current fermentation limitations

Chemicals production by fermentation presents a number of challenges in terms of overcoming the product-mediated toxicities for microorganism's producers, that limits the yields and productivity, by-products formation that lead to reduction in the desired product yields and increase purification costs, minimizing the nutrient and energy costs of fermentation processes, and achieving efficient and economical recovery of the pure chemicals without leading to wastes and high costs.

Carbohydrate fermentation is considered as an interesting alternative process to produce chemicals from fossil resources, simply referring to feedstocks nature which is renewable; however, it has many limitations. First, whole fermentation occurs in a defined pH range, between 6.0 and 7.2 with an optimal pH at 6.8 for *A. succinogenes* [172]. A higher cell growth results from higher pH, however increasing by-products formation; while under pH of 6.0, bacterial growth is limited might be due to an increase of cell maintenance requirements [191]. Many researches indicate that as cells cease to ferment glucose, pH decreases and the major

product formed is succinate [192] as a dissociated molecule, unfortunately, referring to its pKa values which are 4.20 and 5.61, leading to the main problem of most downstream separation methods that entail the undissociated form of SA [193]. During fermentation, formic and acetic acid are produced as well as succinic acid. It is therefore necessary to regulate the pH by alkaline solution addition to stand the process at its optimal pH and hence leading to the equivalent salt succinate otherwise, cell flocculation occurs and the osmolarity is affected; cell productivity is dwindled as energy is consumed for cell maintenance [194]. One other bottlenecks of fermentation process is that when more energy is required to maintain cells, production increases and hence product inhibition takes place, leading to cessation of cell growth and synthesis [195]. Another limitation occurs in media when end product becomes toxic for cell due to its accumulation such as in the fermentation of carvone from carveol [196]. The end product inhibition is considered as a limitation in SA production using *A. succinogenes*, as shown in some studies [197–199]. Till today, the target suggested by the US Department of Energy ($2.5 \text{ g L}^{-1} \text{ h}^{-1}$) is not reachable yet, since the highest productivity seen from this organism is around $1.7 \text{ g L}^{-1} \text{ h}^{-1}$ in batch mode [128, 200]. More recently, the continuous and repeat-batch biofilm fermentation of *A. succinogenes* allowed a significant increase in succinic acid productivity ($22 \text{ g L}^{-1} \text{ h}^{-1}$) [201]. An increased production of SA is allowed by addressing end product inhibition. By-products formation have a similar effect to product inhibition, which prohibit the product formation at high concentrations, as is the case in SA fermentation; by-products mainly formate and acetate can minimize SA production by taking away the carbon dedicated to the main product, decreasing its yield and affecting downstream process [202, 203]. A study done in 2008 [198] revealed that formic acid is the most important inhibitor of both, cell growth and SA production. One solution could be the application of genetic engineering; indeed, specific genes are expressed, allowing to reduce or eliminate unwanted products. However, the idea of altering organism can result in decreasing growth and production rates, as it has been shown in the case of genetically engineered *E. coli* [204, 205]. Because of these limitations, the applications of these genetically modified strains in fermentation at large-scale are not feasible. Taking into consideration substrate concentration level, neither a high concentrations of glucose (over 100 g L^{-1}), nor a low one (20 g L^{-1}) could lead to high cell growth and succinate production [206]. Many studies were done on optimal substrate and its concentration showing that the optimal initial concentration of glucose as substrate was between 50 g L^{-1} and 60 g L^{-1} [198]. To ensure that there is an optimum production of such substrate, fermentation should be monitored steadily; knowing that with time, the concentration of glucose decreases and should be renewed, this situation creates a challenge.

Some studies reveal that there is an increase of the production of succinic acid, even if they worked with a constant glucose concentration, showing that the monitoring of glucose at constant concentration is not a crucial issue. Actually, SA production from butane remains still economically efficient and can compete with these fermentation technologies [28]. Another challenge rises concerning the fermentation cost and the budget dedicated for the improvement of the considered process as declared by the US DOE [3]. To be competitive with petrochemical production, a target productivity should be $2.5 \text{ g L}^{-1} \text{ h}^{-1}$ or more. Downstream process also stands as the largest expense in SA production (can account for up to 60% to 70%) and considered as a major area for improvement [207].

VI. SA downstream separation methods

SA production can be divided in two key steps; fermentation of substrate (carbohydrates) into SA and its recuperation [208]. As mentioned before, as an input, SA is required in its non-dissociated form, and this where expenses took place [209]. The major challenge poses in the overall process is the step of separation and purification due to the problems with fermentation mentioned above. However, it is the major way to enhance the process from an economic point of view [210]. Till now, no single approach meets all requirements (product recovery, concentration, acidification and purification) to have SA from succinate in the fermentation media [126, 211]. Up to now there are few methods converting the dissociated form of succinate to its undissociated form, removing impurities and increasing product purity, that are used at the laboratory scale, including ion exchange, reactive extraction, electrodialysis, crystallization and nanofiltration [212].

Currently, extraction including liquid-liquid extraction, reactive extraction, salting-out extraction, ionic liquids/salts extraction and sugaring-out extraction are evaluated to recuperate SA from fermentation broth media [213]. Based on pKa values, amine-based extraction is a promising method that separates organic acids, as it removes undissociated acids in-situ at mild conditions; thus, in this method, no pre-treatment is needed to act properly [214]. Previous studies have shown that reactive extraction of succinate when used tri-n-octylamine (ToA) extracts SA very efficiently and appear as an excellent separation method. However, complications remain to be solved before this method can be assumed. Only undissociated acids can be extracted using ToA; it is toxic to cells, affecting that way cell growth and production, sensitive to pH fluctuation; so when pH increases, there is a decline of ToA uptake. Consequently, pH should be kept low to make sure that acid is removed from the solution. However, there is no selectivity, acetic acid is removed first; and hence extra fermentation broth

processing is needed after acetic acid removal. It is therefore considered as a pre-treatment step more than a process for SA removal from media [215]. Other methods of downstream extraction and separation of SA need to be inspected. An additional step was evolved to separate and purifies SA, such as crystallization and vacuum distillation, since that byproduct have been eliminated, making it simpler, reaching 99.8% final purity [125].

Ion-exchange technology using resins have been demonstrated to be promising technology when used to recuperate from supernatant, lactic acid without altering the pH of the system [216]. It involves the captures of cations with an ionic resin; a cationic resin is used to capture lactate. Researcher suggested to use this method with SA as a prior step to crystallization [217]. Nevertheless, few publications discuss the purification of SA using ion-exchange resins. According to Chen et al [217], highly acidic ion exchange resin utilization followed by a weak basic exchange resin, can eliminate impurities, cations and anions allowing a purified flow with very low concentrations of sulphate, nitrogenous and impurities. However, ion exchange resins process requires an additional purification step to remove cells and acid due to the low selectivity of the resins.

Glassner et al [218] suggest to use electrodialysis, a process involving an electric potential difference incorporated with membranes to separate molecules that are anionic or weakly ionic from those that are ionized. In the fermentation broth, electrodialysis targets the succinate ion in its dissociated form, and leaving behind other components either non-ionic or very weakly ionic, such as proteins, amino acids and carbohydrates. When fermentation occurs, through the electrodialysis system, liquid from the reactor could be run, the succinate ions are removed and the residual fluid including cells can be recycled back into the bioreactor [219]. Electrodialysis could be combined with other separation methods as stated by [220]. Electrodialysis is a method having a significant potential, with some glaring shortcomings in this system in industrial scale. One of main electrodialysis bottlenecks is the energy requirement, which seems to be a bit counterintuitive to use it, since SA production in fermentation goal as an eco-environmentally friendly process, was to reduce and substitute fossil fuels relying that come from non-renewable resources [221]. Also, electrodialysis selectivity raises an issue because acetate residues still remained in succinate stream and should be removed for a high purity of SA product.

Other separation process suggested by Glassner et al [218] is precipitating SA out of solution. After the completion of fermentation, solids are centrifuged and separated from broth; then broth is processed with calcium hydroxide, creating a solid that precipitates out of solution, calcium succinate which is removed from broth and washed with RO water [214]. The commonly and simplest method for SA separation is precipitation; however, it is the most

inefficient from an eco-environmental standpoint. As an example, the pH of the system is neutralized and buffered in fermentation by supplying chemicals such as calcium hydroxide, and to recuperate SA, large amounts of sulphuric acid are added to the solution, creating SA from calcium succinate, which needs to be washed by a huge quantity of water (raising an environmental issue and making precipitation as an unwanted choice for SA separation); an acidification step to remove contaminants slurry and solid waste and producing calcium sulphate, make this process expensive and unfit for commercial applications [222].

A relatively recent development in separation process is nanofiltration, a new method for separating out the different byproducts of the fermentation broth from succinate. A study done by kang et al [223] show how nanofiltration membranes can separated succinate, formate and acetate; products of glucose fermentation by *A. succinogenes*. First individual components tests were completed, with increasing components numbers up to a quaternary ion solution, and highest retention of succinate was seen in final test. Nanofiltration is a promising method, but still there are some issues that are not addressed, like membranes price, membrane fouling and separation method application in real fermentor broth [224, 225].

Each of these five separation process have a positive side which can be compounded by integrating steps for higher SA recovery than individual methods, however, some defects remain requiring a new process to resolve the issue mentioned above. A perfect process should prevent cells destruction, can be done in-situ and requires a minimal chemicals supplement.

VII. EF as alternative of fermentation

Biological processes are affected by low productivity and conversion efficiency which are primarily thermodynamic and cellular metabolic limitations [226]. To overcome some of these constraints, electrochemical energy derived by electrodes polarization in the fermentation medium can be used. Electrofermentation (EF) is an emerging technology that could open new frontiers in biorefinery using a wide range of substrates, electroactive biocatalysts, and selective bio-based products [227]. The electrochemical energy generated at the electrodes can externally induce a shift from balanced to unbalanced fermentation extending the possibility of achieving theoretical maximum yields [228]. However, such bioelectrochemical conversions require a continuous current flow to maintain the redox conditions and ensure good productivity. The electrode polarization influences the microbial dynamics of the system, and this adds new opportunities to observe specific electroactive bacteria and their interspecies interactions with new metabolic functions [229]. The main precept of EF is controlling electrochemically the

microbial fermentative metabolism [230]. The processing technology of fermentation in electrochemical cell lead to drives a hybrid metabolism that allow electrons exchanging with solid electrodes as alternative oxidizing/reducing equivalents. This process can be implemented in mono-compartment or in bi-compartment [231]. During EF, electrodes either as electron donors (cathodic EF) or acceptors (anodic EF), are employed to modify the medium by changing redox balance of fermentation and to overcome the metabolic limitations that allow unbalanced fermentation [190]. An electrochemical control has a significant effect on microbial metabolism, cellular regulation of pure culture and on the interactions and the selection of interspecies in mixed bacterial populations [232].

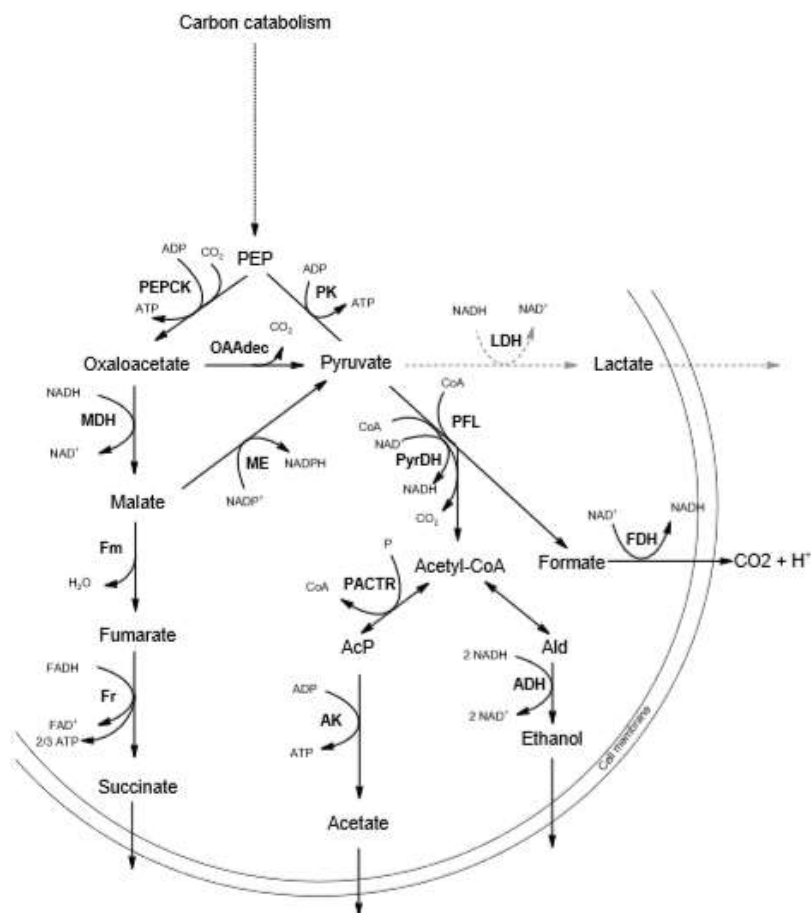
This method features several advantages as providing 1) an oxidative and reductive environment to breakdown or to extend carbon chain, 2) stabilise/optimize fermentation metabolisms by controlling the imbalances referring to substrate purity, byproduct formation and accumulation 3) pH control by transport of ionic products from the broth, and 4) target products extracted by a selective membrane 5) improve microbial biomass yield and increased ATP synthesis [227, 228].

EF process can solve the problems such as microbial growth problems and can lead to an enhancement of productivity, yield, and concentration by adding electron carriers [231] such as NAD^+ and NADH [233] [234]. EF method can be considered to solve the problems encountered in the industrial scale production of valuable chemicals such as ethanol, butanol, and 1-3 propanediol [190].

1. Theoretical conversion from fermentation to Electrofermentation

Fermentation is a metabolic process in which organic and inorganic compounds serve as both electron donors and electron acceptors [235]. Process occurs under aerobic and anaerobic conditions to maintain adenosine triphosphate (ATP) by glycolysis, when there is no oxidative phosphorylation [236]. During fermentation, pyruvate/phosphoenolpyruvate is metabolized to different compounds, such as short-chain fatty acids (SCFAs) [237], alcohols [238], polyols [239], organic acids [200], amino acids [58], polysaccharides, biodegradable plastic components [97], and industrial enzymes in addition to dihydrogen (H_2) [240, 241]. In fermentation, the only energy extraction pathway is glycolysis. The nicotinamide adenine dinucleotide (NADH) produced in glycolysis step cannot drop its electrons off to turn back into NAD^+ [67]. For this purpose, after glycolysis, extra reactions take place to regenerate the electron carrier NAD^+ from the NADH produced in glycolysis [242]. Thus, NADH drops its

electrons off with an organic molecule (such as pyruvate, the end product of glycolysis). Decrease in NADH allows glycolysis to keep running by ensuring a steady supply of NAD^+ [243]. In the first step of fermentation the organic compound is converted to pyruvic acid (pyruvate at neutral pH) during glycolysis. Pyruvic acid (pyruvate) is a cellular metabolite found at the biochemical junction of glycolysis and the tricarboxylic acid cycle, has both keto and carboxyl groups in its structure, so it is a potential precursor for many chemical productions during fermentation [244]. Different microbial inocula can reduce pyruvate to a wide range of end products [245]. To convert an organic compound to pyruvic acid, first it must lose electrons which are picked up by NAD^+ to form NADH [246]. If the glycolysis of glucose molecule is exemplified, the glucose, a pentahydroxy-aldehyde, is converted to the pyruvic acid which is an organic acid, by giving electrons [244] (Fig.4), so it can be said theoretically that this process can take place at the anode of an electro-fermenter because the electron is released in this stage. In other steps of fermentation, it is observed that pyruvic acid gains electron. For example, in succinic acid production, firstly, pyruvic acid is converted to oxaloacetate followed by malate, fumarate and finally succinate. Succinate conversion takes place by taking electrons from the medium (Fig.4). This means that glucose (an aldehyde) is converted to succinate (organic acid). When the oxidation states of organic compounds are examined, it is seen that the aldehyde is reduced to organic acid. Since this reaction is an electron consuming reaction, it is possible to say that this reaction can take place at the cathode of an electro-fermenter.

Figure 4. Carbon metabolism in *Actinobacillus succinogenes* [247]

2. Introduction of electroactive bacteria

Many of the bacteria are generally known to grow aerobically using oxygen as a terminal electron acceptor (TEA) to generate ATP, whereas others are known to grow at low concentrations of oxygen (anoxic) or in the absence of oxygen (anaerobic) [248]. Anaerobic bacteria can use various kinds of TEA-like inorganic compounds such as sulfates, nitrates, iron, DMSO, manganese, etc. Bacteria which gain energy by donating electrons to metal oxides resulting in the reduction of these metal ions are termed as metal-reducing bacteria [249]. These bacteria help in a redox cycling of metals, immobilization of radionuclides, and degradation of contaminants in various environments such as lakes, sediments, aquifers, etc. [250]. They have evolved strategies to transfer the electrons directly to an external electrode or metal ore during their metabolism, which is difficult considering that the cellular membrane is resistant to transfer of charged molecules due to its non-polar (lipid) nature. This extracellular electron transfer (EET) ability of bacteria has shown to be involved in various environmental phenomena such as biogeochemical cycles, pollutants removal, etc. *Geobacter sulfurreducens*

and *Shewanella oneidensis* are considered as the model organisms for studying the characteristics of electroactive bacteria [251, 252]. In addition to oxygen and nitrates, these two bacteria are also known to use iron, manganese, sulfur, and nickel ions as TEAs. Despite identification of many electroactive bacteria (EAB) by high-throughput sequencing analysis, only a small number of EAB were isolated and studied for their EET mechanisms [233]. Specific growth requirements and functional gene markers for identification of the EAB and tools to isolate them are under investigation.

3. Electrodes Materials

In bio-electrochemical system, conventionally, carbon-based electrodes such as graphite, graphite flakes, carbon cloth, carbon mesh, carbon felt, etc. are currently being used for economic viability [253]. Surface modification methods can be interesting in such systems and may include several physicochemical treatments. Plasma treatments applied for metallic surfaces, organosilane coating, chemical modifications of carbon nanotubes for microbial adsorption as well as excellent electrical conductivity, nitric acid treatment, and covalent-immobilization with neutral red molecules for enhancing electrical conductivity are studied [254]. These modifications entail permanently altering the properties of surfaces by chemical or physical means and consequently changing its interaction with the environment and affecting microbial attachment [255]. Two dimensional (2D) and three dimensional (3D) electrodes have also been studied which facilitate open macroporous structure with a large surface area for bacteria attachment and enables the electrostatic interactions [256]. 3D electrodes have good conductivity, large surface area, and suitable surface properties for bacterial attachment and can influence towards higher electron transfer rates. 3D electrodes facilitate EET between the bacteria and electrode interface. Compared with conventional and 2D electrodes, modifying and coating a 3D macroporous electrode is promising in the domain of EF. EF can also be involved in providing specific alterations to the electron transfer proteins which help towards improved and controlled electron kinetics towards overall product output efficiency [257]. The electron carriers involved in the electron transfer during the microbial metabolism are C-type cytochromes, ferrioxin, rubredoxin, hydrogenase/dehydrogenase, etc. [251, 258].

4. Microbe –electrode interaction

Bacteria living in suspension can accept electrons from the surface of insoluble metal forms or electrodes by interacting with metal substrate briefly [259]. Whereas the bacteria living in the biofilm of an electrode or mineral ore face challenge depending on location of bacteria in biofilm. Bacteria located at the surface of an electrode or mineral ore have the highest

availability of terminal electron acceptor, while having the lowest availability of nutrients because of the several layers of biofilm hindering the diffusion of nutrients to the electrode surface. Contrarily, in the case of bacteria living on the surface of biofilm, nutrients are easily available, but there is less contact with electrode surface due to the biofilm presents between electrode and bacteria. Biofilm housed bacteria living on the surface have several ways to transport electrons from the biofilm surface to the electrode surface, which can be differentiated into direct, indirect and mediated mechanisms. Direct electron transfer (DET) mechanisms involve direct contact between the bacteria and the electron acceptor such that electron transfer proteins and various cytochromes on the outer membrane surface could make contact and transfer electrons onto the electrode directly [260]. Indirect electron transfer involves the utilization of extracellular wire-like appendages such as pili that allow electron flux from the electroactive bacteria to an electron acceptor. These extracellular wire-like appendages are also known as nanowires and can conduct electricity at several cell length distances [261]. In mediated electron transfer, soluble redox mediators from either cellular metabolism or external sources are utilized to shuttle electrons between the electrode and bacteria. Most of the mediators can move across the cellular membrane, making them ideal for shuttling electrons between electroactive bacteria and the electrode. Mediators such as flavins, phenazines, pyocyanins, and siderophores are from the cellular metabolism, whereas humic substances, chemical mediators, and environmental mediators can act as external mediators. All the three mechanisms contribute and function under different environmental stresses [233].

The presence of a conductive material in a microbial environment along with external stimulation influences the observed rates of extracellular electron transfer (EET) providing process specificity and flexibility [262, 263]. Fig. 5 depicts the microbe -electrode interactions in the presence of electron carriers showing influence on electron transfer. Electrodes development with higher efficiency to conduct oxygen reduction reaction (ORR) kinetics at cathode leads to higher reducing equivalent utilization for product synthesis [264, 265]. EF relies on the interaction between biocatalysts and electrodes on the energy conservation with applied/in situ potential on the electrode surface. The effective microbe-electrode interactions facilitate in increasing the electrocatalytic rate and regulating bacterial metabolic routes during EF towards achieving higher product synthesis [266].

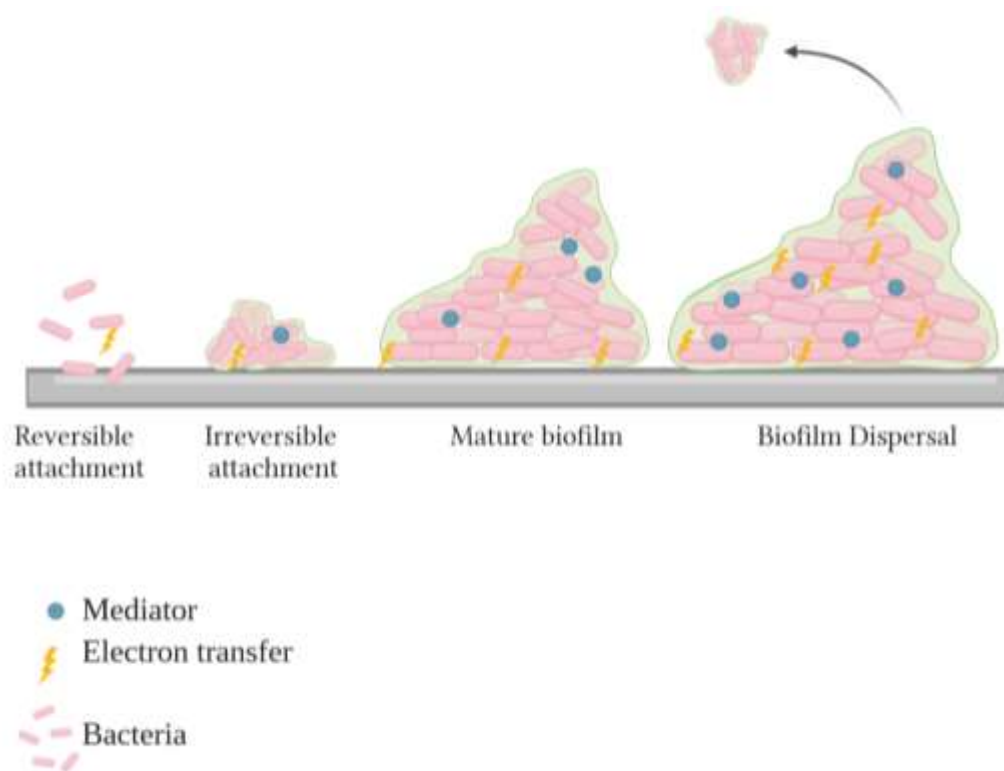


Figure 5. Schematic representation showing bacterial behavior on electrode surface (this picture is made by me using Biorender software)

5. Applied potential

Various studies have shown the importance of the application of external stimulation (anodic/cathodic) in controlling the metabolism of microorganisms for driving a biological process towards a specific product synthesis [267]. The potential inputs (positive/negative) on the electrodes influence the reactions taking place at anode/cathode and are necessary to drive the reaction towards definite product synthesis [187]. The difference in oxidation and reduction potential of anode and cathode influences the reaction kinetics and product output [185]. The energy content in the adenosine triphosphate (ATP) of microbe is equal to the oxido-reductive potential difference, also called Gibbs free energy [231]. It determines the energy that a biochemical reaction requires (endergonic) or releases (exergonic) for product synthesis. Energetics determines the potential that needs to be supplemented during the biochemical reactions [268, 269]. The exergonic voltage generated as a difference of oxidation and reduction potentials requires an additional potential input in EF to neutralize the system energetics for higher energy utilization. It is also similar in the case of endergonic reactions which require higher energy inputs and need to be stoichiometrically redox balanced with potential

supplementation based on the system performance [249]. Most bacteria are negatively charged and hence imposing a positive potential to the working electrode can accelerate the biofilm formation due to the electrostatic interactions [270]. To facilitate the reductive reaction, a negative potential should be imposed [271]. The reactions in EF are anode-/cathode-specific depending on the potential inputs and can either occur in single or dual chambered reactors. Depending on the electrode utilization and the applied/in situ potential, the inoculum gets adapted to the biosystem for enriching specific exoelectrogenic microbial strains.

The redox potentials applied to the biocatalysts affect mainly C-type cytochromes and electro products outcome [234]. The electrons flow from an electron donor with lower redox potential to an electron acceptor with higher oxidation potential. Electrical stimulation (positive and negative potentials) during the EF can possibly change the directions of the reducing equivalents (e^- and H^+) involved in the microbial electron transport chain. This change of directions imposed on the electron transfer channels can strictly make a difference on the electro product synthesis in EF process by having a control on the flow rate of the reducing equivalents. EF have been reported as a very promising technology in both pure and mixed culture systems (table 7); it has been reported for the production of electrofuels, bioelectricity, biohydrogen (H_2) and bioethanol with co-products formation, including formate, lactate, and propionate using food waste as substrate during the bio-electro fermentation process [272, 273].

Table 7. Some value added electro-fermentation products

Microorganisms	Substrate	Applied Voltage	Products	Ref
Mixed microbial cultures	Single or Mixed compounds (glucose, ethanol)	-0.7 V/Ag/AgCl	Isobutyrate	[274]
Mixed enriched culture	CO ₂	-0.9 V/Ag/AgCl	CH ₄	[275]
Methanobacterium palustre	CO ₂	-0.8 V/Ag/AgCl	CH ₄	[276]
Mixed microbial cultures	Food waste	-0.6 V/Ag/AgCl	Acetate, butyrate, propionate	[277]
<i>Corynebacterium glutamicum</i>	Glucose	-1.25V/Ag/AgCl	Lysine	[226]
Electroactive bacteria	wastewater	-*	biohydrogen	[278]
Mixed culture	glycerol	-1.1 *	1,3- propandiol	[279]
Mixed culture	Food waste	0.44 *	Biohydrogen, bioelectricity, bioethanol	[272]
Mixed culture wastewater treatment plant	CO ₂	-1 to -1.15 V/Ag/AgCl	CH ₄ , acetate	[280]
Mixed enriched culture	Sodium acetate	-0.7 V/Ag/AgCl	CH ₄ , acetate	[281]
Mixed enriched culture	CO ₂ , H ₂	-0.8 V/Ag/AgCl	acetate	[282]
<i>Clostridium</i>	CO ₂	0.197 V/Ag/AgCl	Butyrate, ethanol, butanol	[283]

- Two electrodes electrochemical system, potential difference imposed between anode and cathode.

VIII. Conclusions

At present, platform chemicals are mainly produced from petroleum-based raw materials and they have an undeniable commercial importance. However, with the decline of these resources and the looming dangers of dramatic CO₂ emissions, the sustainable production of platform chemicals using biomass-based feedstocks is becoming inevitable. In this context, Current rationale around the concept of platform molecules is discussed in the present review survey. The screening of bio platform molecules, less expensive feedstock, maximum substrate utilization, the development of more efficient microbial strains by genetic engineering, integrated upstream and downstream processing technology and the green production are the major areas of platform chemical discussed in the literature and which need further research. Future perspective to control redox potential fermentation profile is attractive, using genetically modified strains featuring tailor-made redox potential balance that subjected to tight regulation through precise redox potential level. At different levels of redox potential, metabolic flux profiles will be quantified to locate potential bottleneck for strain improvement and to reach a maximum production of various desired metabolites. Redox potential–controlled fermentation technologies thus anticipated. Techno-economic analysis and life cycle assessment based sustainability studies can be used for comparative evaluation of electro-fermentation process with conventional processes.

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Chapter II: Materials and methods

Preamble

This chapter is describing research material and methodology. This chapter also helps to present wide-ranging logic to select methods, cases and tools used in this research work. It will also assist to choose appropriate methodology and make guidelines for material collection. Materials finding and searching important articles are also very important towards the research study so, also included in this chapter. This section gives readers and researchers information on where they can access the materials that you used in this research. Thus, this chapter covered research process, primary research output for proposal purposes in the context of implementation of integrated biorefinery with an overview of qualitative and quantitative methods, data collection, recording and analysis.

Experimental study of succinic acid production

I. Chemical and gas

Unless otherwise specified, all chemicals used were of reagent grade and were purchased from various suppliers as Sigma-Aldrich or Merck. Ultra high purity Nitrogen (N₂) gas was supplied from France.

II. Microorganism and inoculum preparation

1. Pure culture maintenance and preservation

Once *A. succinogenes* has been grown in pure culture, it becomes necessary to maintain the viability and purity of this microorganism, by keeping the pure culture free from contamination. Strains can be maintained by periodically preparing a fresh culture from the previous stock culture. The culture medium, the storage temperature, and the time interval at which the transfers are made, vary with the species and must be ascertained beforehand. Pure cultures can be successfully stored at 0-4°C, either in refrigerators or in cold-rooms. This method is applied for short duration (2-3 weeks). However, since repeated sub-culturing is time consuming, it becomes difficult to maintain a large number of pure cultures successfully for a long time. In addition to the risk of genetic changes as well as contamination. However, some methods do not need frequent sub-culturing; *A. succinogenes* was preserved on TSA Petri dishes and agar slant. They were incubated for 48 h, and then stored in a refrigerator. Periodically, the agar slant cultures were transferred to a fresh media each 4 months.

2. Pure culture

Native *Actinobacillus succinogenes* DSM 22257 in pellet form was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Institute DSMZ-German Collection of Microorganisms and Cell Cultures), and used in all experiments. As per bacterium supplier recommendation, inoculation medium was prepared by thoroughly mixing 3 g of Trypton Soya Broth (TSB) in 100 mL of distilled water whose composition consisted in (g L⁻¹): 15 g Trypton, 5 g Soybean peptone, 2.5 g D (+) Glucose, 5g Sodium chloride (NaCl) and 2.5 g Dipotassium phosphate (K₂HPO₄). The bacterium was revived in 0.5 mL of inoculation medium and preserved on Tryptic Soya Agar (TSA) slant as described below. Cells were incubated at 37°C for 24-48 h in an incubator (VWR® INCU- Line) which was the time required for the microorganisms to enter the exponential growth phase. 15 mL of TSB in 25 mL screw-capped sterilised glass vials was used for this purpose. During the microbial growth, 1 mL sample was

withdrawn from the culture medium to determine the optical density using spectrophotometer. Cells were incubated until their optical density reached 1 at 660 nm.

The yeast species, *Saccharomyces cerevisiae* baker's yeast CLIB 95, used in this study was obtained from CIRM, France. *S. cerevisiae* was maintained at 4°C on Sabouraud glucose agar 4% slant whose composition consisted in (g L⁻¹): 40 g glucose, 10 g peptones, 15 g agar, then cells were incubated at 28 °C for 24 h in an incubator.

3. Case of co-culture

Depending on the experimental set-up, the populations was perfectly mixed at a ratio of (1:1 v/v) and co-cultures were carried out in 200 mL volume range at 37° C and 150 rpm for 48 h and population densities was measured at 660 nm. Cells from co-culture were centrifuged (1800 g, 4°C and 5 min) in a centrifugation (Thermo-Fisher, scientific, HERAEUS Megafuge 16R), the suspension obtained in 10 mL of KCl 150 mM was used for inoculation.

4. Pre-culture

At the end of the incubation period, cells were centrifuged aseptically suspended in 10 mL KCl (150 mM). For the inoculum preparation, 2 mL of suspended culture cells were transferred to 250 mL Erlenmeyer flasks containing 200 mL of culture medium. The inoculated flasks were incubated in a rotating shaker (New Brunswick, INNOVA 40, NJ, USA) at 37° C and 150 rpm for 48 h. Cells from pre-culture were centrifuged aseptically in the same conditions mentioned above and the suspension obtained after harvesting cells and re-suspending in 10 mL of KCl 150 mM was used for inoculation. Figure 1 represents a Schematic diagram of the inoculation procedure of *A. succinogenes* to small anaerobic reactors (SARs). (A) Untrained cells (initial cells before incubation) are inoculated to small tubes and agar plates, (B) 4-6 were incubated at 37°C, (C) the best SAR from each experiment was selected and (D) centrifuged, cell suspensions with KCl were spread to new TSB and then grown to be used for the pre-culture experiment. Cell cultures can be preserved in cryopreservation vials and stored at 4°C. At the end of the pre-culture experiment (after 48 h) (E), cells from the best pre-culture from the next experiment, centrifuged and resuspended in KCl were ready to be used for (SARs) experiments.

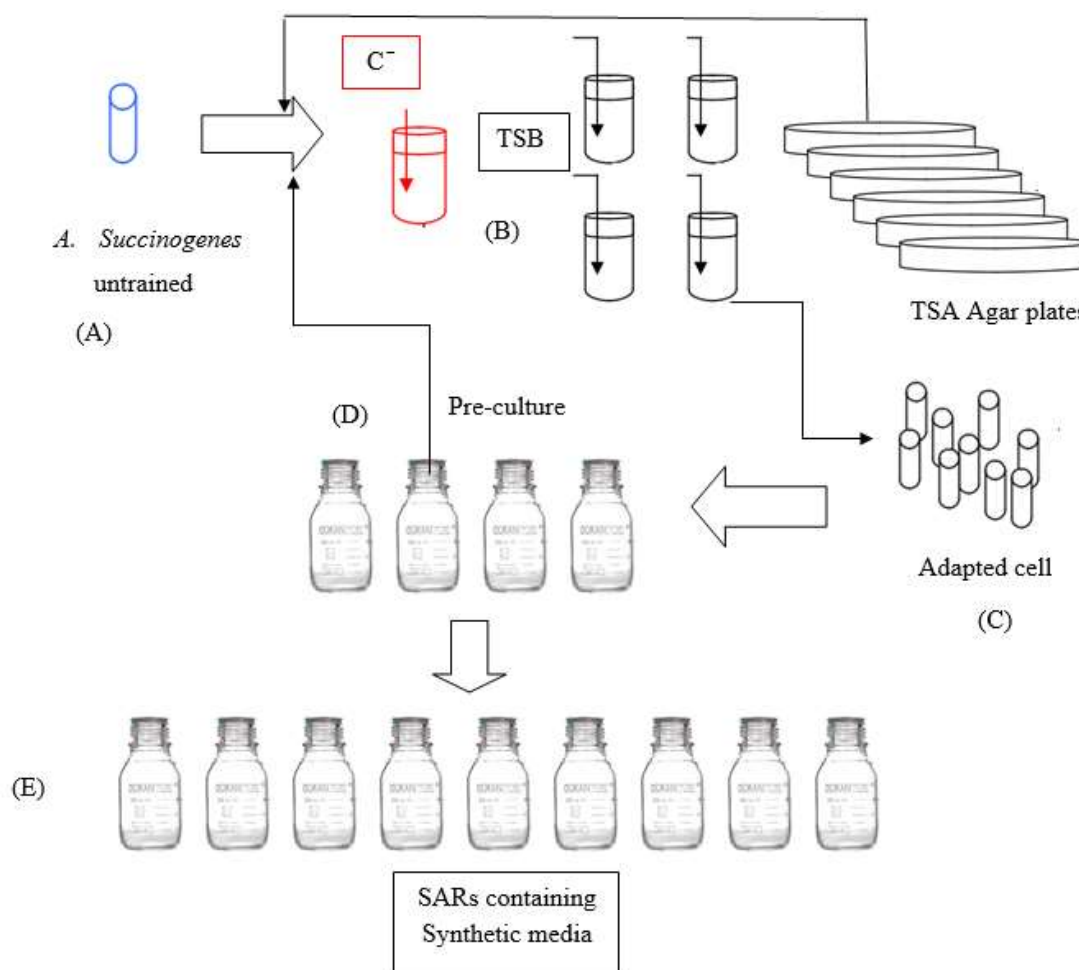


Figure 1. Schematic diagram of the inoculation procedure of *A. succinogenes* to anaerobic bottles (250 mL).

III. Fermentation

Fermentation media consists of major and minor components, where the major components including carbon and nutrient source contained (per liter): 32 g glucose, 32 g fructose and 0.53 g NH_4Cl ; and minor components including inorganic salts, buffers, cofactors, dissolved oxygen, and other dissolved gases, contained (per liter): 3 g K_2HPO_4 , 3 g KH_2PO_4 , 1.25 g $NaCl$, 0.3 g $MgCl_2 \cdot 6H_2O$, 0.3 g $CaCl_2 \cdot 2H_2O$, NaH_2PO_4 , Na_2HPO_4 . Fermentation media was prepared as stock solution and sterilised by filtration on 0.22 μm sterile membrane filter (Sartorius), when setting up the fermenters. Phosphate was added immediately before the inoculation to avoid precipitation.

1. Fermentation conditions

CO_2 fixation is required to enhance succinic acid production. Therefore, CO_2 was supplied to the fermentation broth. Excessive amount of $MgCO_3$ up to 10 g L^{-1} was added as a buffer medium to maintain the pH between 6.8 and 7. Fermentation temperature of 37°C and agitator

speed of 150 rpm were maintained throughout the incubation period. Samples (5 mL) from the fermentations were taken in aseptic conditions at various time points in order to follow bacterial growth, sugar consumption, and the production or uptake of acids (e.g., succinic, formic, acetic, and lactic acid), and other compounds (e.g., ethanol).

2. Experimental design

Batch fermentations were performed out in three different volumes capacity. All equipments were sterilised before use. The tests for this study were carried out in two-types of glass bottles (Duran borosilicate 250 and 1000 mL) with a round shaped floor. The third reactor was a 3 L fermentor (New Brunswick BioFlo®/CelliGen® 115 Benchtop Fermentor & Bioreactor); Fig 2 represents the schematic diagram of the fermentor. A working volume of 1800 mL was considered and it was operated at a temperature of 37°C, pH between 6.8 and 7 with an agitator speed of 150 rpm. Fermentation medium was fed with 1 % v/v inoculation medium into the reactor after the temperature and pH had stabilized. The culture was sparged with N₂ (g) into the fermentor for 10 min at approximately 0.2 volume of gas. volume⁻¹ of liquid minute⁻¹ (vvm) in the beginning of fermentations to maintain anaerobic conditions. During the fermentation, Samples of 10–20 mL were withdrawn aseptically from the reactors at regular time intervals. The samples were centrifuged at 1800 g for 5 min. The supernatant was filtered through 0.22 µm syringe filters and analysed using HPLC for measuring sugars and succinic acid concentration. The fermentation setup and equipment are detailed in table 1. It consisted of two units: Master control and utility station. Fermentation broth entered the reactor. The working volume of the fermentation in the reactor was approximately 54% of total volume. The temperature was controlled by the hot plate and a thermocouple that was inserted into the thermowell at the top section of the reactor. pH was measured by inserting and sealing the bottom of the electrode which was connected in a cylinder casing. The controller, to which the electrode was connected, controlled the pH by intermittently activating the pump that fed 0.1 M KOH solution.

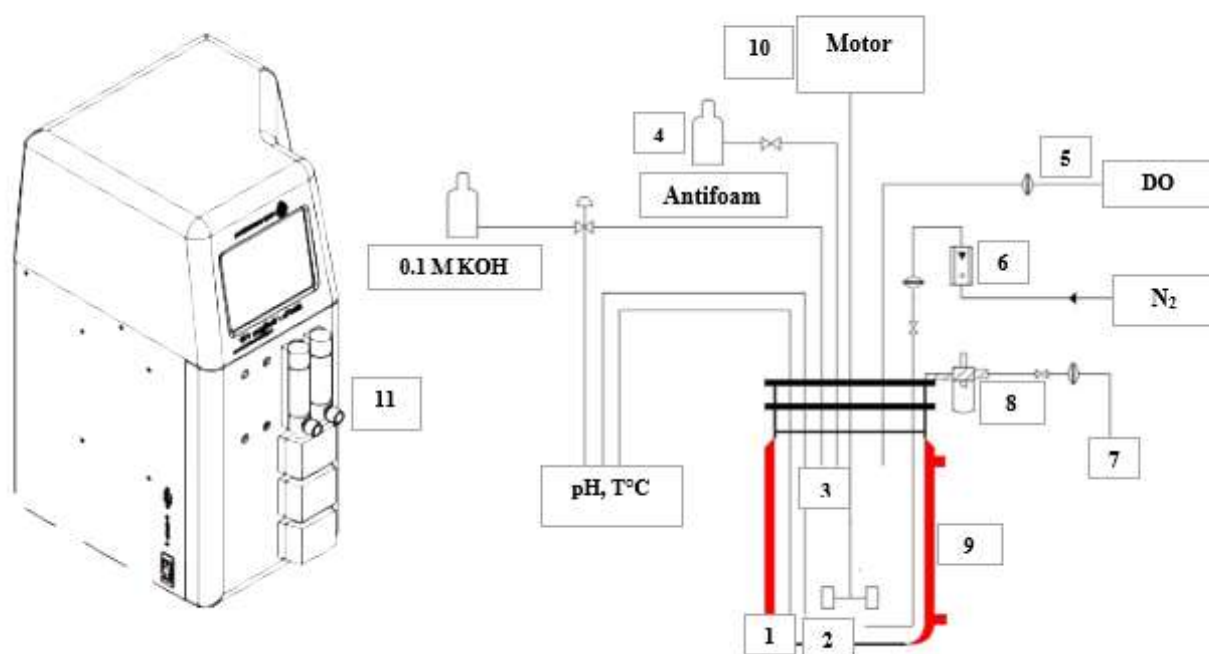


Figure 2. Real (a) vs Schematic (b) Diagram of a Bench-Top Bio-fermentor; (1) pH control, (2) temperature control, (3) alkali input for pH control, 0.1 M KOH, (4) antifoam input, (5) DO prob, (6) N₂ input, (7) sampling tube, (8) sampling vial, (9) electrical heater jacket, (10) Motor and (11), Master control Station. Filters that were used here were 0.22 μ m (SARTORIUS).

Table 1. Equipment used in the experimental setup

Equipment description	Details
Master control station (touch screen)	Fermentation and cell culture monitoring and control
Vessel total volume (Vessel stand with stainless steel head plate)	3 L
Working volume	1.8 L
Temperature control	PID for heating and cooling Heat-blanketed External heating blanket and immersed stainless steel cooling coil. Platinum probe
Agitation control	PID control; manual, automatic, or cascade settings
pH/DO control	Potentiometric DO probe, pH probe
Aeration	Rotameters for gas flow mixing and sparging
3 Fixed peristaltic pumps	Perform acid or base addition (feeding)
Antifoam control	The controller operates the antifoam-assigned pump that adds chemical defoamer into the vessel as needed
Septum	For the safe addition of inoculation cultures

3. Batch fermentation

Batch fermentations consisted of microaerobic and anaerobic phases. Under microaerobic fermentation conditions, the dissolved oxygen level was 100% of air saturation at the time of inoculation and under anaerobic fermentation conditions, nitrogen gas was bubbled through the medium for 5 min to remove oxygen before inoculation, since succinic acid production occurs in anaerobic conditions. During the fermentations, samples were aseptically withdrawn under a laminar flow cabinet (FLUFRANCE- Model; Cytair 125) for optical density measurement, sugars and organic acids analysis after 24, 48, 72 and 94 hours of culture. The ‘working volume’ setup was completely sealed, except for inoculum introduction and for sampling. The inoculum (prepared similarly to stock cultures: see section 2.3) was first transferred to a sterile tip and inoculum was then inserted into bottles.

IV. Bioelectrochemical system (BES) configuration also called Electro-fermentation (EF)

1. Biocatalyst

Actinobacillus succinogenes was used as parent inoculum to start up the bioreactors. The strain was incubated overnight in TSB medium, and pre-cultured for 48h in TSB medium for activation at 37°C prior to inoculation and the resulting biocatalyst (bacteria) was used as inoculum. The pre-cultivated cells were inoculated in the bioelectrochemical single chamber with an initial cell concentration of $OD_{600} = 0.03$.

2. Electro-fermentor and Electrodes

Five Neck round glass bottles (Quickfit®, England) was used to fabricate single chamber bench-scale anaerobic reactors (total/working volume 250/200 mL). The reactors were operated in batch mode with suspended growth configuration with constant mixing (250 rpm) using a magnetic stirring bar at a temperature of 37 ± 1 °C. The individual reactors were operated as control (C-Without electrodes) and with Applied potential difference (AP-with electrodes with potential of 0.3 V/SCE imposed between the graphite felt working electrode and a saturated calomel electrode (SCE) as reference electrode). The Applied potential was used based on the energy scale of a Fumarate/Succinate couple redox for anode and cathode electrode. Graphite felt was used as electrode material for the working electrode and for the counter electrode. The two electrodes had the same dimensions (3.5 x 3.5 x 1.0). Prior to start, the set-up with the electrodes was autoclaved at 121 °C to prevent contamination. The distance between electrodes was approximately 2.5 cm. Each bioreactor was designed to have sampling port, N₂ sparging, electrical connections input ports (top) and leak proof sealing was employed to ensure anaerobic microenvironment in the system.

3. Media

The composition of media used in the electro-fermentation was the same to that used in the fermentation and sterilised by filtration using 0.22 µm sterile filter (mentioned above section 2.5).

4. Operation

Batch fermentation start after inoculation to explore the viability and the performance of the fermentative bacteria strain, *A. succinogenes* with an electron supply through a polarized electrode by imposing an electrical field during the process of electro-fermentation. The bioreactors operating in batch mode for 48 h was presented in Figure 4. The anaerobic conditions in the bioreactors were maintained by sparging N₂ gas into the solution. All the bioreactors were operated at temperature (37 ± 1 °C). At the end of each batch operation, the electrical current was stopped and the set-up was autoclaved to ensure the security of workers.

5. Experimental set-up

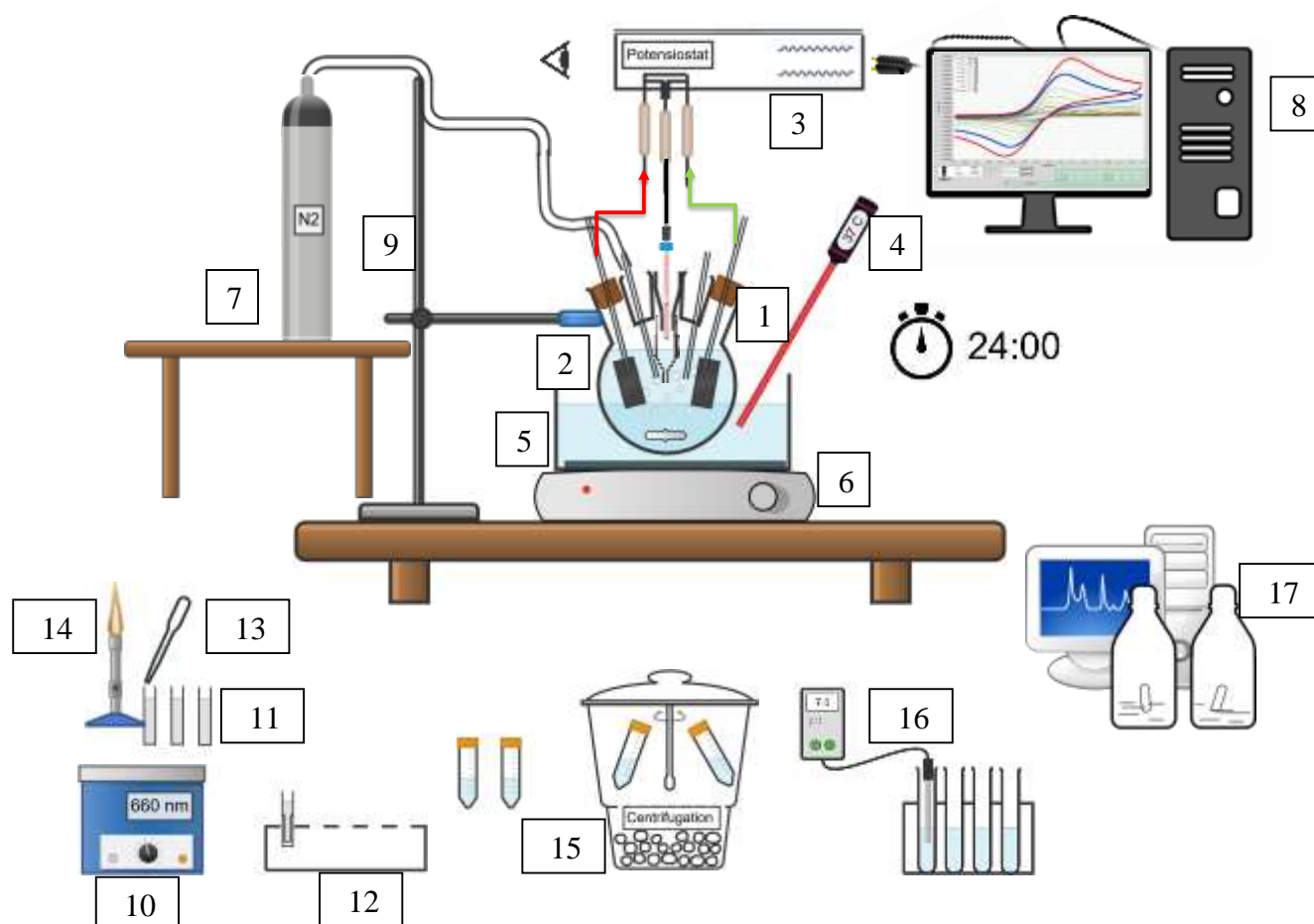


Figure 3. Set-up of an electrochemical cell (1) Round bottle flask with five neck, (2) anode and cathode electrode, (3) potentiostat, (4) Thermometer, (5) tank containing water, (6) Hotplate magnetic stirrer, (7) N₂ gas cylinder, (8), PC, (9) Retort stand, (10) spectrophotometer, (11) cuvette, (12) test tube rack, (13) pipette, (14) Bunsen burner, (15) centrifugation containing centrifuge tube, (16) pH meter, (17) HPLC.

6. Cyclic voltammetry

Current potential curves were plotted with a BioLogic SP 150 potentiostat unit equipped with the EC-Lab-V10.36 software package. The electrochemical experiments were implemented in a typical three electrodes system with a saturated calomel electrode (SCE) as the reference electrode, a platinum wire as a counter electrode and vitreous carbon ($S=3.14 \text{ cm}^2$) as the working electrode at 40 mV.s^{-1} scan rate. The working electrode was carefully polished with sand paper (European # P4000, Struers, Ballerup, Denmark) and rinsed with distilled water before each run. All experiments were conducted under nitrogen atmosphere at room temperature.

V. Analytical methods

This section describes the analytical methodology that was followed throughout this study for the quantitative measurement of the involved components and the qualitative characterization of cell growth in liquid suspension. Fermentation samples were monitored approximately each 24 hours by sampling volume point of around 5 ml (± 0.5 ml). For HPLC analysis, samples were analysed two times and average values and standard deviations were extracted for each one. Fermentation samples were analyzed for optical density (OD) immediately after sampling while the rest of the sample was centrifuged in a centrifuge at 1800 g for 5 minutes using 50 ml Nalgene tubes. The supernatant was decanted in a new annotated culture tube 15 mL (VWR) and stored in the refrigerator at -18°C pending assay. Remaining solids like MgCO_3 and cells were precipitated and were then removed. MgCO_3 also acts as a carbon source and the carbonate concentration CO_3^{2-} was calculated by a titration using 0.2 mol L^{-1} of H_2SO_4 at the end of the fermentation process.

a. Optical density follow-up

Cell growth was determined by measuring the optical density of the sample at 660 nm (OD_{660}) by using a UV-VIS spectrophotometer (PRIM SECOMAM, Milton Roy, Ivyland, PA, USA). The OD expresses the quantity of suspended cells in the fermentation broth. OD measurements took place immediately after sampling by using 4 ml plastic cuvettes. Sample was homogenized and then analysed. Dionised water was used as blank (zero absorbance).

b. Dry Cell Weight Determination

From each flask, all the culture (about 45 - 50 mL) was loaded into a pre-weighed centrifuge tube (M1) and centrifuged (Thermo-Fisher) at 1800 g for 5 minutes. The supernatant was decanted and the tube with cell pellet was then dried in incubator at 105°C overnight. The tube and pellet was weighed to determine cell weight (M2). This drying/weighing was continued until constant weight was achieved.

$$\text{Dry matter} = (\text{M2}-\text{M1}) / 50. \cdot 10^{-3}$$

Cell concentration was spectrophotometrically monitored by OD_{660} correlated to dry cell weight; the relationship between optical density and Dry cell weight is described by:

$$Y = 0.0014x + 0.6634$$

$$R^2 = 0.7452$$

where $Y = \text{OD}_{660}$; $x = (\text{dry weight of cells}) \text{ mg L}^{-1}$

DCW measurements were only used as a rough indication of cell mass concentration. *A. succinogenes* growth in TSB medium with an OD660 value of 1 (Abs) had a concentration of 0.24 g DCW/L.

In the optimal media, the relationship between optical density and Dry cell weight is described by:

$$Y = 0.0042x + 0.096$$

$$R^2 = 0.7445$$

Where X (the biomass) is calculated using this equation and the $r_X(dX/dt)$ is also determined to obtain the $\mu = (dx/dt) = r_X/X$

c. pH Measurements

In batch bottles, pH was measured offline from fermentation samples by using a pocket pH meter (pH 3110, pH electrode SenTix® 21). pH was monitored on line by using gel filled pH electrodes (pH METTLER, TOLEDO, Instruments).

d. High Performance Liquid Chromatography (HPLC)

i. Samples preparation

5 mL of fermentation broth was centrifuged (1800 g, 4° C, 5 min). The supernatant was then filtered through a 0.22 µm syringe filter. The filtrate was then either stored on 4°C or analysed by HPLC.

ii. Materials

To determine sugar and organic acids concentration, 20 µL of filtered sample were injected onto a high performance liquid chromatography (HPLC) (WATERS ®) (BIO-RAD, Hercules, CA, USA). HPLC was equipped with ion exclusion column and a Shimadzu RIO 6-A Refractive Index (RI) detector. The mobile phase consisted of 0.01 N sulfuric acid (H₂SO₄) (Merck, MILLIPORE), was degassed beforehand under vacuum to perform by waters pump (Milford, MA, USA). Figure 3 illustrates all the analytical units of the HPLC equipment that was used in this study. The specification of the HPLC system was the following:

Column: Biorad Aminex HPX-87H organic acids column

Column dimension: 300 x 7.8 mm,

Column temperature: 45°C, maintained by oven (Cro-Cir TM, Cluzeau Info-Labo, ste Foy La Grande, France)

Mobile phase: 0.01 N sulfuric acid (H₂SO₄),

Flowrate of mobile phase: 0.7 mL min⁻¹

Detector: Shimadzu RIO-6A refractive Index detector (JAPAN)

The software used was IC Net version 2.3, Metrohm AG, Herisau, Switzerland

Pure standard solutions in ionised water were prepared for glucose, fructose, glycerol, ethanol, fumaric acid, succinic, formic, and acetic acid and retention time, equation, correlation, detection and quantification limits and the coefficient of variation of each component are listed in Table 2.

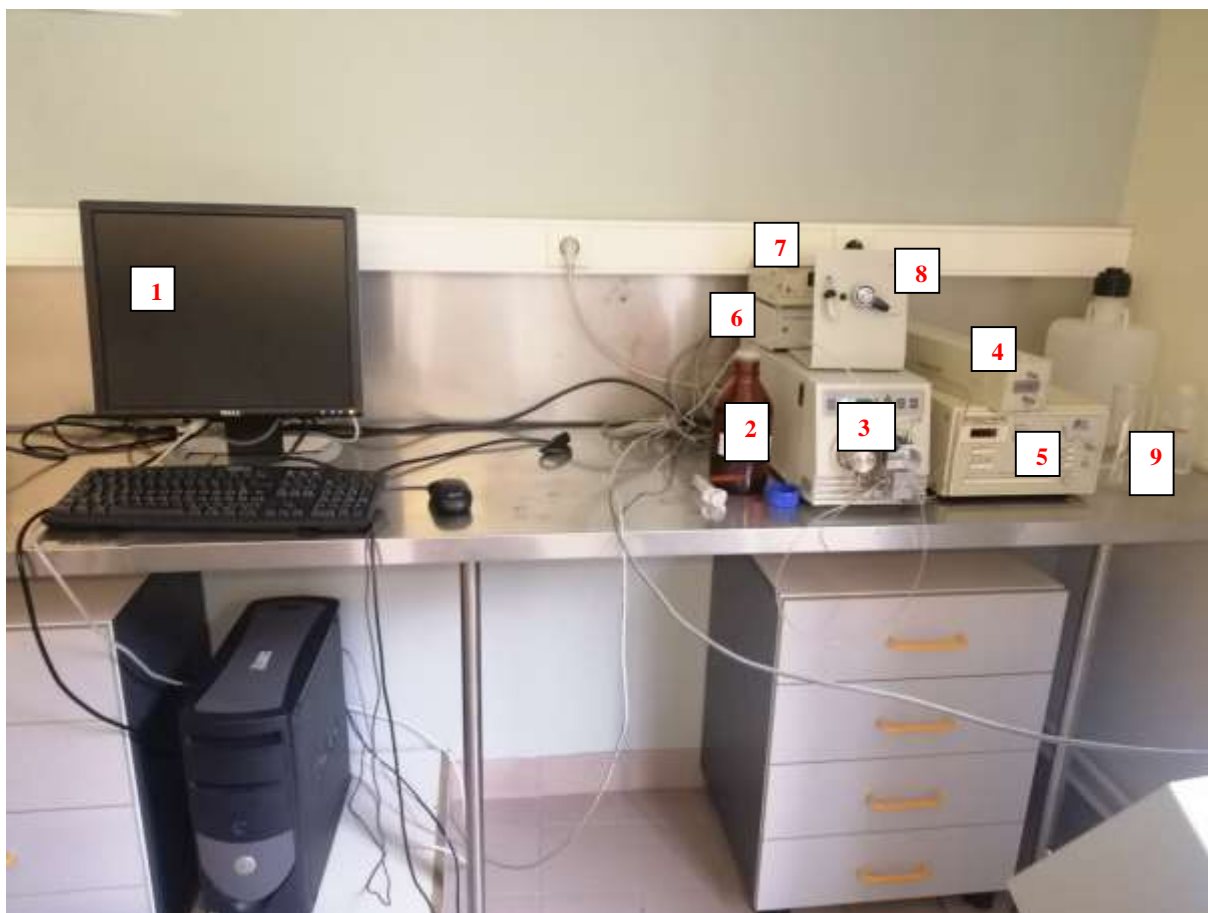


Figure 4. The HPLC apparatus: (1) PC data collection; (2) mobile phase; (3) pump system; (4) column, (5) RI detector (6) integrator, (7) oven, (8) Manual sampler unit, (9) Waste collector unit.

Table 2. Retention time of each component in standard calibration curve

Components	Retention time (tr)	Equation	Correlation (R^2)	Limit of quantification (mol L ⁻¹)	Detection Limit	HPLC validity interval	Coefficient of variation (CV) STDV/Average (%)
Glucose	7.12	$y = 190117x - 0.81$	0.9978	0.001	0.0008	0.001-0.4	0.06
Fructose	7.8	$y = 183540x - 11.5$	0.9989	0.001	0.0003	0.001-0.7	5
Xylose	7.69	$y = 127446x + 25.096$	0.9864	0.001	0.0003	0.001-0.2	0.4
Galactose	7.7	$y = 173619x - 1.2559$	0.9996	0.001	0.002	0.001-0.2	1.8
Sucrose	5.96	$y = 487573x - 95.448$	0.9657	0.01	0.0005	0.01-0.2	0.3
Succinic acid	9.6	$y = 75041x + 17.0$	0.9949	0.001	0.00037	0.001-0.5	0.8
Glycerol	12.16	$y = 158251x + 6.17$	0.9997	0.0021	0.00011	0.0021-0.2	4
Ethanol	17.7	$y = 19721x + 7.52$	0.9974	0.004	0.0003	0.004-1.1	5
Fumaric acid	11.8	$y = 95244x - 2.26$	0.9985	0.0017	0.0008	0.0017-0.0080	0.9
Formic acid	11.4	$y = 22670x + 5.79$	0.9835	0.02	0.004	0.02-0.1	5

In the most cases, $R^2 > 0.96$, which mean a significant correlation between refractive index and the concentration of the chemicals [1] found repeatability values below the maximum acceptable limits for the validation of chromatographic methods. Likewise, the validation of analytical and bioanalytical methods establishes that CV% values less than 10 are acceptable as in our study except for acetic acid.

e. NMR Analysis

NMR (Nuclear Magnetic Resonance) was used to prove and to confirm the production of succinic acid by *Actinobacillus*. The spectra are recorded on a Bruker Avance III 400 spectrophotometer operating at 400.13 MHz for ^1H , equipped with BBFO probe with a Z-gradient coil and a GREAT 1/10 gradient unit. The standard temperature was adjusted to 298 K. All NMR tubes were made as follows:

- Collection of 450 μl of solution and introduction into NMR tube.
- Addition of 100 μl of D_2O to NMR (to remove water)
- Homogenization in NMR tube.
- Spectrum recording: Three experiments per test tube were performed in a systematic way, with the autosampler:
 - 1) Recording a ^1H spectrum in 80 scans
 - 2) Recording of a ^1H spectrum with presaturation of water in 80 scans
 - 3) Fast HSQC to check the ^{13}C allocation of the proton peak at 2.4 ppm

The NMR spectra were manually phased and baseline corrected using MestReNova (Mnova). Each NMR spectrum was used to construct a data matrix by subdividing it into regions having an equal bin size of 0.5 ppm over a chemical shift range of 1-9 ppm.

f. Data Analysis

Feedstock consumption and platform molecules formation were compared in terms of the key performance factors. The dependent variables used were final succinic acid concentration, succinic acid productivity and final succinic acid yield expressed in mol-C succinic acid/mol-C sugars consumed. The analysis of data was carried out by ESP script (EXCEL software program 2016) using T-test ($p < 0.05$) and were reported as mean and standard deviation.

Information about the key performance parameters were collected from various literature sources. Based on the data collection, each key parameter was quantified using the parameter equations.

The key performance parameters are defined as follows:

$$\% \text{ of glucose consumption} = ((C_i \text{ glu} - C_f \text{ glu}) / C_i \text{ glu}) * 100 \quad (\text{Eq.1})$$

$$\% \text{ of Fructose consumption} = ((C_i \text{ fru} - C_f \text{ fru}) / C_i \text{ fru}) * 100 \quad (\text{Eq.2})$$

$$\text{Yield SA} = \text{mol-C SA produced} / \text{mol-C Sugars consumed} \quad (\text{Eq. 3})$$

$$\text{Productivity SA} = \text{g of succinic acid L}^{-1} \text{ h}^{-1} \quad (\text{Eq 4})$$

VI. Conclusion

This chapter was devoted to the presentation of the experimental devices used during the pure culture fermentation, co-culture fermentation and electro-fermentation process, as well as the description of experimental protocols, in order to show the techniques and methodology used during this work

VII. Cited References

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Chapter III: Improvement of succinic acid production; a well- known platform molecule

Preamble

The objective of fermentation is to produce chemicals that can be used in wide applications. Therefore, to develop and optimise the key parameter which influences the process, it is important to understand the mechanisms of fermentation. Based on the information presented in Chapter 1, production of succinic acid is preferred to biological synthesis because it uses renewable resources and the reaction does not require high temperatures or expensive catalysts. A number of bacterial strains exist which can produce succinic acid, but *Actinobacillus succinogenes* has been chosen for this research as it is the most robust and, therefore, most likely microorganism to be used in industrial-scale bioproduction. Here, we perform multiple fermentation experiments in order to optimise the succinic acid bioproduction using MgCO_3 as inorganic carbon source, in addition to one of C4- tricarboxylic cycle acid; fumarate. The addition of fumarate allows succinic acid bioproduction to continue without end product inhibition. Chapter 3 hopes to develop an understanding fermentation experiments to produce succinic acid and how the production will be affected by the inhibition of growth by the product in the medium components.

Improvement of succinic acid production; a well-known platform molecule

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Abstract

Many studies demonstrated the feasibility of succinic acid (SA) production under anaerobic conditions by some ruminant microorganisms, however, this work provides a key insight into the improvement of SA production by *Actinobacillus succinogenes* and the inhibitory conditions caused by the product during fermentation. Kinetics and yields of succinic acid production in the presence of sugars in a relevant synthetic medium were investigated. For an equal mixture of glucose and fructose used as substrate at 0.4 mol L^{-1} with the addition of fumaric acid (FA) as enhancer, and under proved optimal conditions (pH 6.8, $T = 37^\circ\text{C}$, sparging with N_2 for 5 min and 1 % v/v of biomass), about 0.5 mol L^{-1} of succinic acid was obtained; while the theoretical production of succinic acid is 0.74 mol L^{-1} . This concentration corresponded to an experimental yield of 0.88 (molC SA/molC sugars consumed anaerobically) and volumetric productivities of 0.48 g-SA/L/h .

Keywords: platform molecules; succinic acid; fermentation; metabolic pathway; mass balance.

I. Introduction

Succinic acid (SA), which is a biomolecule, has been well-known as a platform chemical and as a highly versatile building block, used in a variety of industrial applications namely, surfactants, green solvents and pharmaceutical compounds, given to its linear and saturated structure [1, 2]. Succinic acid is at the top list of 12 building-block and holds the title of being the only special product with an annual market demand of about 710 kilotons with a net value of \$115.2 million, and expected to exceed \$1.8 billion (768 million MT at \$2.3/kg) by 2025 [3, 4]. Previously, SA was made primarily by catalytic hydrogenation of maleic anhydride, a finite fossil-derived chemical at a current price of 2.94\$/Kg [5]. Actually, the society and the industry are becoming more aware from petrochemical processes on environmental footprint, and concern has increased to find alternative routes for durable productions of chemicals [6]. Today, the world is at a critical changeover as we move ahead into bio-economy, while simultaneously reduce the dependence on finite fossil fuels leaving behind a petroleum based economy [7]. According to this scenario, petroleum refineries could be progressively replaced by bio-refineries as governments pursued to generate renewable energy, bio-fuels and bio-derived chemicals [8]. The preferable approach of bio-renewable chemicals is the fermentation process using microbial-based generation and cheap renewable resources, that requires simple conditions of pressure and temperature to produce chemicals by several pathways [9]. There is a great interest in producing biologically derived succinic acid, potentially at commercial scale, with possible process implementation at different levels (such as feedstock selection, process and operation setup, microbial strain and a novel bio-way) and leading to high concentrations of the accumulated product (Lee et al., 2019; Pinazo et al., 2015). Emphasis has been put on the use of pure or simple fermentable substrates in bio-derived chemicals production; however, to reduce the process fees, low-cost and/or waste organic materials can be promisingly considered [12]. A tremendous research was made to develop a biological process for SA production as an end-product of energy metabolism under aerobic [2], micro-aerobic [13] and/or anaerobic conditions [14]. For this purpose, several natural or mutant microorganisms were considered, mainly including *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, and *Escherichia coli*, especially, *A. succinogenes* 130 Z, a facultative anaerobe isolated from the bovine rumen [15]. Conventional production

of SA involves anaerobic bacterial fermentation of pure or mixture sugars with the addition of carbon dioxide (CO₂) sources, such as *Actinobacillus succinogenes* [15, 16]. This latter produces SA as an end-product at higher concentrations than other strains without genetic tool modification [17]. The highest succinate concentration (105.8 g L⁻¹) has been produced by *A. succinogenes* FZ53 mutant using glucose with a yield and productivity of 0.82 g-SA/g-glucose and 1.36 g L⁻¹ h⁻¹, respectively [18, 19]. Succinic acid production by *A. succinogenes* is implemented in biorefineries employing cheap and complex renewable raw materials, which offer complete nutrient fermentation medium at the less expensive cost compared to commercial nutrient sources [20]. *Actinobacillus succinogenes* native strain could be used but requires an anaerobic environment (nitrogen bubbling) and the presence of dissolved CO₂ concentration in the fermentation broth as MgCO₃, which definitely control the metabolic flux of carbon, and the activity of enzymes including phosphoenolpyruvate carboxykinase (PEPCK), that are the crucial steps for succinic acid bioproduction by succinate-producing bacteria [21].

Succinic acid produced as end fermentation product via some intermediate compounds of tricarboxylic acid (TCA) cycle reductive branch, including oxaloacetate (OAA), malate (MAL), and fumarate (FUM) under anaerobic fermentation [22]. The reduction of fumarate is the main source of succinate accumulation during fermentation, and in *A. succinogenes*, the formation of succinate is strictly required in particular in this organism, taking advantage of its unique incomplete TCA cycle, which natively terminates at SA [23, 24].

The main features to exploit *A. succinogenes* to produce SA are the utilisation of a wide variety of feedstock containing carbon sources. Indeed, *A. succinogenes* can consume a broad range of C5 and C6 sugars and other carbon sources, including mono and disaccharides as glucose, xylose, arabinose, mannose, galactose, fructose, sucrose, lactose, cellobiose, mannitol, maltose and glycerol [25–27]; it shows efficient fermentative pathway even with sustainable raw materials [28], as well as suitable tolerance to inhibitors.

The objective of the current study was to improve the high-value products from native strain of *A. succinogenes*, succinic acid by the addition of one of C4 TCA metabolites. Precisely, the consumption of substrate (glucose and fructose), conversion of carbon partitioning and the pathway toward the high-value products from the carbon flux to

optimize the output of the metabolic network were used as a mean to improve succinic acid production as follows. Based on the conversion rate of substrate, the C4 TCA metabolites and the central path anaerobic fermentations were carried out to upgrade the native strain of *A. succinogenes* and to verify the tolerance and to confirm the inhibitory action of *A. succinogenes* against SA. Finally, fumaric acid (FA) was used as enhancer to improve the production of succinic acid from *A. succinogenes*, according to consumption rate of carbohydrate, production rate and the metabolic pathway for products with biomass formation, a mass balance of experimental data obtained from anaerobic batch fermentation is presented.

I. Material and methods

1. Chemical and gas

All chemicals used were purchased from various supplier as Sigma-Aldrich or Merck. Ultra high purity Nitrogen (N₂) gas was supplied from Linde- GAS, FRANCE.

2. Microorganism and inoculums preparation

Native *Actinobacillus succinogenes* DSM 22257 in pellet form was purchased from DSMZ -German Collection of Microorganisms and Cell Cultures, and used in all experiments. As per bacterium supplier recommendation, the bacterium was revived in 0.5 mL of inoculation medium and preserved on Tryptic Soya Agar (TSA) slant. Cells were incubated in 15 mL of Tryptic Soya Broth (TSB) in 25 mL sterilized glass vials at 37°C for 24-48 h in an incubator (VWR® INCU- Line), which was the time required for the microorganism to enter the exponential growth phase. Cells were incubated until their optical density reached 1 at 660 nm and then centrifuged aseptically (1800 g, 4°C and 5 min) in a centrifuge (Thermo-Fisher, scientific, HERAEUS Megafuge 16R) and then resuspended in 10 mL KCl (150 mM). For the inoculum preparation, 2 mL of suspended culture cells were inoculated into 250 mL anaerobic bottle containing 200 mL of broth and placed in an incubator shaker (New Brunswick, INNOVA 40, NJ, USA) at 37° C and 150 rpm for 48 h. Cells from the preculture were centrifuged aseptically in the same conditions mentioned above and the suspension obtained after harvesting cells and re-suspending in 10 mL of KCl 150 mM was used for inoculation.

3. Fermentation media and experimental design

Fermentation media consisted of major and minor components. The major components included the carbon sources (glucose, fructose) at various concentrations and the nitrogen source, 0.53 g L⁻¹ NH₄Cl. The minor components included inorganic salts, buffers, cofactors, namely (per liter): 3 g K₂HPO₄, 3 g KH₂PO₄, 1.25 g NaCl, 0.3 g MgCl₂·6H₂O, 0.3 g CaCl₂·2H₂O, 1.5g NaH₂PO₄, 1.5g Na₂HPO₄. Initial fermentation pH media was adjusted to 6.8, then, the media prepared was sterilized by filtration on 0.22 µm sterile membrane filter (Sartorius), when setting up the fermenters. A series of experiments were conducted using a modified fermentation medium added with fumaric acid (FA) as co-factor in various concentrations and the relative percentage change which refers to the

difference of succinic acid concentration between the experiments was calculated using the following formula.

Relative percentage :

$$\% \text{ change} = (\text{final SA} - \text{initial SA}) / \text{initial SA} * 100$$

All equipments were sterilized before use. Experiments were carried out in glass bottles 250 mL volumes capacity. A working volume of 200 mL was used and operated at a temperature of 37°C, pH between 6.8 and 7 and an agitation speed of 150 rpm. The inoculum size for the batch fermentation was 1% (v/v).

Experiments for determining the inhibitory effects of the products were carried out in Petri dishes (PD), each containing 15 mL of defined medium TSA with several concentrations of succinic acid.

4. Fermentation conditions and sampling

Carbon dioxide (CO₂) fixation is required to enhance succinic acid production. Therefore, CO₂ was supplied to the fermentation broth by the addition of MgCO₃. Two sets of MgCO₃ concentrations (0.1 and 0.2 mol L⁻¹) were added as a buffer medium to maintain the pH between 6.8 and 7. MgCO₃ also acts as a carbon source, and the concentration present in the media was determined by a simple titration of CO₃²⁻ ions by H₂SO₄ at a concentration of 0.2 mol L⁻¹ at the first fermentation time (T₀) and at the end of fermentation. A fermentation temperature of 37°C and an agitation speed of 150 rpm were maintained throughout the incubation period. During the fermentation, samples of 5–10 mL were withdrawn aseptically from the glass bottles at regular time intervals in order to follow the optical cell densities, sugar consumption, and the production or uptake of acids (e.g., succinic, formic, acetic, and lactic acid), and other compounds (e.g., ethanol). Batch fermentations, consisted of microaerobic and anaerobic phases. Under microaerobic conditions, the dissolved oxygen level was 100% of air saturation at the time of inoculation and under anaerobic conditions, nitrogen gas was sparged into the bottles for 5 min to remove oxygen before inoculation; however, succinic acid production phase occurs in anaerobic conditions.

5. Analytical methods and data analysis

Cell growth was followed by measuring the optical cell density at 660 nm (OD₆₆₀) using a Spectronic 601 spectrophotometer (Milton Roy, Ivyland, PA, USA). From each flask, all the culture medium (50 mL) was loaded into a pre-weighed centrifuge tube (M1) and

centrifuged (Thermo-Fisher) at 1800 g for 5 minutes. The supernatant was decanted and the tube with cells pellet was then dried in an incubator at 105° C overnight. The tube with the pellet was weighed in order to determine cell weight (M2). This drying/weighing was continued until a constant weight was achieved. It turned out that a sampling interval of 24 h was difficult to sustain operationally over a total culture period of around 4 days, it was decided that a 96 h sampling interval would be considered.

Cell concentration was monitored by spectrophotometry using OD₆₆₀ correlated to dry cell weight (DCW); the relationship between optical density and Dry cell weight is described by:

$$Y = 0.0003x + 0.0001$$

$$R^2 = 0.98$$

where Y = OD₆₆₀; x = (dry weight of cells) mg L⁻¹

DCW assessments were used as a rough indication of the biomass concentration. An OD₆₆₀ value of 1 (Absorbance) corresponded to 333 mg L⁻¹ DCW *A. succinogenes*, deduced from a standard calibration curve. Samples were centrifuged and filtered through a 0.22 µm membrane filter, and the concentrations of fermentation products and carbon substrate were determined according to the protocol described in [29] and verified by NMR. Feedstock consumption and platform molecules formation were compared in terms of the key performance factors. Some factors were assigned in terms of percentage; the dependent variables used were final SA concentration, productivity and yield expressed in molC succinic acid/molC sugars consumed. Information about the key performance parameters were collected from various literature sources. Based on the data collection, each key parameter was quantified using the parameter equations. The key performance parameters are defined as follows:

$$\% \text{ of glucose consumption} = ((C_i \text{ glu} - C_f \text{ glu}) / C_i \text{ glu}) * 100$$

$$\% \text{ of Fructose consumption} = ((C_i \text{ fru} - C_f \text{ fru}) / C_i \text{ fru}) * 100$$

$$\% \text{ of Difference} = ((\text{FA consumed} - \text{SA produced}) / \text{FA consumed}) * 100$$

$$\text{Yield SA} = \text{molC-SA produced} / \text{molC-Sugars consumed}$$

$$\text{Productivity SA} = \text{g of succinic acid L}^{-1} \text{ h}^{-1}$$

$$dS/dt = \text{Substrate consumption rate during fermentation time} (S \text{ initial} - S \text{ final}) / t \text{ final}$$

dP/dt = production rate during fermentation time (P final – P initial)/ t final

$Y_{p/s}$ (dP/dS) = yield coefficient of succinic acid on sugars (g SA/g sugars)

$Y_{x/s}$ (dX/dS) = yield coefficient of biomass on sugars (g biomass/g sugars)

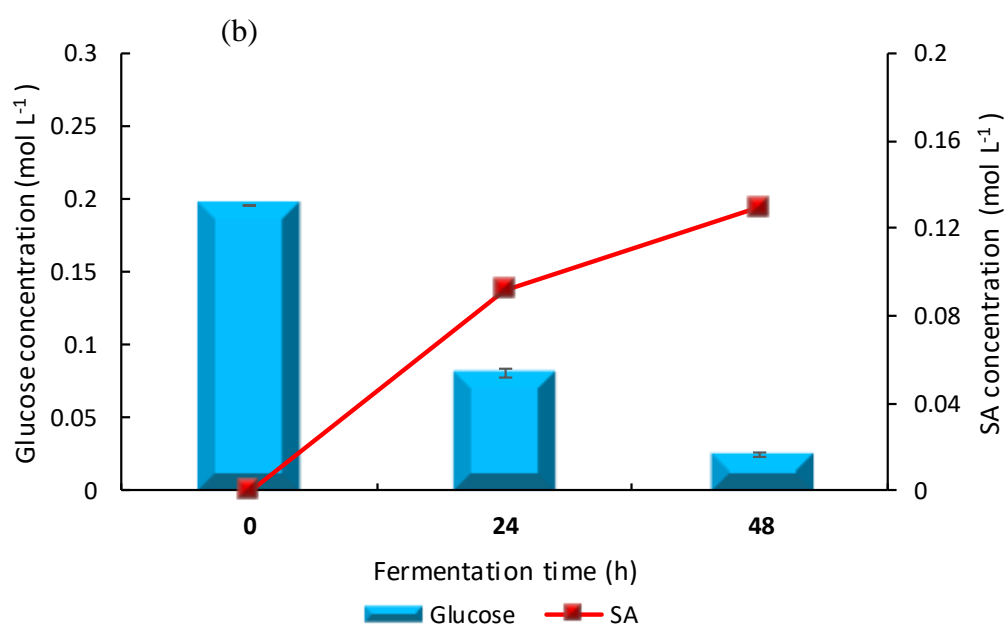
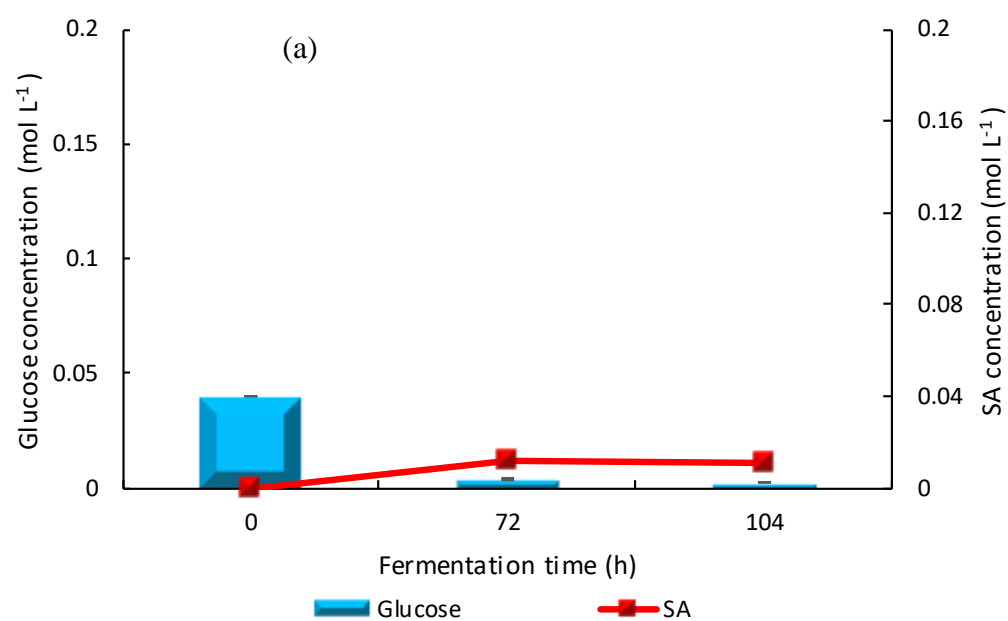
$Y_{p/x}$ (dP/dX) = yield coefficient of succinic acid on biomass (g SA/g biomass)

II. Results and discussion

1. Results from 250 mL Shake Flask Reactors using glucose as carbon source in micro-aerobic and anaerobic conditions

Fermentations of *A. succinogenes* in micro-aerobic conditions did not show any succinic acid production, while it revealed low yields of (< 0.25 mol-C SA/mol- CGLU) and productivities (< 0.012 g-SA/L/h), when 0.04 mol L^{-1} of glucose was used as the only carbon source in anaerobic conditions, SA was produced at low levels, recording a value of 0.01 mol L^{-1} (Fig 1-a). Further evidence is provided by [30] who have tested glucose intake among various carbon resources by *A. succinogenes*, demonstrating that SA is formed even when this substrate was added as the sole carbon feedstock in anaerobic conditions. Furthermore, *A. succinogenes* cannot produce SA in a synthetic media that contains glucose as the sole carbon source in microaerobic and aerobic conditions [31]. Biomass yield was less than 0.03 g-DCW/g-GLU , while analogues runs with different glucose concentrations displayed higher biomass yields ($>0.06 \text{ g-DCW/g-GLU}$). A series of experiments were performed in synthetic medium to improve the process. MgCO_3 was screened to be a key factor in SA production [20], and hence it was added to the fermentation medium as a neutralizer and to redirect the metabolic flux at 0.1 mol L^{-1} concentration.

Figure 1-b illustrates the results obtained from batch fermentation experiments in small anaerobic reactors (250 mL flask) during fermentation in the presence of MgCO_3 . Fermentations were conducted with different amounts of initial glucose concentration (0.05 , 0.19 and 0.26 mol L^{-1}). As shown in Fig 1-b succinic acid concentration reach it maximum value and record 0.14 mol L^{-1} , moreover, yield and productivity increased by 3.5, 0.8 times and exhibited maximum values of $0.93 \text{ molC-SA/molC-GLU}$, 0.17 g-SA/L/h respectively, when compared to the results obtained in the absence of MgCO_3 (Fig 1-a). Also, biomass yield increased from 0.02 g-DCW/g-GLU to 0.05 g-DCW/g-GLU .



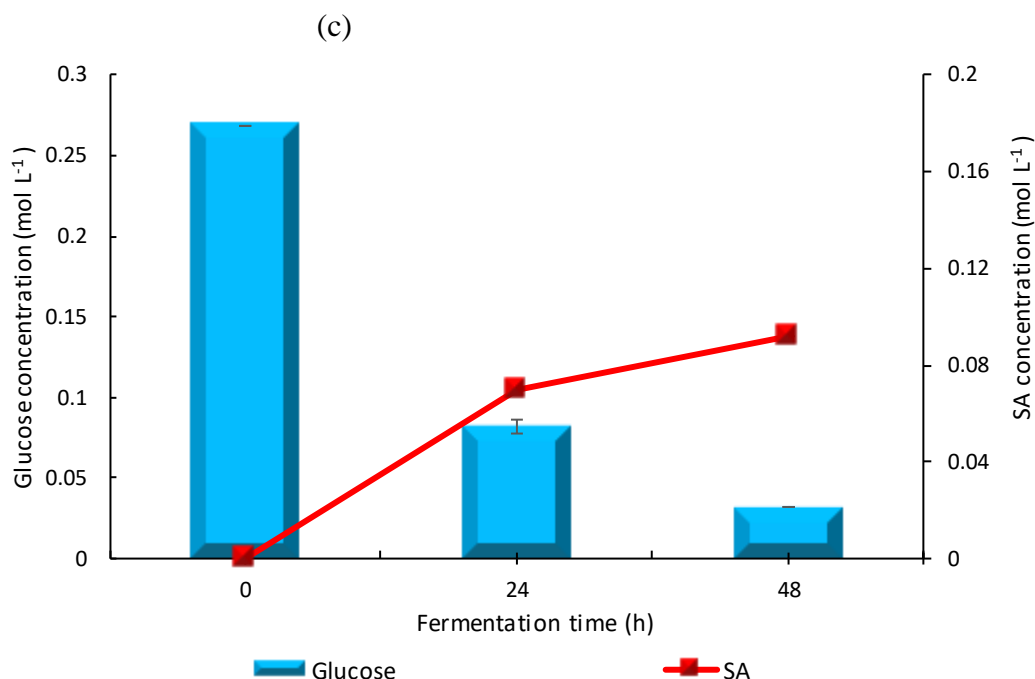
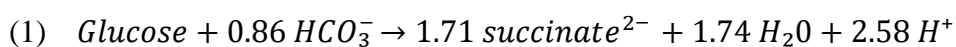


Figure 1. (a, b and c): succinate formation in 250 mL small shake flask for different glucose concentrations (0.04, 0.19 and 0.26 mol L⁻¹ respectively) in anaerobic conditions at 37°C.

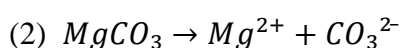
As shown in Figure 1-c, no further major progression was noticed in succinate concentration, yield and productivity at the end of the experiments when 0.26 mol L⁻¹ glucose was added. [32] reported for the first time the substitution of CO₂ gas by MgCO₃ in the fermentation of succinic acid. Theoretically, 1.71 mol of succinic acid is produced per mol glucose in the presence of CO₂ sources based on the available electron as per the following equation (Eq 1)[33]:

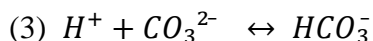


Experimental yields could be restricted by the pathways used and by the carbon converted to biomass and alternative products. The molar yield obtained from the experiments presented in Fig 1-B was about 0.84 mol SA/ mol glucose.

The percent yield was calculated and show a value of 49%, meaning that 49% of glucose was converted to succinic acid after 48 hours of fermentation.

When MgCO₃ was added, HCO₃⁻, CO₃²⁻ and CO₂ would become in equilibrium in the fermentation broth according to the following equations:





Consequently, the insoluble MgCO_3 supplement caused turbid broth, cells spreading which avoid cell flocculation and indeed it increases the dissolved concentrations of HCO_3^- , CO_3^{2-} and CO_2 in the broth which influence carbon flux and the activity of phosphoenolpyruvate carboxykinase enzyme, to redirect the flux toward SA biosynthesis [14], and because of this properties, MgCO_3 plays an essential role in improving succinic acid production. The analyze of MgCO_3 concentration at the end of fermentation, recorded a value of 0.016 mol L^{-1} , which means that the carbonate minerals were consumed when compared to the initial carbonate concentration. However, MgCO_3 could not be used as CO_3^{2-} donor because few reports indicates that CO_3^{2-} is directly used as substrate by SA producer's microorganisms [32].

2. Results from 250 mL anaerobic bottles using fructose or glucose and fructose as carbon source in anaerobic conditions

A fermentation profile of the fructose-succinic acid system was shown in Figure 2. Succinic acid was the main product present in the media. Moreover, no ethanol nor lactic acid was detected in any sample. Yields, productivities, optical cell densities variation, succinate concentrations at the end of the fermentation time for several substrate concentrations are illustrated in Table 1.

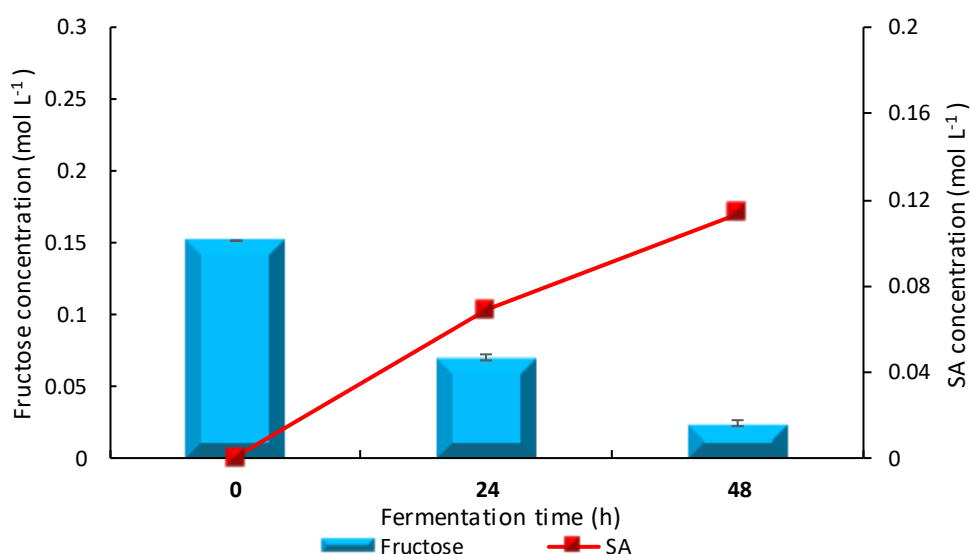


Figure 2. Fermentation profile of the fructose-succinic acid system in 250 mL anaerobic shake flask for a fructose concentration of 0.15 mol L⁻¹ in anaerobic conditions at 37°C.

Table 1. Comparison of succinic acid produced from glucose and fructose after 48 h (A: medium containing glucose, B: medium containing fructose) in 250 ml anaerobic bottles with a volume of 200 mL.

Initial sugar concentration (mol L ⁻¹)		Consumed Sugar (%)		SA (mol L ⁻¹)		A _{λ.660 nm}		Yield (molC/molC)		Productivity (g L ⁻¹ h ⁻¹)	
A	B	A	B	A	B	A	B	A	B	A	B
0.05	0.04	91.90	99.70	0.04	0.025	0.13	0.17	0.90	0.67	0.33	0.06
0.19	0.13	87.24	92.44	0.14	0.07	0.41	0.18	0.94	0.59	0.39	0.17
0.26	0.15	88.05	83.44	0.09	0.11	0.49	0.38	0.40	0.89	0.22	0.27

Fructose was mostly consumed during the first 48 hours. The highest carbon yield (C-mol/C-mol) was found at 0.15 mol L⁻¹ initial fructose concentrations, with a maximum value of 0.92 molC-SA/molC-FRU. This should most likely be related to the highly reduced state of glucose and fructose in addition to glycerol, which unlike the other sugars, promotes the generation of succinic acid [34]. Prior studies also pointed out a high yields in SA production when mutants bacterial strains were used [4, 35, 36]. The feasibility of SA production via *A. succinogenes* co-utilizing glucose and fructose simultaneously as carbon sources was evaluated. By combining these two sugars, they are consumed more quickly than when a single-sugar was used (Fig.3). Moreover, we demonstrate that the process can adapt to changing concentrations of these two sugars. As long as a substrate is present in the medium, biomass grew up exponentially till around 70 h; then due to substrate limitation, biomass enters in the stationary phase. The highest SA production in co-fermentation was 0.22 mol L⁻¹ (equivalent to a yield of 0.94 molC-SA/molC-sugars and a succinic acid productivity of 0.27 g-SA/L/h) observed in a mixture with a ratio of 50 % fructose and 50 % glucose in the presence of 0.2 mol L⁻¹ of MgCO₃ (Fig.3 a). The presence of 0.2 mol L⁻¹ of MgCO₃ was beneficial to promote succinic acid production and giving the maximum of SA concentration (0.22 mol L⁻¹). For this reason, MgCO₃ at 0.2 mol L⁻¹ was considered thereafter (Fig.3b).

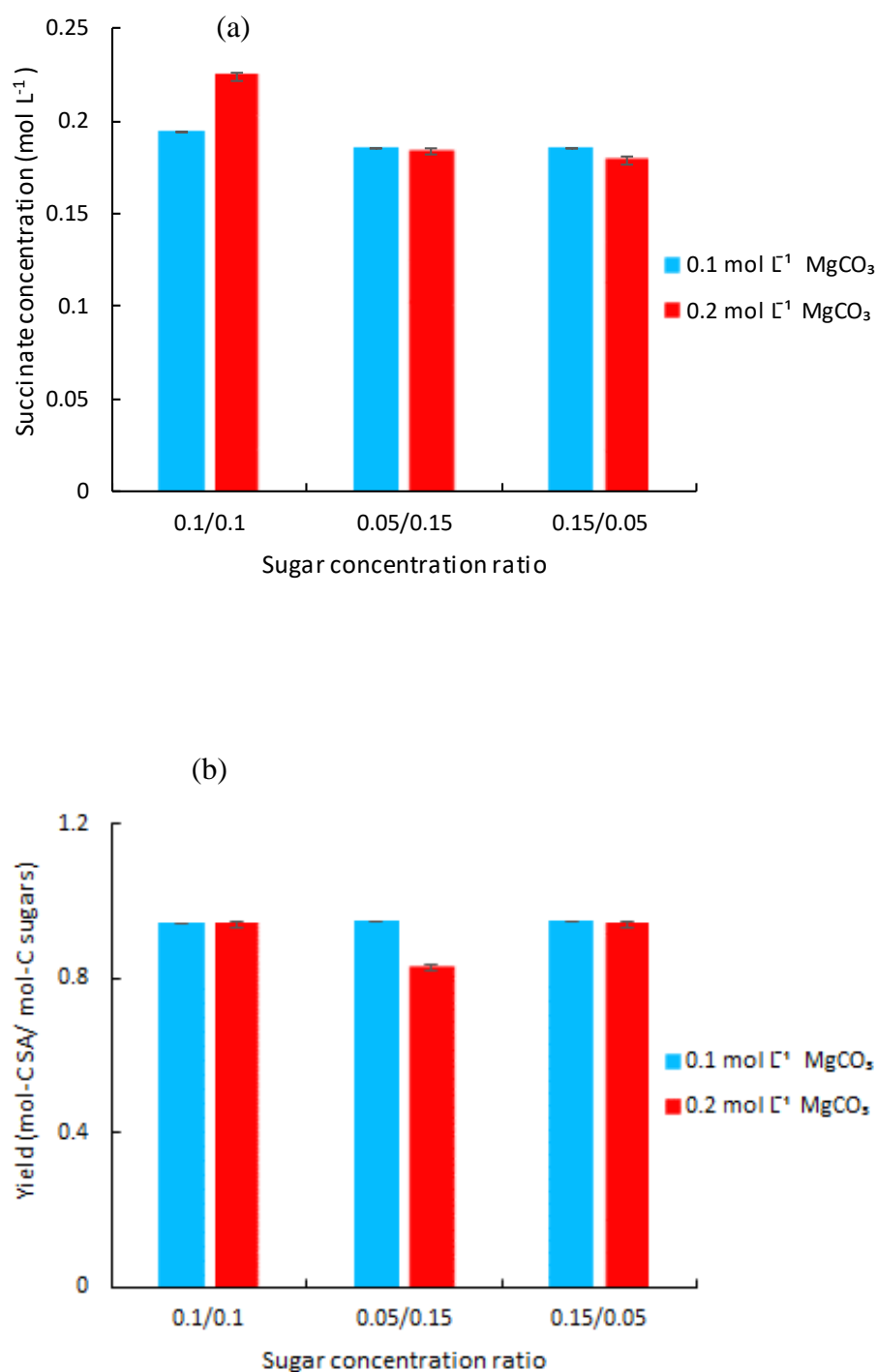


Figure 3: a- succinic acid concentration and –b. Profile of conversion yield during *A. succinogenes* fermentations in 250 mL batch small anaerobic shake flask bottles at 96 hours with different initial sugar concentrations in mol L⁻¹ (with glucose to fructose ratios of 0.1/0.1, 0.05/0.15, 0.15/0.05) The apparent yield refers to the sugars consumed during fermentation.

3. Improvement of succinic acid production by the addition of mediator in 250 mL anaerobic bottles

Most published studies on the improvement of SA production in *A. succinogenes* focused on manipulating enzyme activities through deletion of competing pathways and/or overexpression of the beneficial pathways using genetic tools [1, 37, 38]. Consequently, the purpose of this study was to generate succinate overage from *A. succinogenes* by increasing fumarate reductase activity, through the addition of reduced mediators that transfer electrons into bacterial cells and act as an electron donor for the fumarate reductase. Reduction and oxidation between fumarate and succinate should create a loop into the TCA cycle. We worked on the availability of different fumarate concentrations and our concept was to prove its effect as a mediator. Table 2 present the effect of different initial fumarate concentrations on sugars consumption, optical cell densities, pH, SA formation, yields, productivities and the relative percentage of succinic acid.

Table 2. Effect of Fumaric acid addition on Succinic acid formation at the end of fermentation (A: glucose, B: fructose) in 250 ml anaerobic bottles with a volume of 200 mL.

[FA] g L ⁻¹	consumed sugars (%)		SA (mol L ⁻¹)	A _{λ660nm}	pH	Yield (molC/molC)	Productivity (g L ⁻¹ h ⁻¹)	Percentage (%)
	A	B						
0	97.54	97.04	0.18	0.25	6.00	0.47	0.18	0
0.1	97.99	97.60	0.22	0.25	5.98	0.57	0.21	18.9
0.2	97.94	97.60	0.22	0.24	5.65	0.57	0.22	22.12
0.3	83.81	93.83	0.23	0.28	5.53	0.66	0.22	25.9
0.4	86.74	92.94	0.23	0.18	5.66	0.56	0.23	27.56
0.5	95.76	94.41	0.23	0.13	5.32	0.62	0.23	28.00

The decrease of pH was attributed to the dissociated form of fumaric acid that was initially added into the medium. Results indicate that when FA concentration increased, the net production of succinic acid was higher by 28% compared to experiment done without FA. Also, SA production yield and productivities increased, achieving 0.62 mol-C SA/mol-C sugars and 0.23 g L⁻¹ h⁻¹ respectively with SA concentration up to 0.23 mol L⁻¹ at the end of fermentation (120 h). The fumarate reductase has a key role because it allows electrons transfer from the reduced mediator into bacterial cells. One of the scenarios is that

fumarate is adsorbed at the inner membrane and transfer electrons to the fumarate reductase producing succinate.

A. succinogenes is a wild type succinate producer, which produces SA anaerobically through the reductive branch of the TCA cycle [23, 39]. Succinate production from anaerobic growth with C4-dicarboxylates, such as fumarate requires transporters catalyzing uptake and efflux of C4-dicarboxylates. As a result, a multiple sequence alignment (MSA) of protein sequence was done using UNIPROT databases with a protein sequence found in *E. coli*, which requires transporters for fumarate uptake, showed that *A. succinogenes* have a transport system of 3 transporters for fumarate uptake and expressed during anaerobic growth on fumarate; they are: Asuc_1063, Asuc_1999, and Asuc_0142.

A. succinogenes grows well on C4-dicarboxylates such as fumarate under anaerobic conditions. Supplied fumarate is almost turned into succinate, and more than 90% of the supplied sugars are also transformed to succinate. Under all tested conditions, microaerobic and anaerobic conditions with glucose and fructose or fumarate, succinic acid level was markedly improved in the presence of fumarate (see table 3), if compared to the fermentation using the C6-sugars, leading to a SA concentration of about 0.5 mol L⁻¹ with an experimental yield and productivity of 0.88 mol-C/ mol-C, 0.48 g L⁻¹ h⁻¹ respectively. Anaerobic growth on fumarate was stimulated by the transporter and the major product was succinate with a conversion yield of 95.4%, indicating the involvement of fumarate uptake in succinic acid production, similar to succinate production from glucose and fructose. Fumarate uptake of 100 mg. L⁻¹ shows that the systems had substrate specificity for fumarate but not for succinate.

Table 3. Succinic acid formation from glucose and fructose and/or fumarate at the end of fermentation (A: medium containing glucose,B: medium containing fructose) in 200 mL anaerobic bottles.

Runs	1	2	3
Glucose concentration (mol L ⁻¹)	0.2	-	-
Consumed glucose (%)	97.14	-	-
Fructose concentration (mol L ⁻¹)	0.2	-	-
Consumed fructose (%)	97.01	-	-
FA (mol L ⁻¹)	0.087	0.043	0.087
Consumed FA (%)	100	100	100
SA (mol L ⁻¹)	0.492	0.037	0.083
Yield*	0.99	0.88	0.91
Productivity (g L ⁻¹ h ⁻¹)	0.48	0.036	0.081
Conversion yield (%)	61.87	86.04	95.4

Yield *: Overall yield molC SA/molC substrate

4. Conversion of fumaric acid into succinic acid in *Actinobacillus succinogenes*

In this study, different batch fermentation tests were carried out in 250 mL anaerobic bottles contained glucose and fructose and fumaric acid in different quantities, among which the concentrations of glucose and fructose were equal. On the other hand, a simulated medium, containing fumaric acid as described earlier except the sorts of mixed sugars were used for succinic acid fermentation. In this case, batch fermentation tests were further chosen to compare the main kinds of carbohydrates, as glucose and fructose with the fumaric acid to the ferment ability of single carbon feedstock (fumaric acid). Each carbon source was added to keep a constant amount of carbon. Glucose, fructose, and fumaric acid were almost consumed at the end of the fermentation (table 4). The extra SA produced is normally corresponding to the consumed FA added in the media with an error percentage (table 4). The first run corresponded to the control experiments done without the addition of fumaric acid. In this experiment the SA concentration obtained was about 0.053 mol L⁻¹. However, the maximum succinic acid concentration (run 3) produced was approximately 0.24 mol L⁻¹, with almost a similar productivity (0.25 g L⁻¹ h⁻¹),

corresponding to sugars and fumaric acid consumption. According to this, the fumaric acid consumed was 0.192 mol L^{-1} and then the extra SA produced from fumaric acid when compared to control run was 0.187 mol L^{-1} with an error percentage of 2.6%, indicating that fumaric acid can be used to directly produce SA in the presence of mixed sugars. Succinic acid concentrations were about 0.24 mol L^{-1} , 0.16 mol L^{-1} and 0.05 mol L^{-1} , obtained from an initial equal concentration of a mixture of glucose and fructose (0.05 mol L^{-1}), with 0.2 mol L^{-1} , 0.1 mol L^{-1} or without fumaric acid, respectively. For the fermentation using fumaric acid as sole carbon source, SA concentration and productivity were relatively low when compared to conversion yield.

Table 4. Efficient succinic acid formation from glucose and fructose and/or fumaric acid at the end of fermentation (A: glucose, B: fructose) in 200 mL culture medium, in anaerobic condition

Runs	1	2	3	4	5
Glucose concentration (mol L^{-1})	0.05	0.05	0.05	-	-
Consumed glucose (%)	99.05	98.87	96.67	-	-
Fructose concentration (mol L^{-1})	0.05	0.05	0.05	-	-
Consumed fructose (%)	97.62	98.09	96.72	-	-
FA (mol L^{-1})	-	0.1	0.2	0.1	0.2
Consumed FA (%)	-	92.55	96.16	99.33	98.92
SA (mol L^{-1})	0.053	0.17	0.24	0.09	0.15
Extra SA (mol L^{-1})	0	0.11	0.187	-	-
Yield*	0.58	0.92	0.85	0.94	0.74
Productivity($\text{g L}^{-1} \text{ h}^{-1}$)	0.05	0.18	0.25	0.09	0.16
Conversion yield (%)	54.63	89.47	85.71	100	76.14
% of difference (absolute value)	-	27	2.6	0	24

Yield *: Overall yield $\text{molC SA/molC substrate}$

However, compared to the fermentation using glucose and fructose, the fermentation using 0.1 mol L^{-1} and 0.2 mol L^{-1} of fumaric acid (run 4 and 5 respectively) as the carbon

source resulted in a final succinic acid concentration of about 0.08 mol L⁻¹ and 0.14 mol L⁻¹ respectively. These batch tests led to a yield of 0.94 and 0.74 molC- SA/molC- FA with 0.09 g L⁻¹ h⁻¹ and 0.16 g L⁻¹ h⁻¹ succinic acid productivity, respectively, confirming that the carbon obtained from fumaric acid can be used for the biomass formation (growth) and is crucial and efficiently used to produce SA during fermentation culture. Fumarate reductase (FRD) is a key enzyme activated and synthesized under low oxygen conditions. In *Actinobacillus succinogenes*, fumarate reductase induced by anaerobic growth is expressed in the last step of anaerobic fermentation [30], allowing the released electrons to an awaiting fumarate to be reduced into succinate in the anaerobic pathway. The reduction of fumarate is the main source of succinate during fermentation, and under anaerobic conditions, it is strictly required for the formation of succinate [40]. According to the metabolic flux of *Actinobacillus succinogenes*, OAA converts into succinate via MAL and FUM as intermediates [41]. The succinate yield is strongly related to Nicotinamide Adenine Dinucleotide (NADH) availability [42] and hence to fumarate. Subsequently, a flux distribution that supports high fumarate and NADH presence is in favor of succinate production [42–45].

5. Mass Balance Analysis (MBA)

MBA is a way to determine what the maximum capabilities of an organism are in a steady state conditions for a given carbon substrate. In addition, MBA are profitable for flux distribution, biomass formation as well as metabolite synthesis and it is influenced by environmental conditions. The accuracy of data was evaluated by performing general mass balances which consist on the calculation of stoichiometric amount of substrates required (glucose and fructose in our case) to produce the metabolite relying on elemental balances ($C_{in} = C_{out} + C_{accumulated}$), and compare this amount (theoretic) to the obtained (measured) amount of metabolites produced. Table 5 presents kinetics of multiple fermentation runs with the mass balance closure. Since glucose and fructose have the same C, H and O ratio, they were combined into a single amount in the calculation and the mineral carbon obtained from MgCO₃ was excluded. The percentage closure of the mass balance is calculated as the ratio of the experimental C_{SA} , and DCW produced to the obtained amount of sugars consumed. A value under 100 indicates that more carbon was consumed than accounted for metabolites and biomass production.

The maximum production rate was seen when an equal mixture of sugars with fumaric acid addition was tested, recording a value of $0.48 \text{ g L}^{-1} \text{ h}^{-1}$; while the maximum yield was obtained from the co-fermentation when the mass balance closed on average of 82%.

Table 5. Kinetics and mass balance analysis for different runs. Run 1: glucose in different concentrations; run 2: Fructose; run 3: equal mixed sugars of glucose and fructose; run 4: equal mixed sugars of glucose fructose with FA; run 5: fumaric acid

Runs		1		2	3	4	5
Fermentation time (h)	104	48	48	48	96	120	96
Glucose concentration (mol L ⁻¹)	0.05	0.2	0.26	-	0.1	0.2	-
Fructose concentration (mol L ⁻¹)	-	-	-	0.15	0.1	0.2	-
Fumarate concentration (mol L ⁻¹)	-	-	-	-	-	0.087	0.2
dS/dt (g L ⁻¹ h ⁻¹)	0.06	0.64	0.86	0.48	0.34	0.66	0.23
dP/dt (g L ⁻¹ h ⁻¹)	0.01	0.34	0.24	0.29	0.27	0.48	0.18
Y _{p/s}	16.4	54.1	28.5	60.7	79.4	72.8	77.8
Y _{x/s}	≈0	1.69	1.3	2.2	2.7	1.47	1.00
Y _{p/x}	-	31.8	21.5	26.8	130.2	49.10	89.8
% closure of mass balance	16.4	55.7	29.8	62.9	82.1	74.2	78.8
Yield (mol-SA/g-DCW)	-	0.26	0.18	0.22	0.25	0.41	0.65
(SA) ratio (o/t)	14.7	48.2	25.6	54.5	71.0	65.3	76.1

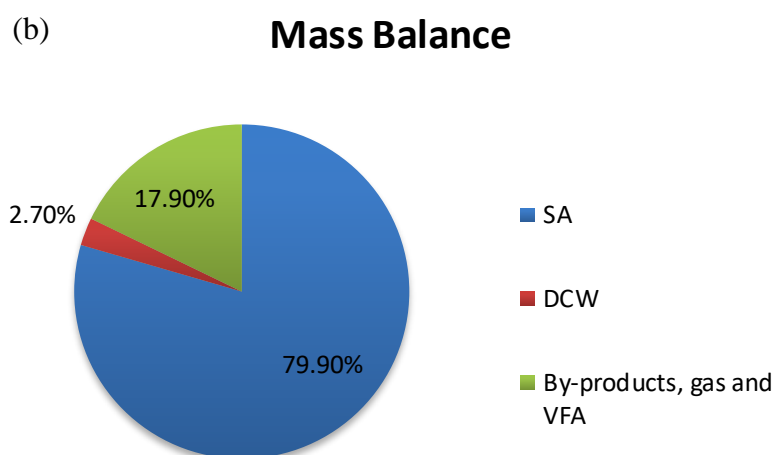
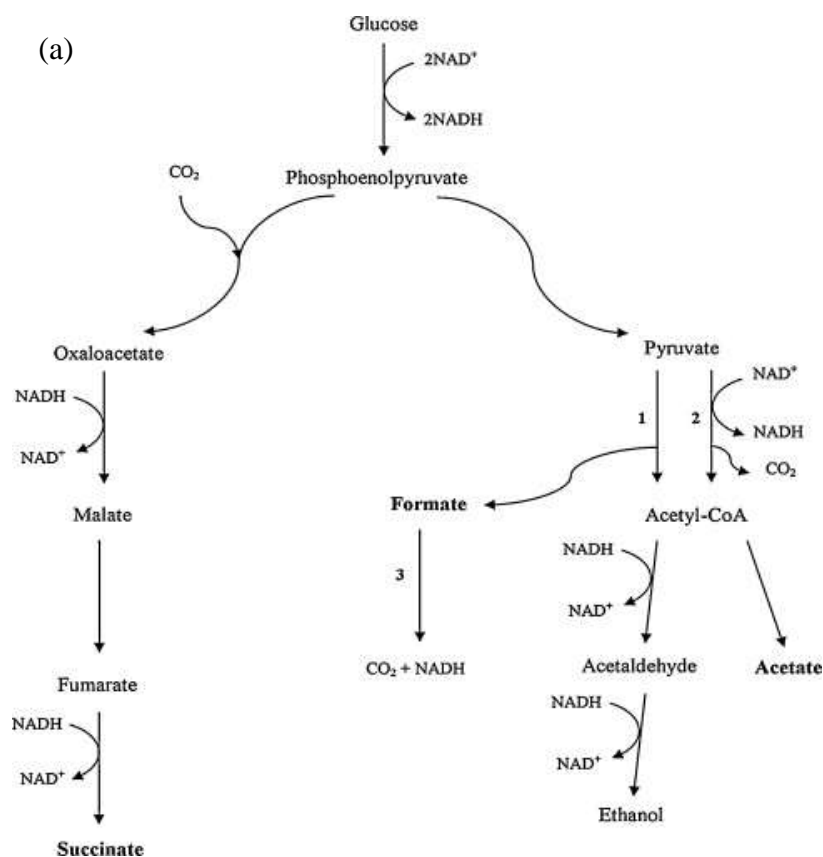
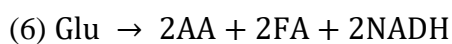
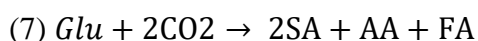


Figure 4. Carbon flux (a) and mass balance (b) at the end of co-fermentations (96 h) carried out in 200 mL anaerobic bottle flasks using an equal mixture of 0.1 mol L⁻¹ of glucose and fructose. The metabolic network of *A. succinogenes* representing the pathways of metabolites [52]. (1) pyruvate formate lyase (PFL), (2) pyruvate dehydrogenase (PDH), and (3) formate dehydrogenase (FDH).

Fig 4 present the mass balances in co-fermentation which closed to 79.9% on average suggesting that more sugars were consumed than metabolite produced. The sample taken at the end of fermentation (96 h) present 2.7% of DCW closed the MBA to 82.1% and a rest of 17.9 % distributed between by-products, gas and volatile fatty acid (VFA). The incomplete mass balances closure attributed to undetected metabolites does not detract from the process relevance of the obtained results. Writing a mass balance for each intracellular metabolite leads to collect the metabolic reactions and pathways occurs in *Actinobacillus succinogenes* (see annex 1) to understand the mechanism of production of the end products and the by-products. Natural *A. succinogenes* strain produces SA by Phosphoenolpyruvate (PEP) pathway and TCA cycle reductive branch (C₄ pathway, Fig 4). Through these pathways, NADH was completely consumed; thereby flux toward SA is restricted due to satisfy the demand of redox balance leading to by-products formation such as formic and acetic acid through the pyruvate pathway to balance the carbon metabolism. *A. succinogenes* pyruvate metabolism can take place in two different pathways, particularly the pathways of pyruvate formate-lyase (PFL) or pyruvate dehydrogenase (PDH). When PFL flux is taking place, the Y_{AAForA} is 1.0 mol/ mol because the Acetyl-CoA generated from pyruvate is transformed into acetic acid (Fig 4). However, when PDH route takes place, CO₂ and NADH were formed instead of formic acid; thus, Y_{AAForA} turned to zero and an extra reducing power (NADH) output is increased. Likewise, the Y_{AAForA} become zero when the PFL pathway occurs in concurrence with formate dehydrogenase (FDH), leading to CO₂ and NADH production resulted from formic acid breakdown by FDH. The following reactions performed when pyruvate was transformed via the PFL pathway:

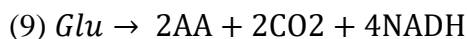
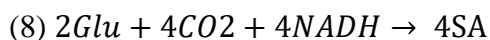


Eq. (5) presents SA formation and Eq. (6) indicates acetic and formic acids formation. The integration of the two equation (5 and 6) yields the net redox balance, Eq. (7):



Theoretically, biomass formation is considered as zero and all the carbon is converted to products. Eq. (7) presents the theoretic value for $Y_{SA, AA}$ which is 1.0 mol/ mol = 1.97 g/

g and 0.66 g/ g for $Y_{SA,Glu}$. When pyruvate is converted through the PDH pathway, the reactions that take place are the following:

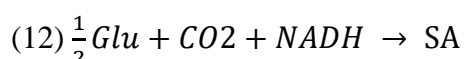


Basically, Eq. (8) represent the Eq. (5) times two, to fit the redox requirements and Eq. (9) presents the acetic acid production. As above, integration of equation (8) and (9) yields the net redox balance in Eq. (10):



Theoretically, no biomass was formed and from equation (10), the $Y_{SA, AA}$ maximum value is 2.0 mol/mol equal to 3.93 g SA/g_{AA} and 0.87 g/g for $Y_{SA,Glu}$. Similarly, when PFL is active with concurrence of FDH, formic acid can be transformed into CO_2 and NADH giving 3.93 g/g as maximum value for $Y_{SA, AA}$ when biomass growth and maintenance state are negligible. When biomass is formed, a part of substrate (glucose and/or fructose) will be addressed to the biosynthesis metabolism flux, affecting that way redox balance with a lower yield than mentioned before. The maximum yield observed in this study ($Y_{SA, S}$) achieved 0.79 g/g, exceeding the theoretical yields for glucose, fructose (0.66 g/g) but not for the co-fermentation with (1.02 g/ g) or without (1.12g/ g) fumaric acid addition. The highest $Y_{SA, S}$ values published in *A. succinogenes* fermentation was 0.76 g/g [46], 0.69 g/ g [47], 0.59 g/g [48] and 0.94 g/ g [18]. It should be pointed out that in most cases, the literature related to the fermentation of *A. succinogenes*, sugar consumption, metabolite concentrations or yields, are not provided and thus metabolic network analysis cannot be assessed. The transhydrogenation also called the net formation of NADPH/NADH is related to biomass generation and may account for the redox imbalance. To settle the redox balance, it is helpful to determine the surplus ‘extra’ NADH needed at high $Y_{SA, AA}$ as a function of substrate demand. The yield of ‘extra’ NADH is expressed by moles of NADH produced per mole of substrate consumed mol/mol (pathway presented in Fig 4). In our study, at the end of fermentation, the carbohydrates consumption was 69.84 g $L^{-1} h^{-1}$ and led to 1.18 g L^{-1} of DCW. [49] assumed the biomass molecular composition ($CH_{1.8}O_{0.5}N_{0.2}$) and from the metabolic

pathway given in Fig 4, the NADH generated via biomass formation (0.0027 mol/ mol), via glycolysis (0.77 mol/ mol) and via PDH/FDH pathway (unknown in our case) but theoretically (1 mol/ mol) is only 77.27% must require to produce 58 g/ L of SA. The ‘extra’ NADH implies the surplus reducing power formed by biomass and by PDH/FDH pathway, as a whole may considered the extra NADH needed and delivered to please the redox balance. Many papers reported the highest yield ($Y_{SA, Glu}$ to 1.12 g/ g) and discuss the ability of generating NADH from TCA cycle oxidative branch by the glyoxylate shunt, increasing thereby the C_4 pathway flux without by-product formation (Van Heerden et Nicol 2013b). Yet, TCA cycle functional enzymes leading to the glyoxylate shunt (citrate synthase and isocitrate dehydrogenase) are lacked in *A. succinogenes* [51, 52]; hence, these pathways are ruled out as NADH additional origins. A study done by [53] discussed the ability of *Bacillus subtilis* to sustain the metabolic activity with non-significant biomass formation, leading that way to an overflow in NADPH through the pathway of pentose phosphate (PPP) which further transformation into NADH via transhydrogenase. Similarly, when (PPP) takes place, the carbon produced from glucose-6-phosphate, and/or fructose-6-phosphate is completely recycled and channeled into this pathway in *A. succinogenes*, which possess the transhydrogenase as mentioned above, suggesting the conversion of excess NADPH to NADH. The present net reactions could occur:



Eq. (11) presents the SA formed via (PPP) oxidative pathway assuming that the glyceraldehyde-3-phosphate (GA3P) formed from (PPP) is transformed into PEP with one NADH release, and the PEP is thereafter being channelized to SA by C_4 pathway. Eq. (12) presents the SA formed from TCA cycle reductive branch. By combining these two equations, the redox is balanced in the following reaction:



From Eq. (13) SA on glucose maximum yield ($Y_{SA, Glu}$) is 1.12 g/ g obtained with no biomass or by-products formation. Consequently, SA yield maximization can be achieved with an active oxidative PPP as it provides enough reducing power and eliminated the need of C_3 pathway. This mechanism could elucidate the non-detectable by-products

observed in this study. Another data published, dealing with *A. succinogenes* batch fermentations [54, 55], suggest the shift of metabolic flux distribution in favor of SA production reflected by DCW decrease. In the present study, the yield $Y_{SA, s}$ obtained was between 0.16 g/ g and 0.79 g/ g and substrate consumption between 7.2 g L⁻¹ and 79.93 g L⁻¹. DCWs in different situations were between (≈ 0 and 1.18 g L⁻¹) and $Y_{SA, x}$ was also between 0.18 g/ g and 0.65 g/ g. The differences in degree imply a difference in the behavior of *A. succinogenes*. At a high concentration of SA, the $Y_{x, s}$ decrease could be explained by maintenance or non-growth state applied by the cells as mentioned above. The notion of *A. succinogenes* maintenance state entering with multiple flux distribution ties is discussed by [53]. However, an inquiry to PPP when it is active whether or not should be required.

6. Effect of SA on Bacterial Growth

Succinic acid can be produced in relatively high yields from the C6 sugars, glucose and fructose using *A. succinogenes* [43], yet, maximum succinic acid concentration was 0.4 mol L⁻¹ during fermentation. Until now, no study was done on the tolerance of *A. succinogenes* against SA. Consequently, to examine this point, time-courses of SA consumption at different initial concentrations of SA (SA_0) are shown in Fig. 5. It can be clearly seen that SA consumption remained constant during the course of culture.

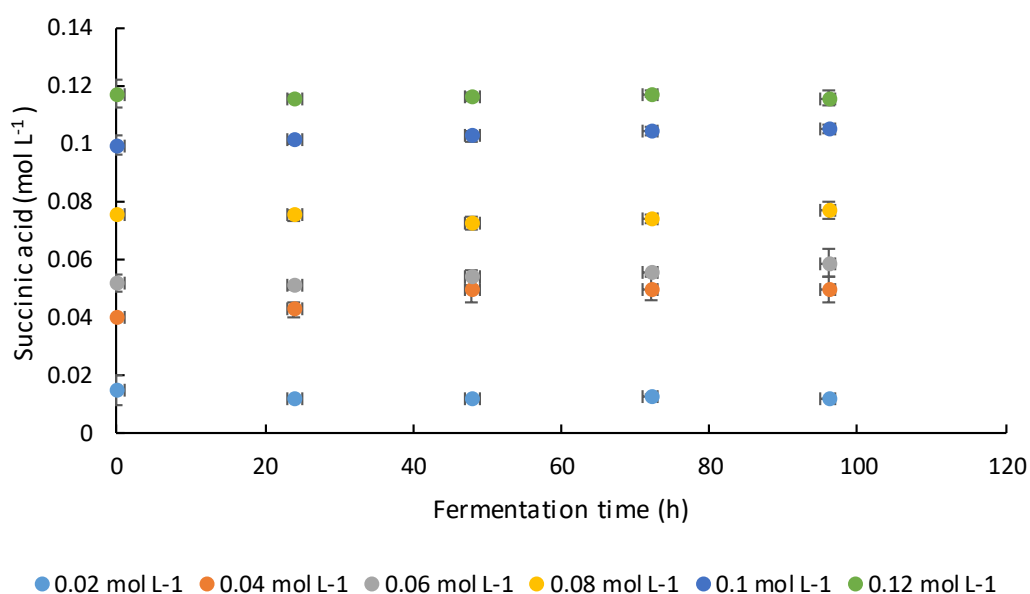


Figure 5. consumption of different concentration of succinic acid in mol L⁻¹ during the course of the fermentation in 250 ml anaerobic bottles with a volume of 200 mL

Therefore, the aim was to obtain more detailed information about the inhibitive forms of the substrate/product as well as about the concentrations that cause inhibition. The product inhibition was tested in batch tests and on Petri dish using TSA media. Each experiment was carried out using a set of six Petri dishes. One of them served as negative control, while the five others contained increased concentrations of succinic acid, in the range 0.1 to 0.5 mol L⁻¹. After 24 h incubation, the strain was developed on TSA containing 0, 0.1, 0.2 and 0.3 mol L⁻¹ of SA; while no development was observed on TSA containing 0.4 and 0.5 mol L⁻¹ of SA (fig. 6). Results displayed the initial critical concentration of SA for *A. succinogenes* biological activity was around 0.4 mol L⁻¹, and at this SA₀ no bacterial growth was observed. In fact, succinate had an important inhibitory effect on cell growth as well as on succinic acid production; to our knowledge, this observation was not widely reported. Cells growth was suitable for inhibition detection caused by succinic acid production [56]. The results obtained from inhibition tests carried out on different Petri dishes showed negative growth starting from 0.4 mol L⁻¹ of SA concentration. This value correlate fairly well with that obtained by [57] who tested the effect of different concentrations of substrate and product on *A. succinogenes* growth and figure out SA critical concentration of above the bacteria cease to grew up for 0.38 mol L⁻¹ of SA, and further support the concept of growth inhibition of *A. succinogenes* by the product in batch fermentations. Although our results differ to some extent from those of [58], who quantified the inhibitory phenomena and critical inhibitory concentration of mixed acids in batch fermentation which was observed at 0.18 mol L⁻¹ for succinic acid using *A. succinogenes*. Even though this value differ from our results obtained and from previous studies, they are consistent with the concept that the accumulation of fermentation products such as weak acids can act as inhibitory factors and decrease considerably the cell growth [59, 60].

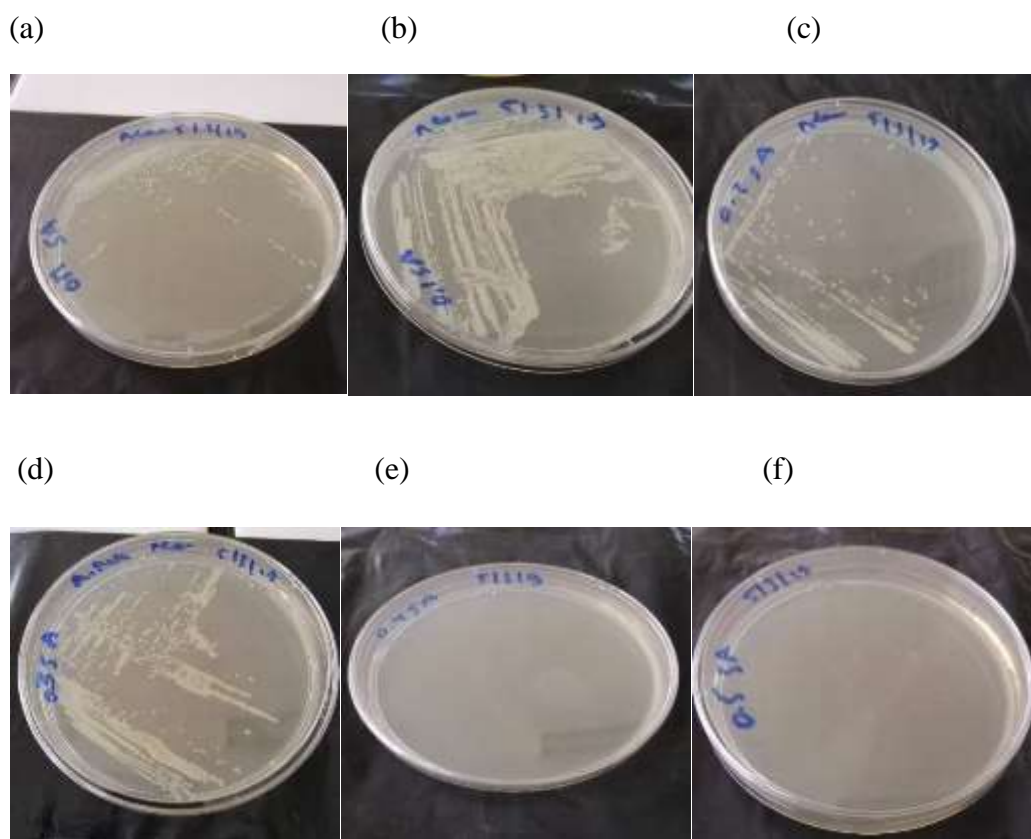


Figure 6. Effect of increased concentration of succinic acid on *A. succinogenes* growth (a: without succinic acid, b: 0.1 mol L^{-1} SA, c: 0.2 mol L^{-1} SA, d: 0.3 mol L^{-1} SA, e: 0.4 mol L^{-1} SA and f: 0.5 mol L^{-1} SA).

III. Conclusions

Among the media used for *A. succinogenes*, synthetic fermentation media containing fumarate gave the highest SA concentration and productivity, 0.49 mol L⁻¹ and 0.48 g L⁻¹ h⁻¹ respectively. Interestingly, the addition of a mediator during the anaerobic fermentation process favored the production of succinic acid with a conversion yield of about 96%. The yields and concentrations obtained 0.88 (molC succinic acid/ molC sugars consumed anaerobically) with 0.5 mol L⁻¹ SA, respectively, were of the same order of magnitude as those reported in the literature. The experimental results pointed out the inhibitory effect of succinic acid on *A. succinogenes* growth.

IV. Perspectives

Succinic acid fermentation performance could be improved by shifting the process from batch mode into continuous mode. Therefore, from a sustainability perspective and industrial side-streams, it is interesting to valorize agricultural by-products, food residues and effluents into building block chemical. To complete this work, scaling-up the process from laboratory scale to pilot plant bioreactors in batch and continuous mode could be carried out to confirm the promising experimental results obtained.

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Appendices

Table S1

Glycolysis	Pentose phosphate
$Glu + ATP \leftrightarrow G6P$	$G6P \leftrightarrow 2NADH + RU5P + CO_2$
$G6P \leftrightarrow F6P$	$RU5P \leftrightarrow R5P$
$F6P + ATP \rightarrow F1,6P$	$R5P \leftrightarrow S7P$
$F1,6P \leftrightarrow DHAP + GA3P$	$GA3P + S7P \rightarrow E4P + F6P$
$GA3P \leftrightarrow DHAP$	TCA Cycle
$GA3P \leftrightarrow 1,3PG + NADH + ATP$	$PEP + CO_2 \leftrightarrow OAA + ATP$
$1,3PG \leftrightarrow 3PG + ATP$	$OAA \rightarrow PYR + CO_2$
$3PG \leftrightarrow 2PG$	$OAA + NADH \leftrightarrow MAL$
$2PG \leftrightarrow PEP$	$MAL \leftrightarrow PYR + NADPH + CO_2$
$PEP \leftrightarrow PYR + ATP$	$MAL \leftrightarrow FUM$
Fructose catabolism	$FUM + NADH + 2/3ATP \rightarrow SUCC$
$FRU + ATP \rightarrow F1P$	Transhydrogenation
$F1P + ATP \leftrightarrow F1,6P$	$NADPH \leftrightarrow NADH$
Biomass formation equation	By-products
Not Available	Not detected

Chapter IV: Improvement of succinic acid bio-production by *Actinobacillus succinogenes* via fermentation and electro-fermentation at lab-scale

Preamble

In the previous chapters, the production of succinic acid and the need for developing novel bioprocesses to produce value added chemicals was discussed. It was also shown that succinic acid bio-process has been studied in anaerobic bottles and there are many areas which can be further explored. In this chapter, the fermentation of glucose and fructose by the bacterium *Actinobacillus succinogenes* for the production of succinic acid was investigated in two different systems (batch fermentation and electro-Fermentation). This bacterium has been studied in our laboratory for producing succinic acid from various chemically defined carbon sources; mainly sugars like glucose, fructose, sucrose, xylose and galactose. Initially, experimental work focused on the production of succinic acid by conducting a series of experiments in glucose and fructose media. Batch fermentations were then performed using *Actinobacillus succinogenes* with different initial conditions in small anaerobic reactors (0.25 L), (1 L) and lab scale bench-top reactors (B-TRs) (3 L). Electro-Fermentations were also carried out by using glucose and fructose ‘synthetic media’ in small anaerobic reactors (0.25 L) and results were compared to those obtained in conventional fermentation. To our knowledge, it is the first related to the electro-fermentation of this microorganism. The employed experimental methodology and results are also described in this chapter.

Improvement of succinic acid bio-production by *Actinobacillus succinogenes* via fermentation and electro-fermentation at lab-scale

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Abstract

Anaerobic fermentations of glucose and fructose were performed by *Actinobacillus succinogenes* 130Z in batch mode using three different volume bioreactors (0.25, 1 and 3 L). Work on the bench scale (3L) showed the best results when compared to the small anaerobic reactor; recording a value of (0.88 mol C-SA/mol-C-sugars and 0.5 g L⁻¹ h⁻¹) of succinic acid yields and productivity, respectively, after 96 h of fermentation. Electrofermentation (EF) was then used to compare with the conventional fermentation. The primary results indicate that during the fermentation process, succinic acid was produced under proved conditions (pH= 6.8, T= 37 °C, and 1% v/v of bacterial biomass using graphite felt under an applied potential of 0.3V), resulting in SA yield (0.57 mol-C SA/mol-C sugars), concentration (0.41 mol L⁻¹) and a volumetric productivity of 1.01 g L⁻¹h⁻¹, after 48 h. The inferences concluded the key role of electrical stimulation on fermentation to enhance the conversion of conventional substrates to targeted product in less consuming time. The SA yields and concentrations obtained were significant and in the order of those reported in the literature.

Keywords: *Actinobacillus succinogenes*; succinic acid; fermentation; Scale-up; Batch culture; Electrode.

I. Introduction

Over the past decades, industrial biotechnology had seen an increase in the production of platform chemicals [1], and one of the most promising contenders was the succinic acid (SA). There are expended studies that emphasize on the SA production as a building block chemical from biomass due to its functionality, its multiple industrial applications and its ability to be converted into several well-known chemicals (e.g. 1,4-butanediol and tetrahydrofuran and γ -butyrolactone) among others [2, 3]. This growth has been done in a part to the increase in reliable scale-up culturing technologies for microorganisms [4]. Industrial fermentations use the process of fermentation to produce the desired product from the microorganism by using their living cells [5]. *Actinobacillus succinogenes* wild type strain is considered as a top-performing succinic acid producer strains and has demonstrated the ability to produce a high concentrations of SA, as well as high yields and productivities [6–8]. Fermentation techniques are formulated using flasks and small lab scale fermenters [9]. However, there can be significant difference between designs and efficiency of small and large-scale fermenters which calls for the determination of proper incubation conditions that are needed to be employed at large scale production tanks, based on information obtained from experiments done with various small fermenters [10]. The process of fermentation is first designed at laboratory level but the actual industrial scale production means quantitative increase of several thousand liters as compare to laboratory fermentation [11]. Such changes can lead to decreased yields and reduce batch-to-batch consistency [10]. A direct changeover from a laboratory scale experiment to industrial production scale may give rise to results against the expectation which means a huge financial loss [12]. So, it is necessary to perform lab scale up experiments before proceeding to full scale plant installation. Additionally, it is vital to maintain optimum and homogenous reaction conditions, minimizing microbial stress exposure and stabilizing their metabolic activity in order to increase product yield and to ensure consistent product quality [13]. The development of bioreactors has provided a scale-up method which is dependable and which reduces some of the cost and labor associated with large-scale cell cultures [14]. Nonetheless, in the industrial biotechnology, cultures must be maintained at much larger volumes in order to produce the desired amount of product [15]. With the increase in production comes a need for successful scale-up strategies in order to more quickly and efficiently convert laboratory results to the industrial scale. While temperature, dissolved oxygen (DO), and pH requirements are independent of volume, parameters such as mixing time and agitation rate need to be

determined specifically for each volume [16]. Performing these runs can be costly and time consuming, hence a reliable method for estimating requisite power and mixing requirements would be valuable to the industry. Developing various innovations to conventional fermentation processes towards higher productivities and yields of platform chemicals with economic benefits is considered a priority for the society. In recent studies, a novel bioelectrochemical approach termed electro-fermentation has been proposed to manipulate the constraint of fermentation. The main envisaged advantage of electro-fermentation is to increase the yield of production of target metabolites and, more in general, to provide an additional tool for controlling and manipulating the spectrum of attainable products in pure and mixed-culture fermentation. Despite some promising results, very limited information is still available on the factors and conditions ultimately affecting the efficacy of electro-fermentation processes.

This work aims to produce SA from different reactors from using synthetic media containing various types of sugars as glucose, fructose, xylose and galactose in 0.25 L small anaerobic reactors. In the following, a setting up of small scale-up system in the laboratory using three different volumes (0.25 L, 1 L and 3 L with and without pH control) for succinic acid production is also designed and the process is described in details. This work aims to increase the scale of fermentation (volume) without compromising yield and focused on providing reliable methods in different forms of bioreactors. The results were then compared to the results of electro-fermentation.

II. Materials and methods

1. Materials

Unless otherwise specified, all media components, fermentation reagents, chemicals and chemicals used in HPLC analysis, were acquired either from Sigma-Aldrich or CARLO-EBRA suppliers.

2. Microorganism

Wild-type strain of *Actinobacillus succinogenes* 130Z (DSM 22257) purchased from German Collection of Microorganisms and cell cultures (DSMZ). Culture samples were stored in 25 mL screw-capped vials agar slant for long term and in tryptic soy broth (TSB) for medium and short term at 4 °C. The Inocula was prepared by reviving a frozen culture from stock bacteria in a 30 g L⁻¹ tryptic soy broth (TSB) and incubating for 24 h at 37 °C then harvesting the pellet in 150 mM KCl and re-incubate in 250 mL sterilized anaerobic bottles containing 200 mL TSB, whose composition consisted in (g L⁻¹): 15 g trypton, 5 g soybean peptone, 2.4 g (D+) glucose, 5 g soduim chlorldide and 2.5 g dipotassium phosphate at 37 °C and 150 rpm for 24-48 h. At the end of the incubation period, cells were centrifuged aseptically (1800 g, 4°C) for 5 min in a centrifuge, then the pellets were suspended in 10 mL KCL (150 mM).

3. Fermentation

Batch fermentations were performed in three different volumes capacity (250, 1000 and 3000 mL) for 96 h. All equipments were sterilised before their use for pure bacterial growth. The tests for batch mode in 3000 mL were performed in a 3 L fermentor (New Brunswick BioFlo®/CelliGen® 115 Benchtop Fermentor & Bioreactor). In addition, BioFlo/CelliGen 115 system can be remotely controlled from a PC via New Brunswick BioCommand optional supervisory software. Samples were analyzed for optical density at 660 nm using a spectrophotometer. Information about the key performance parameters were collected from various literature sources. Based on the data collection, each key parameter was quantified using the parameter equations.

The key performance parameters are defined as follows:

$$\% \text{ of glucose consumption} = ((C_i \text{ glu} - C_f \text{ glu}) / C_i \text{ glu}) * 100 \quad (\text{Eq.1})$$

$$\% \text{ of Fructose consumption} = ((C_i \text{ fru} - C_f \text{ fru}) / C_i \text{ fru}) * 100 \quad (\text{Eq.2})$$

$$\text{Yield} = \text{mol-C produced} / \text{mol-C Sugars consumed} \quad (\text{Eq. 3})$$

$$\text{Productivity SA} = \text{g of succinic acid L}^{-1} \text{ h}^{-1}$$

Sugars consumption rate (rs) and succinic acid production rate (rp) were also calculated as follows:

(rs)= dS/dt: Substrate consumption rate during fermentation time (S initial- S final)/ t final

(rp)= dP/dt: production rate during fermentation time (P final – P initial)/ t final

4. Anaerobic bottles experiments

A 250 mL anaerobic reactor was inoculated with 1% (v/v) pre-culture with a working volume of 200 mL of synthetic media. Fermentation was achieved by introducing the inoculum into a natural media (apple juice) or directly in a synthetic medium (SM) prepared as a medium frequently used for culturing anaerobic succinate producing bacteria [17]. The composition of this medium was, per liter of de-ionized water: 3 g K₂HPO₄, 3 g KH₂PO₄, 1.25 g NaCl, 0.3 g MgCl₂·6H₂O, 0.3 g CaCl₂·2H₂O, 1.5 g NaH₂PO₄, 1.5 g Na₂HPO₄. The pH was adjusted at 6.8 ± 0.2 for all the experiments. Here, the concentration of MgCO₃ is present as a subindex in Molar (mol L⁻¹) (e.g. SM_{0.2} refers to a SM medium supplemented with 0.2 mol L⁻¹ MgCO₃).

5. Bio-fermentor experiments

Batch fermentations were run out in the fermentor. A working volume of 1800 mL was considered and operated at a temperature of 37°C, pH between 6.8 and 7, with an agitator speed 150 rpm were set using automatic controllers in bio-fermenter. Fermentation SM medium was fed with 18 ml inoculation medium into the reactor after the temperature and pH had stabilized. The culture was sparged with N₂ (g) into the fermentor for 10 min at approximately 0.2 volume of gas. volume⁻¹ of liquid minute⁻¹ (vvm) at the beginning of culture to maintain anaerobic conditions. During the fermentation, Samples of 10–20 mL were withdrawn aseptically from the reactors at regular time intervals. The samples were centrifuged at 1800 g for 5 min and the supernatant was analyzed using HPLC.

6. Electro-fermentor experiments

Five round necked glass bottles (Quickfit®, England) was used to fabricate single chamber bench-scale anaerobic reactors (total/working volume 250/200 mL). The reactors were operated in batch mode with suspended growth configuration with constant mixing (250 rpm) using a magnetic stirring bar at a temperature of 37± 1 °C. The individual reactors were operated as control (C-Without electrodes) and with Applied potential difference (AP-with electrodes with potential of 0.3 V/SCE imposed between the graphite felt working electrode and a saturated calomel electrode (SCE) as reference electrode). Graphite felt was used as an electrode material for the working electrode and for the counter electrode. The two electrodes had the same

dimensions (3.5 x 3.5 x 1.0). Prior to start, the set-up with the electrodes were autoclaved at 121 °C to prevent contamination. The distance between electrodes was approximately 2.5 cm. Each bioreactor was designed to have sampling port, N₂ sparging, electrical connections input ports (top) and leak proof sealing was employed to ensure anaerobic microenvironment in the system by continuous N₂ sparging.

7. HPLC Analysis

Concentrations of sugars and products in the medium during the fermentation were measured by HPLC as described in [18].

III. Results and discussion

1. Batch fermentation in 250 mL using various sugars

Succinate production by *A. succinogenes* on carbon sources, such as glucose, fructose, xylose, galactose, sucrose and cellobiose has previously been reported [19–21]. Fig 1 shows the global metabolism of *A. succinogenes*. To the best of our knowledge, there are no publications comparing succinate yield and productivity under the same conditions using pure sources of the most prevalent monosaccharides in apple juice. For this reason, succinic acid production from different monosaccharides was assessed in Duran bottle fermentations. *A. succinogenes* was able to consume all the tested sugars. Glucose was the most quickly consumed and the succinic acid yield on glucose (0.8 mol-C/mol-C) was higher than for the other tested carbon sources. They were 0.77, 0.74 and 0.72 mol-C SA/mol-C for succinate, galactose and xylose (Fig. 2). Nevertheless, a complete conversion was obtained for all these sugars.

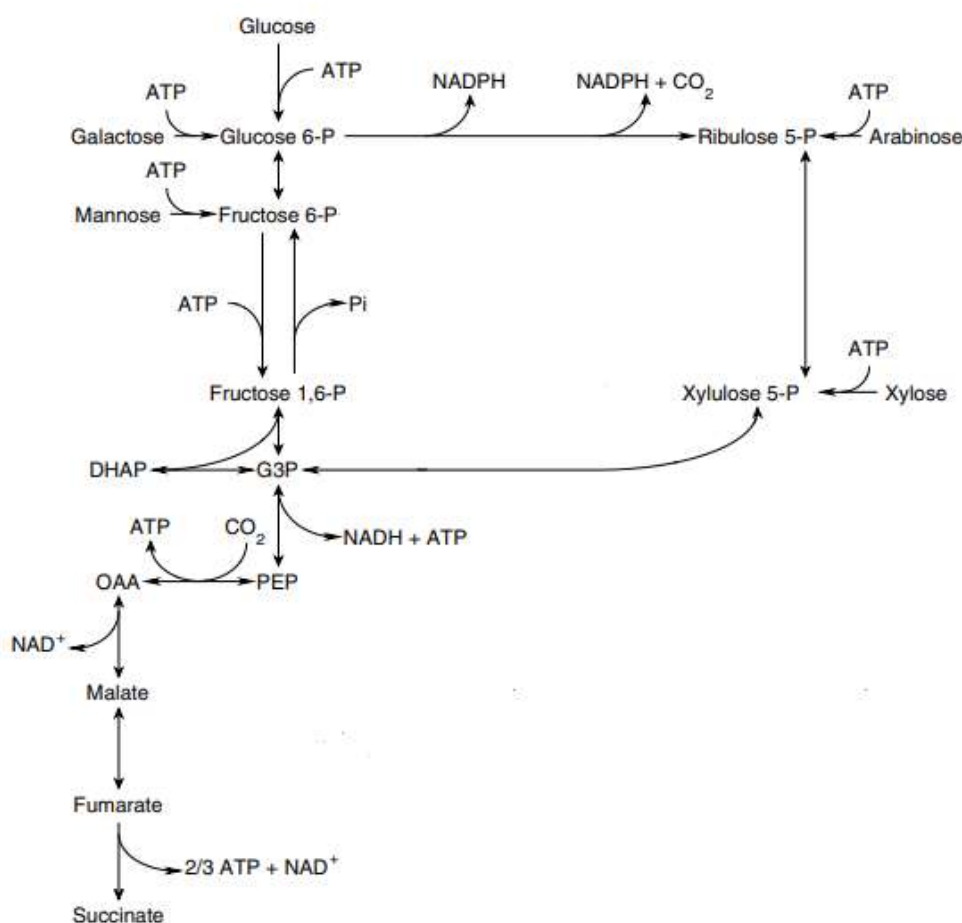


Figure 1. Simplified central carbon metabolic pathways of *Actinobacillus succinogenes*

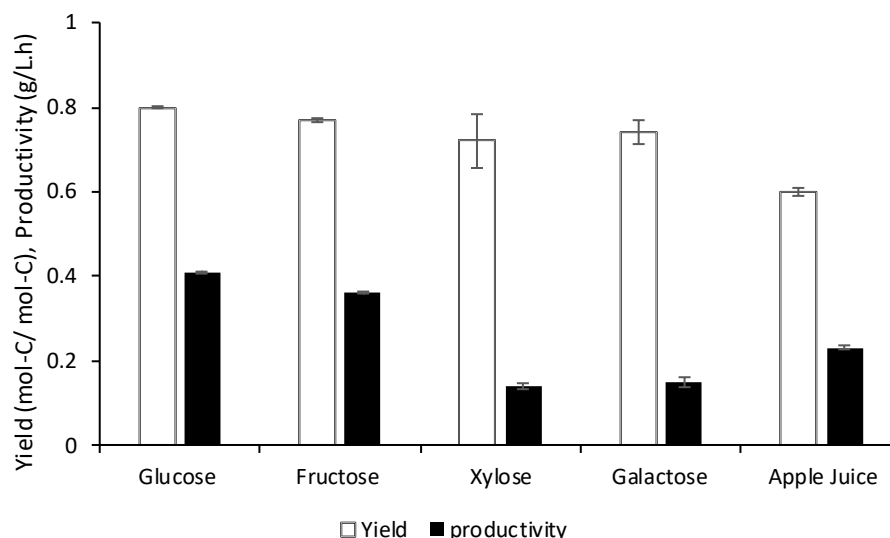


Figure 2. Succinate yield (white bars) and productivity (black bars) of *A. succinogenes* in anaerobic bottle fermentations using different monosaccharides as carbon source. Error bars indicate standard deviation

Succinate fermentation in *A. succinogenes* involves a carboxylation, one C-mol of CO_2 is required for every three C-mol of monosaccharides converted to succinate. If the carbonate is supplied solely as MgCO_3 , this corresponds to 0.93 g MgCO_3 per gram of monosaccharides. Hence, a complete conversion of 0.2 mol L^{-1} of sugars would require approximately the same concentration of MgCO_3 . Succinic acid was produced effectively from glucose, fructose, xylose and galactose at initial concentration of 0.2 mol L^{-1} by *A. succinogenes* in anaerobic mode with carbonate supply by adding defined amounts of MgCO_3 to the medium ($\text{SM}_{0.2}$) as well in apple juice containing glucose, fructose and sucrose (Fig 2). For this reason, glucose and fructose were then chosen as substrate for the rest of the study.

2. Batch fermentation realized in 1 L reactors

Previous experiments in 250 mL anaerobic bottles demonstrated that succinic acid is produced regardless sugar species (Fig. 2). To more deeply investigate succinic acid production, batch cultures were carried out under anaerobic conditions in 1 L reactor employing SM containing glucose and fructose and or mixtures of both sugars to have different initial concentration varied in total between 0.1 and 0.4 mol L^{-1} . Sugar consumption (r_s) for glucose, fructose and their mixtures at different initial sugar concentrations are illustrated in Fig. 3; sugar consumption rates (r_s) were for fructose (0.73 g $\text{L}^{-1} \text{h}^{-1}$), glucose (0.50 g $\text{L}^{-1} \text{h}^{-1}$), and sugars mixture (0.74 g $\text{L}^{-1} \text{h}^{-1}$) at 24h. No sign of diauxic growth was observed, since glucose and fructose were simultaneously consumed. The amount of remaining sugar increased with the initial concentration of initial sugars concentration increased (Fig 3-a). Regarding succinic acid

production (Fig 3-b), maximum yield values were 0.79, 0.83 and 0.67 C-mol SA/C-mol substrate for glucose and fructose, respectively, at 0.1 mol L⁻¹ for pure and/ or mixed sugars.

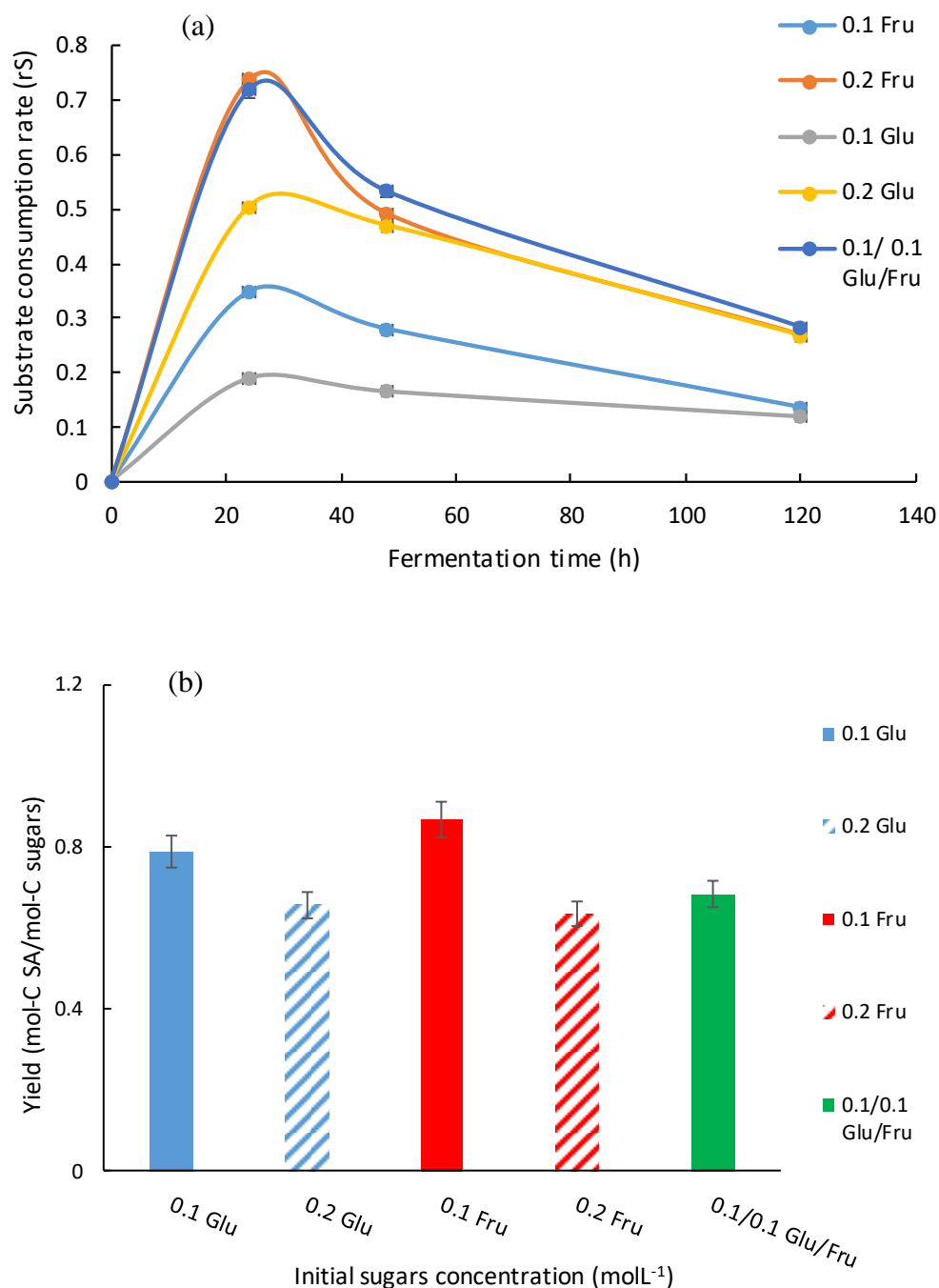


Figure 3. Experimental substrate consumption rate (a) and succinic acid production yield (b) of *A. succinogenes* fermentation at different initial concentrations of glucose and fructose.

Based on the above results as obtained in anaerobic bottles, batch fermentations with equal initial sugars concentrations of 0.2 mol L⁻¹ were also carried out in 1 L anaerobic bottles (Fig.

4). The results indicated that cell growth and metabolites production were similar to those obtained in 250 mL anaerobic bottles; except that the production of succinic acid was slightly higher in 1 L anaerobic bottles. At an initial sugars concentration of 0.2 mol L^{-1} , cells grew very rapidly, and an optical density at 660 nm (OD_{660}) of 0.4 was obtained at 48 h (Fig. 4). However, cell density reached was lower than that obtained in previous work [22].

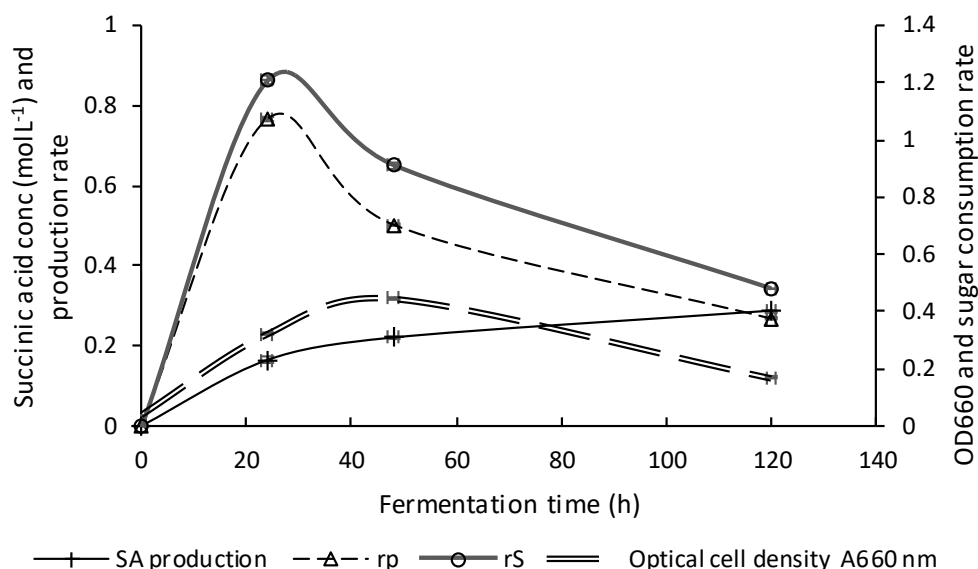


Figure 4. Fermentation rates as (substrate consumption and succinic acid production) with succinic acid concentration and cell density evolution during the fermentation time

3. Fermentation in the Bench-Top Fermenters (B-TFs)

Fermentation runs were also performed in a 3 L capacity fermentor with 1800 mL working volume using the same medium as in 250 mL anaerobic bottles. Fig 5- a displays the results from similar initial sugars concentrations (glucose and fructose equal mixture) with fumaric acid as a co-factor at the ending of experiments. Maintenance production towards the end of the fermentation is slow (fig 5-a), glucose and fructose were simultaneously consumed, however, they present a significant amounts of residual sugars present (0.01 mol L^{-1}). The succinic acid concentration obtained at the end of fermentation was about 0.4 mol L^{-1} . The specific productivity was 14.6 g of succinic acid/ g of dry cell weight at the end of fermentation (after 96 h). Fermentations in B-TFs demonstrated similar behavior as the 250 mL anaerobic bottles. At $t = 0 \text{ h}$; 100 % saturation level of DO indicated highly aerobic conditions; however, at $t = 48 \text{ h}$, a situation between aerobic and anaerobic conditions was obtained, and a slight decrease in the pH was observed (fig 5-b).

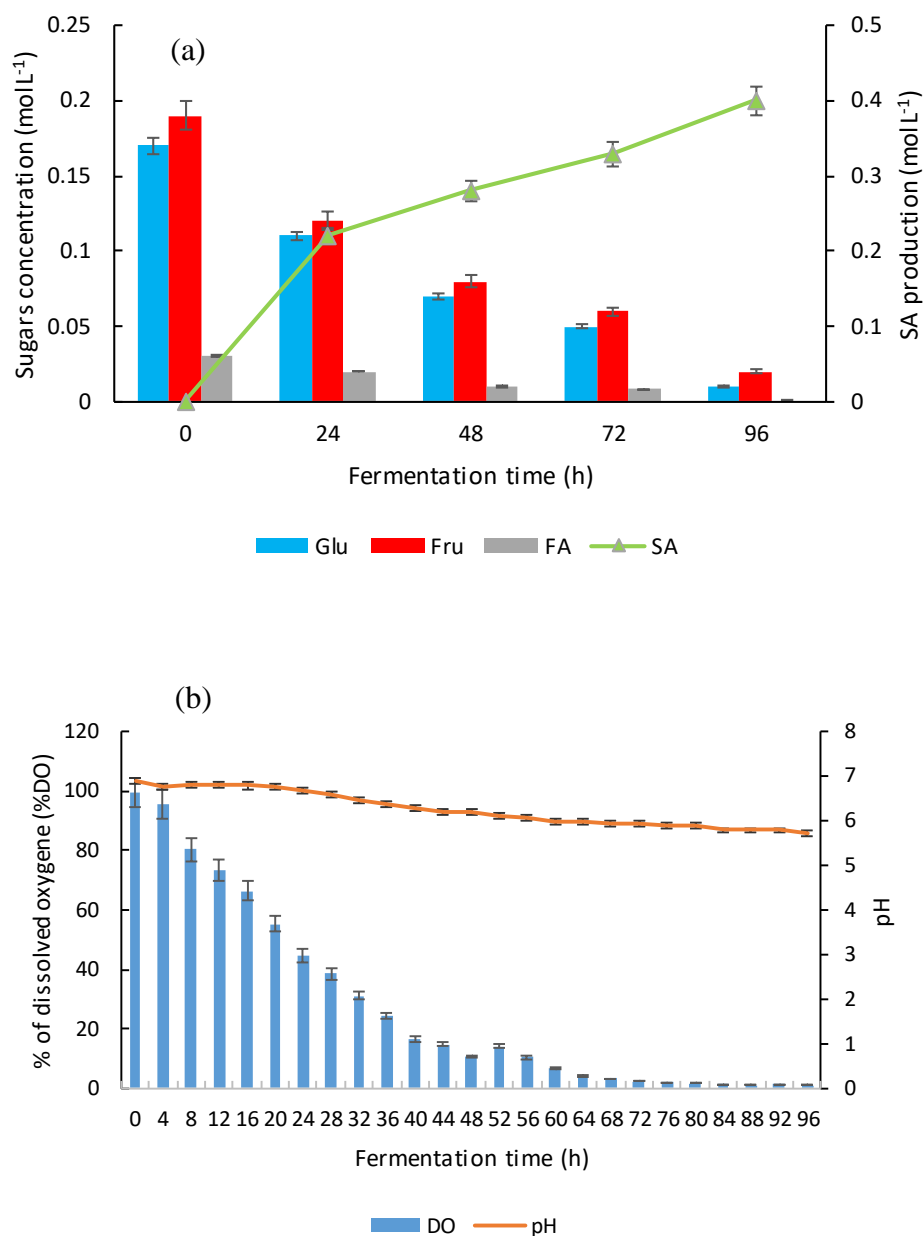


Figure 5. a- sugars consumption vs succinic acid concentration, b- pH. and Dissolved oxygen evolution during fermentation time in 3 L bioreactor performed in anaerobic conditions at 37°C.

Succinic acid production of (0.3 mol L^{-1}) using glucose as the sole carbon substrate in the reactor was lower when compared to the mixture of sugars (data not shown). When a reactor is run in batch mode, important process variables, (e.g., cell mass, pH, dissolved oxygen (DO), substrate concentration) may vary significantly [23]. If the dissolved oxygen level is kept

constant, the aeration efficiency can be used as an indicator of the biological activity and it may also be beneficial for process supervision. For instance, elevated oxygen levels are unfriendly with succinic acid production and therefore the dissolved oxygen variation should be monitored. *A. succinogenes* can grow up in aerobic and anaerobic conditions by modifying its physiology and metabolic pathways to adapt the climate change [24, 25]. Succinic acid production is controlled by regulatory systems that detect oxygen levels and transmit a signal to modify gene expression accordingly [26]. As a result, the concentration of succinic acid increased in anaerobic conditions when compared to aerobic ones. The results given above throw light on the relation between cultivation modes (the dissolved oxygen environment) and succinic acid production, and in the light of this speculation, fermentation of *A. succinogenes* established in microaerobic conditions can be shifted to anaerobic environment when O₂ is completely consumed without nitrogen sparging. The accumulation of succinic acid in the broth might however affect biomass formation, as already described in the literature [27]. It is known that non-dissociated organic acids can penetrate the lipidic membrane of bacteria cells and be dissociated at intracellular pH (6.8), decreasing intracellular pH. As a consequence, energy (ATP) will be required to adjust the intracellular pH and anaerobic microorganisms such as *A. succinogenes* will use more energy to expel protons instead of using energy for biosynthesis and growth [20].

4. pH regulation

The pH culture is a crucial factor in fermentation. In bioreactor, NaOH was used as buffering agent for SA production, along with MgCO₃. Although the use of NaOH could promote a significant accumulation of Na⁺ ions in the broth in addition to NaH₂PO₄ and Na₂HPO₄ present in the media used to neutralize pH and organic acids synthesis. Na⁺ ions are known to be involved in intracellular pH regulation [17], their accumulation resulting in a hyperosmotic environment and subsequently damage of the cell morphology. The effect might be so severe that could actually affect cells even at an early stage of fermentation, reducing the growth rates. Without pH regulation, the pH value in the culture decreased from 6.8 to 5.7 at the end fermentation due to the accumulation of succinic acid. For this reason, it was essential to study the significance of pH on *A. succinogenes* production performances in a bench-top reactor. The effect of pH on SA accumulation was examined by setting-up the pH at 6.8 during the entire fermentation process. When the pH was adjusted to 6.8, no significant effect was shown on succinic acid biosynthesis at the end of the fermentation and the neutral environment appeared favorable to an accumulation of succinic acid (table 1). When pH was controlled, Succinic acid

yields and productivity were increased if compared to experiments carried out in 250 mL anaerobic bottles (0.88 mol C-SA/mol-C sugars and 0.5 g L⁻¹ h⁻¹) respectively, in addition to succinic acid final concentration (0.41 mol L⁻¹). Maintaining the pH fermentation within suitable range for the microorganism, and the selection of an appropriated base to neutralize the produced acid, had a significant effect on the overall SA production costs [28]. Cell density and sugars consumption were therefore almost similar during pH controlled and non-controlled fermentation.

Table 1. Succinic acid production from 0.2 mol L⁻¹ equal mixture of glucose and fructose with 0.2 mol L⁻¹ of MgCO₃ at the end of fermentation time (96h) in 3L benchtop reactor with a volume of 1800 mL.

	Initial sugar concentration (mol L ⁻¹)	Consumed sugars (%)		Consumed FA (%)	SA mol L ⁻¹	Yield (molC/molC)	Productivity (g L ⁻¹ h ⁻¹)
		Glucose	Fructose				
Controlled pH	0.40	96.83	98.26	96.49	0.41	0.88	0.50
Uncontrolled pH	0.40	93.30	91.39	96.07	0.40	0.87	0.49

5. Scale-up study

In this study fermentation techniques are formulated using bottles and small lab scale fermenters. The surrounding environment of *A. succinogenes* may change with the change in scale of production. Different parameters can be observed in number during lab scale fermentation setups. The parameters obtained for the various volumes examined and the impact on the SA production are shown in Table 2. Increase the scale of fermentation (volume) without compromising yield or productivity was seen in 250 and 3000 mL. Results show that the data were representative, when scaled up, similar values of SA concentration and productivity were obtained, as compared to those in 3000 mL. However, in the fermentation done in one liter, results were different, for this reason, identify the factors that contribute to decrease and to rectify it is necessary. This might be due to a lack of scale-up considerations. The speculations that must be considered while developing the process of higher fermentation productions are the same in terms of inoculum development, sterilization, pH, temperature, environmental parameters and agitation.

Table 2. Comparison of scale-up studies

Volume (mL)	Working volume (mL)	SA (mol L ⁻¹)	Productivity (g L ⁻¹ h ⁻¹)	Yield mol-C/ mol-C
250	200	0.4	0.49	0.81
1000	800	0.28	0.26	0.57
3000	1800	0.41	0.50	0.88

Although, previous studies carried out in anaerobic bottles proved that the decrease of cell density and/ or biomass could not be correlated to high sugar concentrations regardless of the sugar nature. Instead, it might be related to the accumulation of succinic acid in the broth, as mentioned in the study of Lin and colleagues [27]. As cell growth stops, flux towards C3 pathway also decreases as there is no further need to maintain such NADH demand via acetate and formate production [8]. Thus, the available carbon is directed to produce SA, and then SA yield increased. The maximum SA concentration is not associated with total sugar consumption, while the production of succinic acid begins to slow down at 52 hours. An inhibitory effect of succinic acid on bacterial growth and its production can be suggested. From the investigated experiments, the highest concentration of succinic acid obtained by *A. succinogenes* was identical to the 250 mL anaerobic bottles experiments, exhibiting a value of 0.28 mol L⁻¹ with a sugars consumption and SA production rate of 1.2 g L⁻¹ h⁻¹ and 0.76 g SA g L⁻¹ h⁻¹ after 48 h, respectively (fig 5-a).

6. Impact of the experimental conditions during cultures in 250 mL anaerobic bottles.

Fig. 6 shows the different variation of metabolites in three different fermentation parameters, in the presence (EF) and the absence (CF) of electrode (continuously N₂ sparging) with conventional fermentation (F) (sparging 5 min N₂ gas), as the mean value of the two different cultures, which were thus regarded to as replicates. Electro-fermentation (EF) showed marked influence on the microbial synthesis of SA compared to conventional fermentation (F) and control fermentation (CF) (Fig.6). An applied potential of 0.3 V was imposed, referring to the range of fumarate/succinate acid oxido-reducing couple. The self-induced applied potential (AP) microenvironment (0.3 V), when compared with conventional fermentation (F) showed an improvement from 0.22 mol L⁻¹ to 0.4 mol L⁻¹ after 48 h, namely 50% enhancement. While, control fermentation (CF) when compared with conventional fermentation (F) showed only a slight increment from 0.22 mol L⁻¹ to 0.25 mol L⁻¹ (13.6%). The apparent changes in electro-

metabolic activity of the fermentative microenvironment are due to electron flux created by the presence of electrode assembly. The increased and controlled electron flow through the electrodes (when a potential was applied) showed productivity enhancement in terms of SA amount. Electrodes functioned as electron donors (anode) and acceptors (cathode) in the fermentative microenvironment. The regulation in electron transfer rates, in terms of acceptance of e^- and H^+ towards the conversion of substrates enhanced product recovery, which is positively influenced by the interactions of electrodes with the microbial environment.

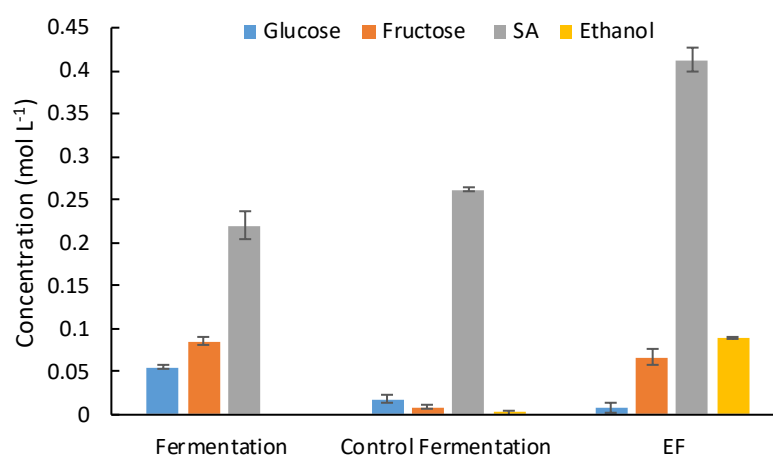


Figure 6. Variation in metabolites composition at different experimental conditions after 48h fermentation done in 250 mL in the presence of $MgCO_3$. Note that in Control fermentation and in EF, N_2 gas was continuously sparged

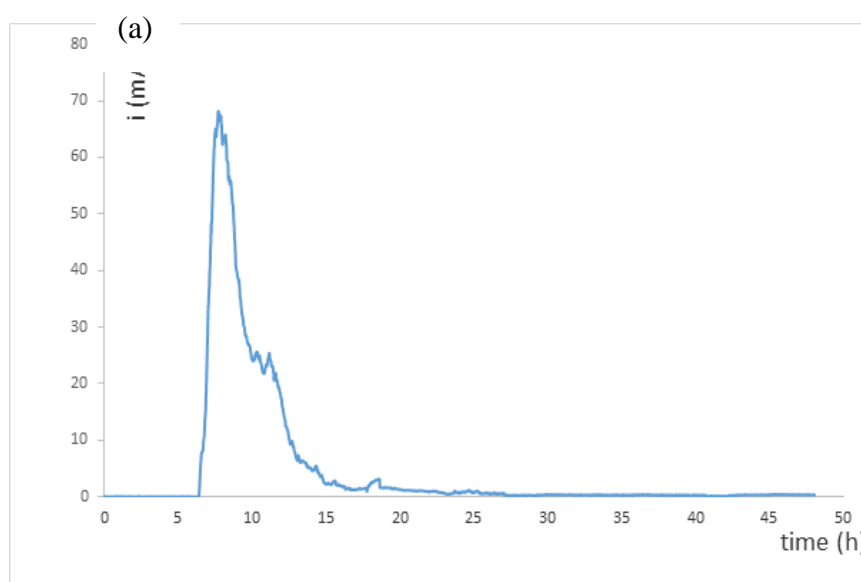
Table 3. Kinetics of succinic acid fermentation in different experimental conditions at the end of fermentation

Experimental conditions	Time (h)	SA (mol L ⁻¹)	r _s (dS/dt) (g L ⁻¹ h ⁻¹)		r _p (dP/dt) (g L ⁻¹ h ⁻¹)	Yield *
			Glu	Fru		
F	48	0.22	1.29	1.18	0.54	0.33
CF	48	0.25	1.43	1.46	0.64	0.34
EF	48	0.41	1.47	1.24	1.01	0.57

*The yield was calculated as the mol-C of succinic acid produced by the mol-C of substrate consumed anaerobically.

Sugar consumption rates profile (r_s) obtained in different experimental conditions were in fact similar for all experimental conditions, namely 2.47 g L⁻¹ h⁻¹, 2.89 g L⁻¹ h⁻¹ and 2.71 g L⁻¹ h⁻¹ for F, CF and EF cultures. Regarding succinic acid production, r_p values obtained were 0.54, 0.64, and 1.01 g L⁻¹ h⁻¹ for (F), (CF) and (EF), respectively (table 3). Maximum SA production

was observed for (EF), suggesting that SA production in (F) and (CF) may be limited by the high sugar concentration or by an insufficient biomass formation. However, sugar consumption rates remained in the same levels suggesting that biomass formation is not limited by sugar uptake but by the absence of electrode. Glucose and fructose uptake in *A. succinogenes* is mediated through phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS), that were identified in previous report [29]. This system is responsible for selective transport of sugar molecules into the cell, accomplished in parallel with sugar phosphorylation, which prevents sugar efflux. Other PTS families have been identified and characterized but the most representative among bacteria is the glucose family, followed by fructose, mannose and lactose families [30]. These data clearly illustrate a change in *A. succinogenes* metabolism as sugar concentration increases, decoupling SA production from cell growth, favoring the C4 reductive pathway and as a result, succinic acid production (Table 3). At the beginning of the electro-fermentation, current intensity was lower than 0.1 mA and suddenly increased up to 68 mA from around 6.4h (Figure 7-a). This increase proved the production of an electroactive compound at this time and its decrease from around 8h seemed to suggest the consumption of this compound.



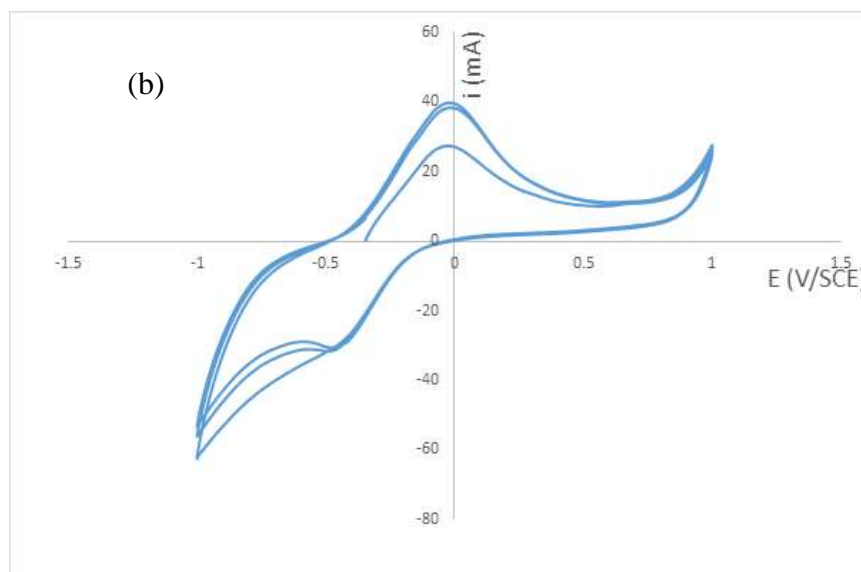


Figure 7. - a: evolution of current intensity during the electro-fermentation, b-: Current-potential curves obtained by cyclic voltammetry with a graphite felt electrode (3.5 x3.5 cm), $r = 40 \text{ mV.s}^{-1}$, under nitrogen atmosphere and $T = 37^\circ\text{C}$ after 48h of electro-fermentation.

At the end of the electro-fermentation, a current-potential curve was plotted by cyclic voltammetry (40 mV s^{-1}) with the same electrochemical system as for the electrolysis. Figure 7-b shows the cyclic voltammograms and highlighted a signal in oxidation around 0 V/SCE and a signal in reduction at around -0.45 V/SCE which correspond to SA. Results of the electro-fermentation showed the production of succinic acid and the presence of ethanol. In order to verify if the signals obtained after electro-fermentation corresponded to succinic acid oxidation and/or reduction, several current potential curves were then plotted (appendices 1 and 2).

The results obtained in these experiments were compared with the results reported in previous work in Table 4 for succinic acid production from different substrates; it is similar to the other cited substrates. Succinic acid production from *A. succinogenes* CGMCC1593 was reported at 0.50 mol L^{-1} which was an increase of 55.5% in comparison to the batch fermentation of 0.32 mol L^{-1} [22]. *Mannheimia succiniciproducens* LPK7 was employed to produce succinic acid from glucose in batch run and increase by a 2.9-fold in succinic acid concentration when compared to fed-batch fermentation (33). For the fed-batch system, the succinic acid concentration and productivity were 0.4 mol L^{-1} , while the values for batch fermentation were 0.1 mol L^{-1} respectively [31]. Cassava roots were employed for SA production using *A. Succinogenes*, and this resulted in concentration, yield, and productivity of 0.78 mol L^{-1} , 1.3, and $1.87 \text{ g L}^{-1} \text{ h}^{-1}$, in batch mode and 1.27 mol L^{-1} , 2.4, and $3.22 \text{ g L}^{-1} \text{ h}^{-1}$, in fed-batch mode

respectively [32]. Corn straw was investigated for succinic acid fermentation by *A. succinogenes* recording a SA value of 0.38 mol L⁻¹, 0.44 mol L⁻¹ corresponding to a productivity of 0.95 g L⁻¹ h⁻¹, 1.21 g L⁻¹ h⁻¹ in batch and fed-batch fermentation, respectively [33].

Table 4. Comparison of succinic acid production by fermentation with previous studies

Micro-organisms	Substrate	Fermentation strategy	Succinic acid production			Ref
			Concentration	Yield ^a	productivity ^b	
<i>A. succinogenes</i> CGMCC1593	Glucose	Batch	0.32	1.25	1.00	[22, 34]
		Fed-batch	0.50	1.2	1.30	
<i>M.succinio- produces</i> LPK7	Glucose	Batch	0.11	0.97	1.22	[31]
		Fed-batch	0.44	1.16	1.80	
<i>A. succinogenes</i> DSMZ 22257	Glucose, Fructose	Electro-fermentation Batch	0.41	0.57	1.01	This study
<i>A. Succinogenes</i> 130 Z	whey	Batch	0.11	1.67	0.61	[35]
<i>A. succinogenes</i> NJ113	Sweetsorghum bagasse	Batch	0.15	0.93	0.98	[36]
<i>A. succinogenes</i> 130 Z	Rapeseed meal	Batch	0.13	0.20	0.22	[37]
		Fed-batch	0.19	0.20	0.33	
<i>A.succinogenes</i> 130 Z	Raw carob pods	Batch	0.07	0.55	1.32	[38]
<i>A. succinogenes</i> ATCC55618	Cassava roots	Batch	0.78	1.3	1.87	[32]
		Fed-batch	1.27	2.4	3.22	
<i>A. succinogenes</i> CGMCC1593	Corn straw	Batch	0.38	1.36	0.95	[33]
		Fed-batch	0.44	1.4	1.21	

^a The succinic acid yield (mol/mol) was calculated as succinic acid concentration (mol) obtained divided by consumed substrate (mol) during the fermentation

^b The succinic acid productivity (g L⁻¹ h⁻¹) was calculated as succinic acid concentration obtained (g L⁻¹) divided by the overall fermentation time (h)

IV. Conclusions

Succinic acid production from *A. succinogenes* using different reactors was evaluated. The results showed that succinic acid can be successfully produced from glucose and fructose in conventional fermentation and in electro-fermentation by *A. succinogenes* in batch mode. SA production was more important in EF than in 3L reactor; while in both cases yields and productivities were similar to those obtained by other authors under the same conditions of batch fermentation. The optimal results in electro-fermentation and in 3L bioreactor at pH controlled at 6.8 were obtained with 0.4 mol L⁻¹ of glucose and fructose leading to 0.41 mol L⁻¹ of SA for 0.2 mol L⁻¹ MgCO₃. However, EF experiment was a preliminary indications and results have to be verified. Further research on the fermentation mode (fed-batch, continuous, biofilm), the bacterial strain used and other parameters such as pH and CO₂ level are needful in order to help to develop a bio-production of succinic acid more cost-effective.

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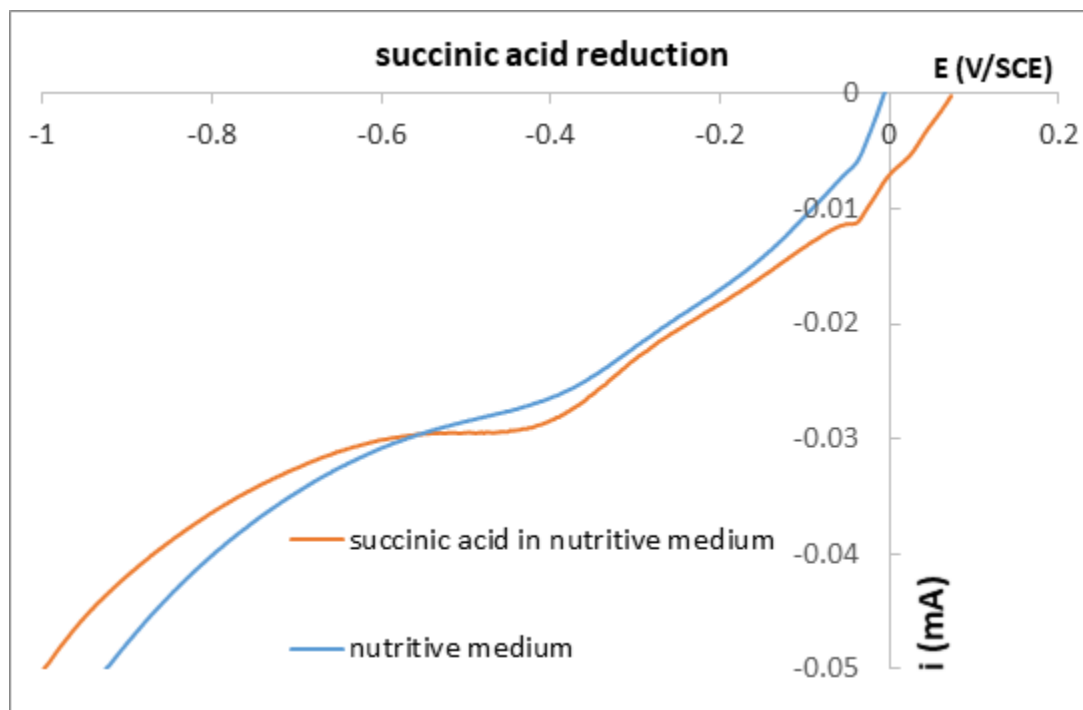
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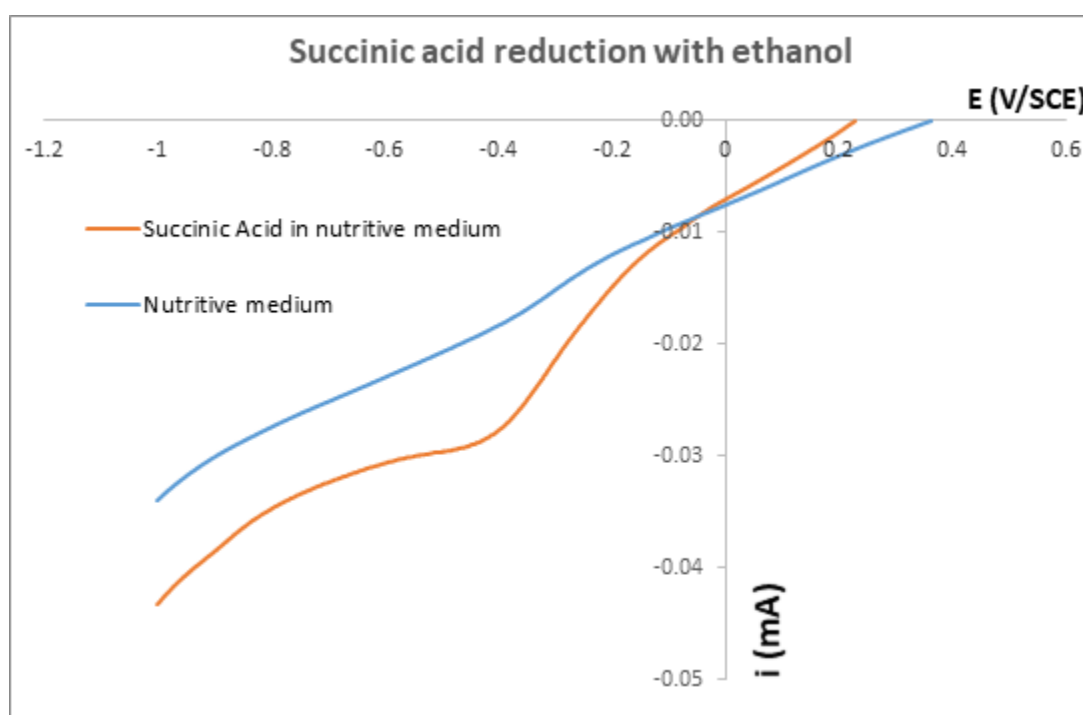
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Appendices



Appendice 1: Current-potential curves obtained by cyclic voltammetry with a vitreous carbon electrode ($S = 3.14 \text{ mm}^2$), $r = 40 \text{ mV.s}^{-1}$, under nitrogen atmosphere, in culture medium with or without succinic acid at pH 5.3 and room temperature.



Appendice 2: Current-potential curves obtained by cyclic voltammetry with a vitreous carbon electrode ($S = 3.14 \text{ mm}^2$), $r = 40 \text{ mV.s}^{-1}$, under nitrogen atmosphere, in culture medium and ethanol with or without succinic acid at pH 5.3 and room temperature.

Chapter V: A new approach of co-culture system to produce succinic acid

Preamble

In the previous chapter, batch fermentations were performed using *Actinobacillus succinogenes* with different initial conditions in anaerobic reactors (0.25 L), (1 L) and lab scale bench-top reactors (B-TRs) (3 L). Electro-Fermentations experiments were also carried out and the results were compared to those obtained in conventional fermentation. The need to develop a new bioprocess was also highlighted. Here we focus on the use of both mono-culture and co-culture strategies of *A. succinogenes* with *S. cerevisiae* to produce succinic acid using C6 sugars as carbon source (glucose and fructose). Under microaerobic and anaerobic conditions, the process was characterized in terms of concentration of sugars, cells density, metabolites, yield, productivities and pH. In each of these cases, tests were carried out in a batch culture. An effort is pointed on the co-culture system to improve the biosynthetic pathway between *A. succinogenes* – *S. cerevisiae* by combining these two strains in a single fermentation process. In the second step, the effect of co-culture microorganisms with different initial proportions was investigated. After this step the effect of temperature on co-culture production profile was also evaluated. At the end, sequential 48h co-culture was tested in order to replace the nitrogen sparging with the MgCO_3 addition.

A new approach of co-culture system to produce succinic acid

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Abstract

Microorganisms can produce a wide range of bio-based chemicals that can be used in various industrial applications as molecules of interest. In the present work, an analysis of the power production of pure culture, co-culture, and sequential culture was performed. Both mono-culture and co-culture strategies of *Actinobacillus succinogenes* with *Saccharomyces cerevisiae* were examined in this study to produce succinic acid using glucose and fructose as carbon source. Cultures were performed in batch mode and a great attention was paid to the co-culture system to improve the biosynthetic pathway between *A. succinogenes* and *S. cerevisiae* by combining these two strains in a single fermentation process. Under microaerobic and anaerobic conditions, the process was characterized in terms of sugars concentration, cells density, metabolites, yield (mol-C products/ mol-C sugars), productivities temperature and pH. The results showed that the process was able to consume glucose and fructose and was able to adapt to different concentrations of the two sugars more quickly than by a single organism and the best results was obtained in sequential co-culture recording 0.27 mol L⁻¹ of succinic acid concentration and a volumetric productivity of 0.3 g L⁻¹ h⁻¹. Under the investigated operating conditions, the combination of these two strains in a single reactor yielded a significant amount of succinic acid (0.70 mol-C SA/ mol-C substrates). Simultaneous and sequential co-culture strategy can be a powerful new approach in the field of bio-based chemical production.

Key-words: fermentation; Co-culture; sequential culture; bio-based chemicals; Succinic acid.

I. Introduction

Interest in bio-based chemicals production from sugars has strongly increased in the last decade, as these sugar-derived can potentially replace oil-derived chemicals such as succinic acid [1]. Traditionally, succinic acid (SA) is derived from fossil sources via hydrogenation of maleic anhydride and considered as a key platform chemical as it is used in the production of a wide range of products, from pharmaceuticals to green solvents, fibers and bioplastics [2]. There is a tremendous opportunity for SA biosynthesis. Interesting advantage for bio-based SA is that its production does not contribute to the accumulation of carbon dioxide (CO₂) into the environment and can therefore, be considered as a "green" chemical [3, 4]. SA fermentation is relatively well studied, by an array of microorganisms and under different conditions process [5, 6]. Conventional production of bio-SA involves anaerobic bacterial fermentation of pure or mixture sugars with the addition of CO₂ sources, such as *Actinobacillus succinogenes* [7, 8]. Previous studies showed that the production of SA could be raised by controlling CO₂ supply in fermentation media, which can significantly add to the product cost [9]. *A. succinogenes* can be used but requires an anaerobic environment (nitrogen bubbling) and the presence of dissolved CO₂ in the fermentation broth, such as MgCO₃, which definitely control the metabolic flux of carbon, and the activity of enzymes including phosphoenolpyruvate carboxykinase (PEPCK), that are the crucial steps for succinic acid bioproduction by succinate-producing bacteria [10]. To build-up a potential method, it is important to improve its efficiency and sustainability. Yet, there are two key tools to improve the process output, that has been used in various fields of study: to optimize the operating conditions and to manipulate/steer the microbial community, which requires improvement of the existing knowledge pertaining to the relationship between microbial community structure and operational conditions [11]. Several studies were reported, evaluated and investigated related to the impact of the operating conditions, in different variations and/or combinations, on SA output such as the pH [12], temperature [13], culture conditions [14] and standardised one or various types of substrates [15]. Aside from the effects of the operating parameters, some studies focused on metabolic pathways such as turn on/off gene pathways [16]. Most previous studies have focused on finding correlations between the operating parameters and SA production [4, 7, 10, 13, 15]; however just a few have investigated relationships with the microbial community in the same environment [17]. Mixed culture biotechnology (MCB) could become an attractive alternative to traditional pure culture based biotechnology that can be established for the production of a narrow spectrum of chemicals and/or bioenergy from a pure or mixed substrates [17, 18].

Mixed-culture fermentations are those in which the inoculum always consists of mixtures of two or more known or unknown species [19]. The mixed cultures may be all of one microbial group, such as bacteria, or they may consist of a mixture of organisms of fungi and bacteria or fungi and yeasts or other combinations in which the components are quite unrelated [18, 20]. The main motivation behind employing co-culture is to examine interactions between microorganisms and to develop novel cell approach techniques to produce platform molecules [21]. Microorganisms that consume sugars sequentially, must have lower productivities for the generation of a product, than if the organisms consumed the sugars simultaneously [22].

In the context of large-scale succinic acid production, the use of both MgCO_3 and N_2 gas may become prohibitively expensive. To overcome these limitations, we suggest a novel strategy for eco-efficient SA production based on a partnership between yeast producing CO_2 , *Saccharomyces cerevisiae* with SA producing bacteria, *A. succinogenes*. The CO_2 produced by yeast respiration during fermentation of sugars could be fixed by *A. succinogenes*; this can be a reliable alternate strategy that is cheap, easy to manage, durable and effectively replace the addition of MgCO_3 and N_2 sparging. Here, we set out to construct a synthetic co-culture of *A. succinogenes* (DSM 22257) and *S. cerevisiae* (CLIB 95) to produce SA by efficient co-utilization of sugar mixtures. The concept centers on the fact that we can readily "design" a co-culture approach, in simultaneous and sequential culture, that could be a bridge between feedstocks and bio-based production. In this paper, we report our findings of this new method for direct SA production, from substrates. Batch data of viability, residual sugars, yield, productivity and SA concentration using *A. succinogenes* – *S. cerevisiae* co-cultures fermentation process are discussed.

II. Materials and Methods

1. Microorganisms and inoculum preparation

A. succinogenes (DSM 22257) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Institute DSMZ-German Collection of Microorganisms and Cell Cultures) and the yeast *S. cerevisiae* CLIB 95 obtained from CIRM (Centre National de Ressources Microbiennes, France).

Bacterial inoculum was prepared as follows: The bacterium was suspended in Trypton Soya Broth (TSB) and preserved on Tryptic Soya Agar (TSA) plates and slant. Cells were incubated at 37°C for 24-48 h in an incubator (VWR® INCU- Line), which was the time required for the

microorganism to reach the exponential growth phase. For yeast species, *Saccharomyces cerevisiae* was maintained at 4°C on Sabouraud glucose agar 4% slant whose composition consisted in (g L⁻¹): 40 g glucose, 10 g peptones, 15 g of agar; then cells were incubated at 28 °C for 24 h in the incubator. To generate bacterium and yeast inocula, culture suspensions were transferred to 50-mL centrifuge tubes, resuspended in KCL 150 mM and incubated in an incubator shaker (New Brunswick, INNOVA 40, NJ, USA) at 37 °C, 150 rpm for 24 and 48 hours for yeast and bacteria respectively.

For the co-culture, at the end of the incubation period, cells were centrifuged aseptically (1800 g, 4°C and 5 min) in a centrifugation (Thermo-Fisher, scientific, HERAEUS Megafuge 16R) suspended in 10 mL KCl (150 mM). For the inoculum preparation, 2 mL of suspended culture cells were transferred into 250 mL Anaerobic bottles containing 200 mL of culture medium. Depending on the experimental set-up, the populations were mixed at a different % v/v ratio of bacterial-yeast populations respectively (0.25;0.75), (0.5;0.75), (0.75;0.25) with equal starting optical density and co-cultures could potentially be carried out in 200 mL volume range at 37° C and 150 rpm for 48 h and population densities was measured at 660 nm. Cells from co-culture were centrifuged and the suspension obtained in 10 mL of KCl 150 mM was used for inoculation.

2. Fermentation media

Fermentation media contained carbon and nutrient source (per liter): 32 g glucose, 32 g fructose and 0.53 g NH₄Cl, and minor components including inorganic salts, buffers and cofactors (per liter): 3 g K₂HPO₄, 3 g KH₂PO₄, 1.25 g NaCl, 0.3 g MgCl₂·6H₂O, 0.3 g CaCl₂·2H₂O, NaH₂PO₄, Na₂HPO₄. Fermentation media were sterilized by filtration on 0.22 µm sterile membrane filter (Sartorius), when setting up the fermenters.

3. Fermentation experiments

Fermentations were executed under optimized conditions at 37°C in 200 mL of synthetic media in anaerobic conditions, by inoculating different biomass concentration of *A. succinogenes* and *S. cerevisiae*. The inoculations were performed in the following way: 1) Co-culture (inoculation of *A. succinogenes* with *S. cerevisiae* simultaneously); 2) Sequential inoculation of *S. cerevisiae* pursued by inoculation of *A. succinogenes* after 48 h were run in a benchtop reactor 3L capacity with a volume of 1800 mL. *A. succinogenes* and *S. cerevisiae* pure cultures fermentations were also run as controls. Both strains were pre-cultured as mentioned previously, and the inoculated volumes were calculated based on of the final volume present in anaerobic bottles. Fermentation temperature of 37°C and stirring speed of 150 rpm were maintained throughout the incubation

period. During the fermentations, samples were aseptically withdrawn (5 mL) from the broth at various time points 24, 48, 72 and 94 in order to follow microbial growth, sugar consumption, and the production or uptake of acids (e.g., succinic, formic, acetic, and lactic acid), and other compounds (e.g., ethanol).

4. Analyses

Cell growth was detected by measuring the optical density (OD) of the sample at 660nm (λ_{660}) using a UV-VIS spectrophotometer (PRIM SECOMAM, Milton Roy, Ivyland, PA, USA). OD measurements were carried out immediately after sampling by using 4 ml plastic cuvettes and deionized water was used as blank (zero absorbance). Sample was homogenized and then analyzed. Samples were collected from the broth every 24 h and centrifuged at 1800 g for 5 min. The supernatants were then transferred to 15 mL tubes and were analyzed according to previously published methods [23]. Briefly, sugars (glucose, and fructose), organic acids (succinic, acetic acid,) and others were detected by HPLC using BioRad HPX-87H column at 45 °C with 0.01 N H₂SO₄ mobile phases flowing at 0.7 mL/min.

5. Statistical analyses

The analysis of data was carried out by ESP script (EXCEL software program 2016) using T-test ($p < 0.05$) and were reported as mean and standard deviation.

III. Results

1. Comparative analysis of production of pure culture of *A. succinogenes* and *S. cerevisiae* in 250 mL batch bottles

In this experiment, two cultures were inoculated as pure cultures in separate batch bottles. The readings were taken for two days. Figure 1a and b show the potential and current comparison between the *A. succinogenes* and *S. cerevisiae* pure cultures. During fermentation, *A. succinogenes* produced SA under anaerobic conditions and no other acids were produced, in particular lactic acid. This result shows the capacity of *A. succinogenes* to ferment glucose and fructose; which are used as carbon source for synthesized and produce succinic acid. [24] report that *A. succinogenes* prefers to consume glucose, but it is also able to metabolize glycerol (by-products of ethanol fermentation process naturally produced by *S. cerevisiae*). Fermentation performance differs for each and every microbiome. Likewise, *S. cerevisiae* produce glycerol, ethanol and CO₂ in the fermentation media. About 0.2 mol L⁻¹ of SA was produced using *A. succinogenes* after 48 h, which corresponding to a yield of 0.6 mol-C SA/mol-C substrate and 0.49 g L⁻¹ h⁻¹ productivity, respectively (Fig 1a). However, when *S. cerevisiae* was cultivated, ethanol and glycerol were the metabolites produced by this microorganism recording a value of about 0.07 mol L⁻¹ for each metabolite (Fig 1b). Notable characteristics of *S. cerevisiae* include their ability to grow and undergo metabolic processes in both aerobic and anaerobic. Under aerobic conditions, *S. cerevisiae* performs cellular respiration through oxidative phosphorylation of pyruvate, producing CO₂. Under anaerobic conditions, *S. cerevisiae* will conduct fermentative metabolism to ethanol and carbon dioxide (as the primary fermentation metabolites) as the cells strive to make energy and regenerate the coenzyme NAD⁺ [25]. Henceforth, *A. succinogenes* and *S. cerevisiae* were chosen for the fermentation study as co-culture.

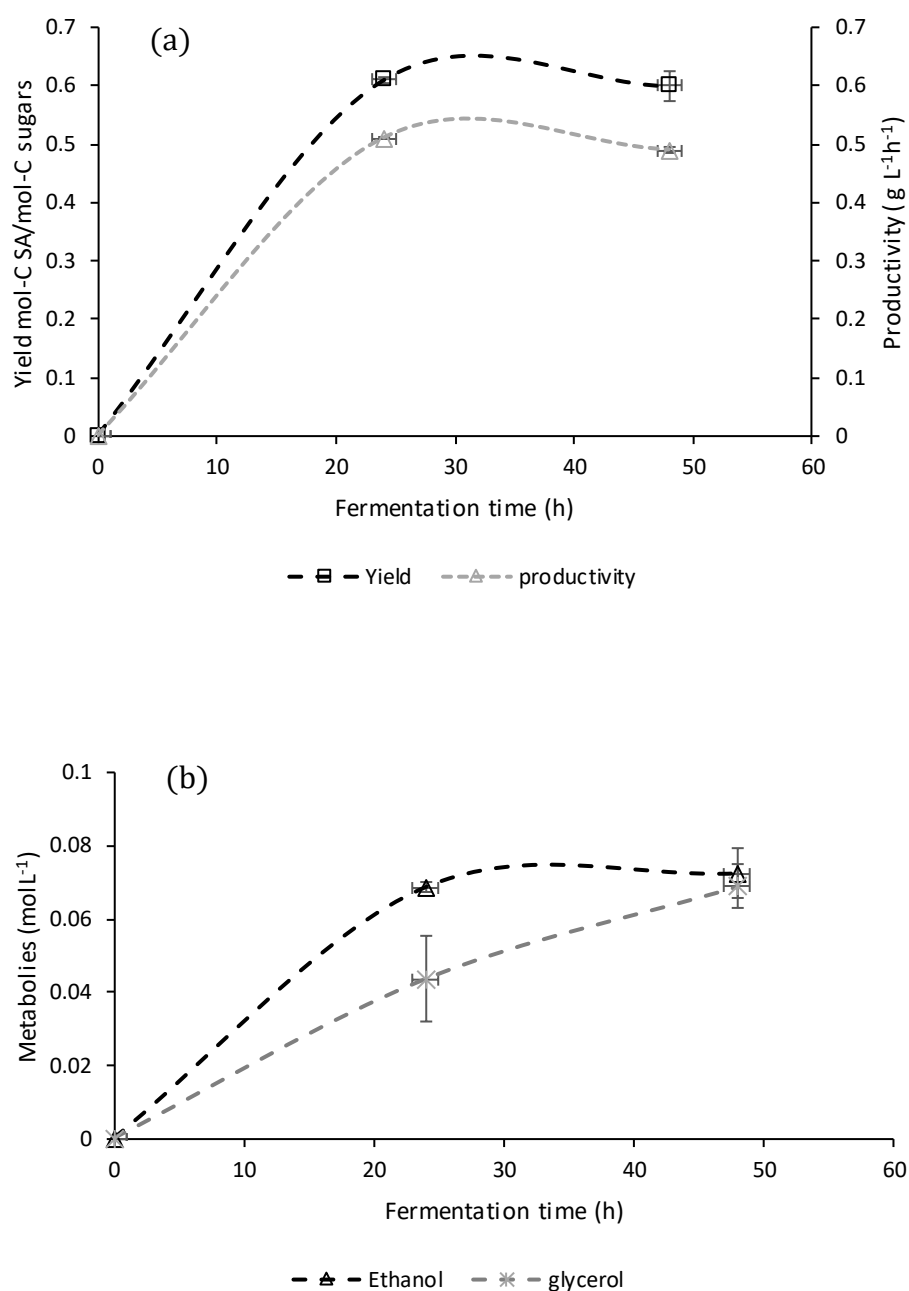
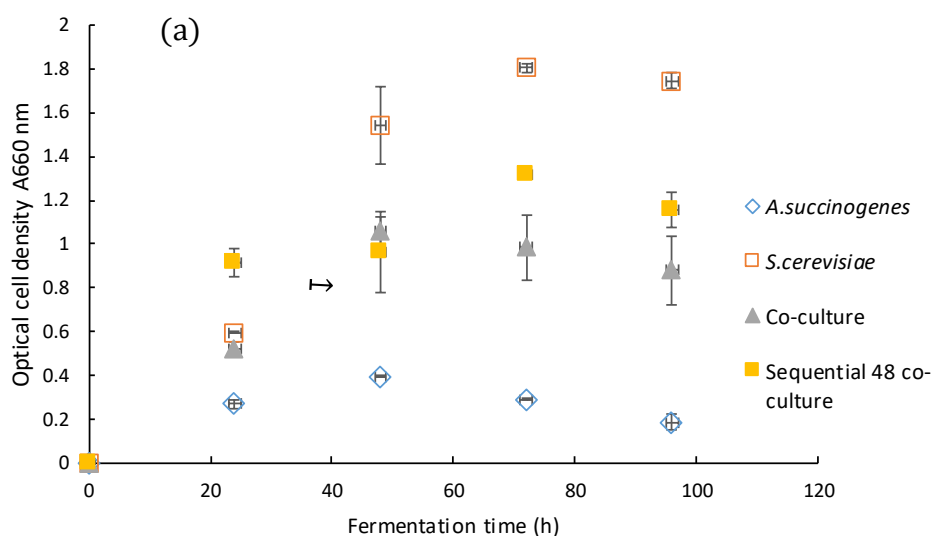


Figure 1. Pure culture fermentations parameters of *A. succinogenes* (a) and *S. cerevisiae* (b).

2. Effect of the inoculation mode on microbial growth during fermentation in 250 mL bottles using a % ratio of (0.5 *A. succinogenes*; 0.5 *S. cerevisiae*)

Fig 2 reveals the microbial evolution (OD_{660nm}) of pure cultures and co-cultures with a % volume ratio of (0.5; 0.5) of *A. succinogenes* and *S. cerevisiae* in microaerobic and anaerobic conditions (Fig 2 a- and b-, respectively). A significant difference was found in cell density

between the co-cultures and the *A. succinogenes* single culture ($p < 0.05$) during the fermentation. The results indicate that the growth of *A. succinogenes* was not limited by *S. cerevisiae* regardless of the combination. On the contrary, *A. succinogenes* decreased the growth but did not cause the death of *S. cerevisiae*. The microaerobic co-cultures showed a slight decrease in their evolution during fermentation in comparison with *S. cerevisiae* pure culture control. The difference between co-culture and the *S. cerevisiae* single culture evolution was not obvious, however, cell density in *S. cerevisiae* was more than that obtained by the co-culture (Fig 2-a). During anaerobic fermentation, when both strains were inoculated together simultaneously (Fig. 2 b), the co-cultures were persisting for the first 3 days with an elevated OD than that obtained from the monoculture of *A. succinogenes*, and then reduced gradually. *Sequential cultures* attained and retained optical cell densities more important to that achieved in the pure culture and in anaerobic co-cultures at the end of the fermentation time. When *A. succinogenes* was supplied sequentially after 48 h, the *S. cerevisiae* as starter was rather the dominant species throughout the fermentation process than *A. succinogenes*.



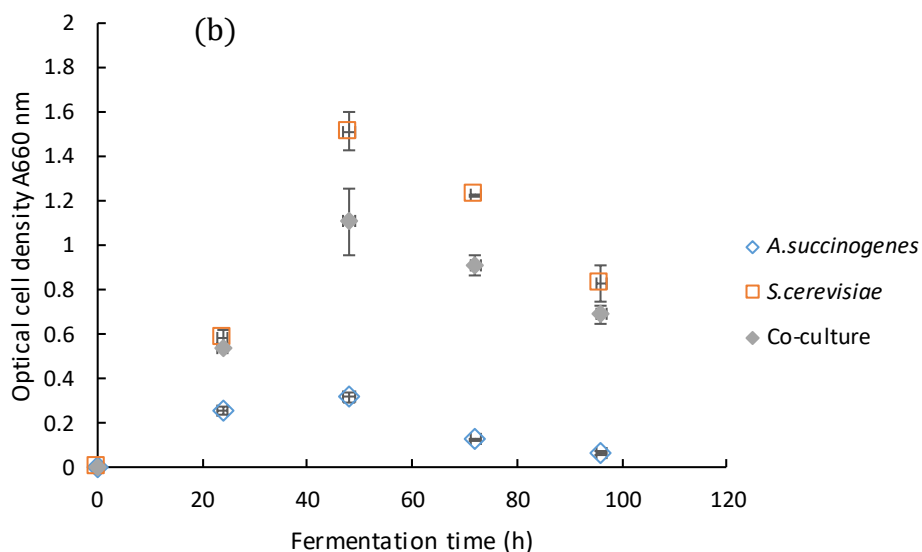
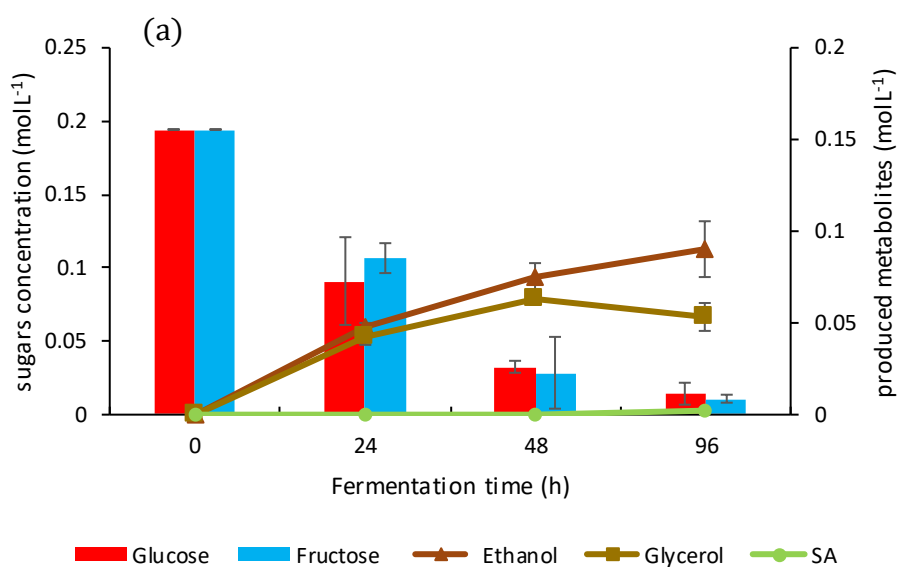


Figure 2. Optical density evolution during fermentations with different modes of inoculation in microaerobic (a) and anaerobic conditions (b) in the presence of $0.2 \text{ mol L}^{-1} \text{ MgCO}_3$ except for the sequential culture. Pure culture fermentations of *A. succinogenes* (\diamond) and *S. cerevisiae* (\square). Co-cultures fermentations with a % ratio of (0.5;0.5) of *A. succinogenes* and *S. cerevisiae* respectively. (\diamond), and co-culture of *S. cerevisiae* with *A. succinogenes* addition after 48 h (\blacklozenge). The arrow (\rightarrow) represent the inoculation of *A. succinogenes* in the sequential culture at 37°C .

3. Effect of MgCO_3 on simultaneous co-culture fermentation

To evaluate the effect of MgCO_3 in the fermentation, a simultaneous co-culture was inoculated with a % ratio of (0.5; 0.5) of *A. succinogenes* and *S. cerevisiae*. Fig 3 a- and b represent the sugars and the products profile evolution with or without the addition of MgCO_3 .



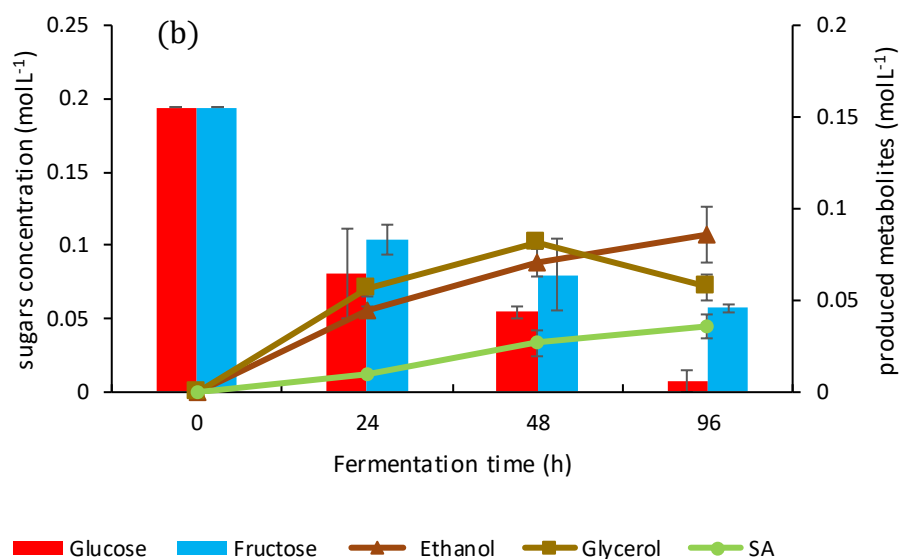


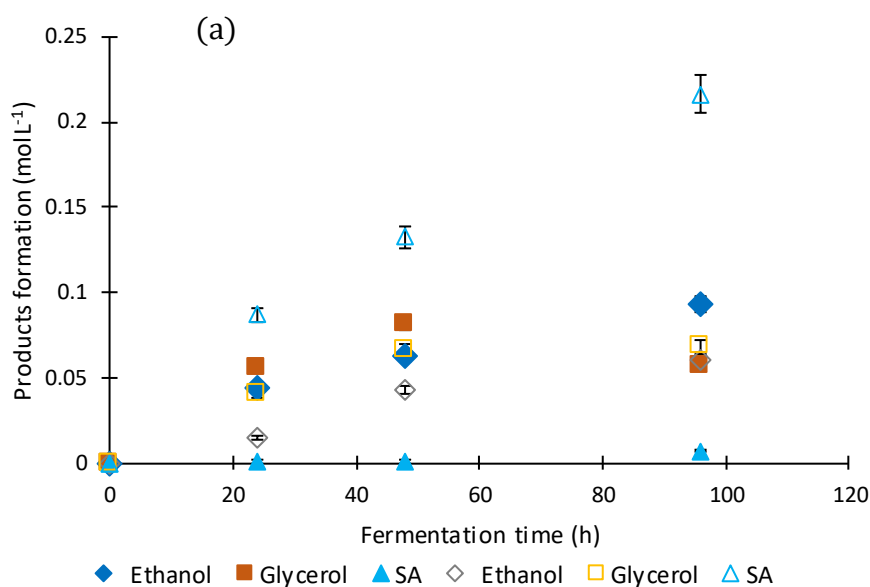
Figure 3. Fermentation kinetics (as sugars consumption and products formation) showing co- cultures fermentation ratio in microaerobic conditions in the absence (a) or in the presence (b) of 0.2 mol L⁻¹ of MgCO₃ during fermentation at 37°C for a % volume ratio of (0.5; 0.5), of *A. succinogenes* and *S. cerevisiae* respectively.

As shown in Fig. 3, sugar utilisation was not significantly different in (a) and (b). The glucose and fructose levels decreased significantly in microaerobic co-culture fermentation until more than 91% consumed. These data confirmed that the co-culture system can consume both glucose and fructose, whereas about 0.1 mol L⁻¹ and 0.06 mol L⁻¹ ethanol and glycerol were produced respectively, and the produced amounts were nearly similar irrespective of the presence of MgCO₃ supplement. SA production was monitored and found to remain negligible throughout culture in the absence of MgCO₃ (Fig 3-a); while 0.04 mol L⁻¹ SA was produced in the presence of MgCO₃ (Fig 3-b), corresponding to a yield of 0.1 mol-C SA/ mol-C substrates and a productivity of 0.05 g L⁻¹h⁻¹ confirming the need for a mineral carbon source. The evolution of CO₂ depends on the extent of sugars being metabolized by *S. cerevisiae* and utilised by *A. succinogenes*. The divergence in SA concentration observed between its two values could be attributed to the release of CO₂ by *S. cerevisiae*, indicating the stimulation of CO₂ production and yeast metabolism during the stage of fermentation. Therefore, the assimilation of the inorganic carbon source showed the potential of the two strains for use as co-culture in microaerobic fermentation to increase the persistence of CO₂ and to improve SA production process quality (supplementary table S1). The CO₂ production in the co-cultures correlates with the transcriptional up-regulation of the glucose fermentation pathway genes from *S. cerevisiae* [26] and the consumption rates correlate to the fixation of CO₂ to produce SA from *A.*

succinogenes [27]. The stimulatory effects that occur during fermentation correspond to the direct response of *S. cerevisiae* and *A. succinogenes* to the presence of each other.

4. Influence of the ratio of *S. cerevisiae*- *A. succinogenes* on the performance of simultaneous microaerobic and anaerobic co-culture during fermentation.

The variation of biomass ratio in the culture medium may affect significantly the values of fermentation parameters calculated from the measured concentrations of the substrates and/or products. As shown in the above results, SA could be produced by co-culture of *S. cerevisiae* and *A. succinogenes*. To evaluate the co-culture fermentation on the SA production, co-culture of *S. cerevisiae* and *A. succinogenes* were provided to the media at different % v/v ratios (0.25; 0.75; 0.75; 0.25) and compared to their mono-cultures. Fig. 4 reveals the differences between the concentrations of metabolites in the fermentation with a different biomass ratio in the co-culture without MgCO_3 . To investigate the impact of biomass ratio on the performance of fermentation, the metabolites in the culture were compared in microaerobic and anaerobic conditions. For both inoculation ratio, SA production from co-cultures fermentations was only observed in anaerobic conditions and was significantly reduced in microaerobic conditions (see fig 4 a, and b).



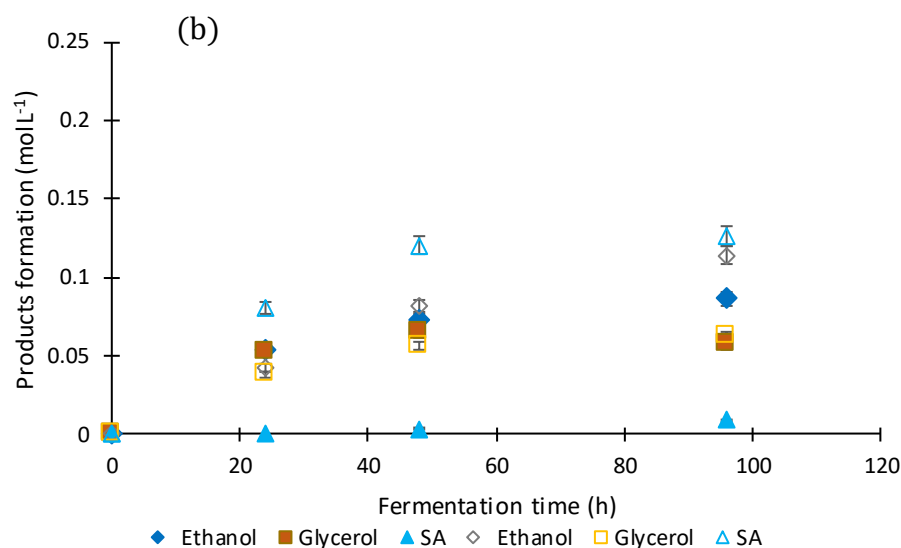


Figure 4. Product time-courses during co-cultures in microaerobic and anaerobic conditions without MgCO_3 during fermentation at 37°C ; a- for a % volume ratio of (0.25; 0.75), and b- for a % ratio of (0.75; 0.25) of *S. cerevisiae* and *A. succinogenes* respectively. The filled character represents the products obtained from fermentation in microaerobic conditions (-♦-) ethanol; (-■-) glycerol (-▲-) SA and the empty character represent the products obtained from anaerobic conditions (-◇-) ethanol (-□-) glycerol (-△-).

The maximum value of SA was obtained in anaerobic conditions from a co-culture % ratio of (0.25; 0.75) of *S. cerevisiae*- *A. succinogenes*, 0.23 mol L^{-1} (Fig 4-a); while only 0.03 mol L^{-1} SA was produced in microaerobic conditions regardless the biomass ratio corresponding to $0.28 \text{ g L}^{-1} \text{ h}^{-1}$ and $0.037 \text{ g L}^{-1} \text{ h}^{-1}$, respectively of SA productivity, wherein the fermentation activity of the *A. succinogenes* strain was limited. Regarding the ethanol formation, there was a substantial reduction in ethanol concentration earned from all the co-culture at the end of fermentation time (96 h) compared to that obtained from a co-culture % ratio of (0.75; 0.25) of *S. cerevisiae*- *A. succinogenes*. The variation of the ratio of co-culture shows that the total production of (0.75; 0.25) of *S. cerevisiae*- *A. succinogenes* was obviously slower than obtained (0.25; 0.75) of *S. cerevisiae*- *A. succinogenes*. However, results show that the highest value of SA was obtained in anaerobic mono-culture of *A. succinogenes*. Biomass concentrations deduced from Dry cell weight (DCW) decreased at the end of fermentation (96 h) for all co-cultures and recorded a maximum value of 0.40 g L^{-1} , 0.49 g L^{-1} for figure 4-a, and -b, respectively, as a result of the increased product formation and substrate limitations which provoked a drastic decrease of the microbial biomass.

5. Influence of the inoculation mode

To investigate the effect of inoculation mode on co-culture fermentation, the two strains were inoculated simultaneously and/or in different orders (table 1) with a constant culture time for all experiments. In microaerobic co-cultures, significant amounts of SA were produced; while no production was observed in pure culture of each strain. In addition, the comparison of the simultaneous and the sequential co-cultures showed a slightly higher amount in the case of the sequential co-culture.

Table 1. Influence of the mode of inoculation on the fermentation profile at the end of fermentation time (96h). Data are duplicates from two independent experiments represented as a mean \pm standard deviation.

Fermentation parameters		Microaerobic				Anaerobic	
Inoculation modality	t_0	Pure culture AS	Pure culture SC	Simultaneous Co-culture	Sequential 48 co-culture	Pure culture AS	Simultaneous Co-culture
OD	≈ 0	0.39 ± 0.002	1.54 ± 0.17	1.05 ± 0.06	1.32 ± 0.01	0.36 ± 0.04	0.96 ± 0.04
pH	6.8 ± 0.02	6.15 ± 0.08	5.04 ± 0.07	5.65 ± 0.15	5.24 ± 0.08	5.2 ± 0.02	5.32 ± 0.02
Substrate (mol L⁻¹)							
Glucose	0.2 ± 0.005	0.11 ± 0.005	0.01 ± 0.0001	0.04 ± 0.01	0.06 ± 0.009	0.02 ± 0.003	0.02 ± 0.001
Fructose	0.2 ± 0.002	0.12 ± 0.00016	0.014 ± 0.0006	0.02 ± 0.003	0.10 ± 0.001	0.03 ± 0.005	0.02 ± 0.002
Products (mol L⁻¹)							
SA	n.d	-	-	0.09 ± 0.007	0.12 ± 0.0006	0.3 ± 0.01	0.17 ± 0.003
Ethanol	n.d	-	0.147 ± 0.005	0.07 ± 0.003	0.071 ± 0.0007	-	0.06 ± 0.01
Glycerol	n.d	-	0.025 ± 0.0005	0.02 ± 0.002	0.013 ± 0.007	-	0.04 ± 0.001
Productivity (SA g L⁻¹ h⁻¹)							
YSA	-	-	-	0.11 ± 0.008	0.14 ± 0.0008	0.3 ± 0.005	0.20 ± 0.004
Y ethanol	-	-	0.51 ± 0.003	0.41 ± 0.006	0.34 ± 0.002	0.84 ± 0.01	0.44 ± 0.003
Y glycerol	-	-	0.06 ± 0.002	0.08 ± 0.002	0.02 ± 0.003	-	0.13 ± 0.01

AS: *A. succinogenes*, SC: *S. cerevisiae*, n.d. none detected; Y: yield mol-C products/ mol-C sugars

As mentioned above, SA was produced by *A. succinogenes* in anaerobic fermentation while ethanol and glycerol were produced by *S. cerevisiae*. In all the experiments, the co-culture of

A. succinogenes and *S. cerevisiae* produced more metabolites than in pure cultures. The SA obtained from the sequential 48 h cultures revealed a remaining in sugar content, while it turns to fall in the co-culture fermentations profile that were analogous in order of metabolites to that obtained from the fermentation of *S. cerevisiae* pure cultures. Data showed significant production of SA in all co-cultures, with a decrease of pH recording a value of 5.56, 5.32 for microaerobic and anaerobic co-culture and 5.24 for sequential co-culture. SA produced from co-cultures in microaerobic and anaerobic conditions was significantly lower when compared with the anaerobic control *A. succinogenes*, while the glycerol amount produced, and rose in the sequential fermentations, wherein the fermentation performance of *S. cerevisiae* strain was important (table 1). Considering the organic acid production, an increase in SA levels was seen in the sequential culture at 48 h when compared to the *co-culture*, while that from the pure fermentation of *A. succinogenes* in *anaerobic* culture was greater when compared to those obtained by the simultaneous and the sequential 48 h aerobic co-culture.

For the sugars contents present in the fermentations medium, data displays a high consumption of the glucose and fructose; accounting by 85%, 90% of both microaerobic and anaerobic simultaneous co-culture and by 60% for sequential 48 h co-culture fermentations with respect to the control of *A. succinogenes*. On the other side, the microaerobic co-cultures lead to a decrease in pH, with succinic acid production, which was significantly detected, even if it remained significantly below the control level from *A. succinogenes* anaerobic fermentation, a behavior reflected that the co-culture of *A. succinogenes* with *S. cerevisiae* generally showed the ability to produce SA, even in microaerobic conditions.

Sequential 48 culture led to $0.14 \text{ g L}^{-1} \text{ h}^{-1}$ SA production rate in lab-scale (1.8 L), reaching of about 85% substrate conversion efficiency, recording an overall yield of 0.69 mol-C of added chemical produced/ mol-C sugars consumed, with 54% of carbon content belong to succinic acid. The results emphasize the efficiency of the co-culture for the biological conversion of sugars to SA and might work in parallel to other succinic acid production processes such as the chemical process, or anaerobic fermentation. Even though the system differs from earlier studies, the results are consistent with those obtained by [7, 24, 28] in terms of SA concentration, yield and productivity. By way of summary, this provides a process for the batchwise fermentation of succinic acid, wherein a carbon dioxide from *S. cerevisiae* is maintained in a fermentation vessel then withdrawing the broth from the vessel and re-filled the vessel with newly substrate, and prepare it for the reception of a subsequent batch of *A. succinogenes* to produce SA by fermenting the sugars.

6. Effect of the fermentation temperature

Fig. 5 (a- and b) present the evolution of the concentration of sugar during the fermentations in *S. cerevisiae* and *A. succinogenes* controls cultures and in the co-cultures at the different optimal fermentation temperatures of 28 °C and 37 °C, respectively. At both temperatures, the co-culture fermentations evolved more gradually than in the pure strain culture fermentations expect for the *S. cerevisiae* at 28°C. The biomass progression in terms of the OD, pH, residual sugars concentrations and product concentrations in the pure culture and the co-cultures fermentations are given in table 2 at 28 and 37°C. In the pure culture, *S. cerevisiae* achieved its highest optical density value in 16 hours (around 1.2), which remained almost constant till the end of fermentation (4 days), and the remaining sugar was below 0.02 mol L⁻¹. Once the two microbial strains were co-inoculated (*A. succinogenes* and *S. cerevisiae*), the *A. succinogenes* cell concentration was less than that of *S. cerevisiae*, and reached a value of 0.38 and 0.36 at 28°C and 37°C respectively, when the fermentation almost over (after 96 h). Thus, at these fermentation conditions (28 °C), *A. succinogenes* has shown an inhibitory activity in the production of SA even in anaerobic conditions (0.07 mol L⁻¹ SA) when compared to the amounts obtained at 37°C. The compositional profiles of parameters collected in pure and co-culture fermentations at 28°C and 37°C are listed in Table 2. At 37°C and contrarily to 28°C, the co-cultured *A. succinogenes* and *S. cerevisiae* showed a decrease in the amount of ethanol and remaining sugar, when compared to the control of *S. cerevisiae* alone. With respect to *S. cerevisiae* controls, the co-culture mode indicated a notable increase in the ethanol production at 28°C, whereas a reduction in ethanol was seen at 37°C. Regarding the total production activity, the levels significantly increased from the co-culture at both 28°C and 37 °C, in comparison to their relevant *S. cerevisiae* controls at 37 °C. This increase in ethanol at 28°C (0.08 mol L⁻¹) was significantly higher than at 37°C (0.05 mol L⁻¹). There was a substantial increase of glycerol formed, when the co-cultures were run at 28°C (0.06 mol L⁻¹) with regard to the control. The coupling of *A. succinogenes* –*S. cerevisiae* in microaerobic co-culture at 37 °C, have a positive impact on the sugar consumption, resulting in SA formation with levels of 0.22 mol L⁻¹, with respect to SA levels obtained from *A. succinogenes* pure cultures (0.3 mol L⁻¹). This suggests that SA production in microaerobic co-culture is primarily impressed by *S. cerevisiae* coupling and by the different temperature conditions.

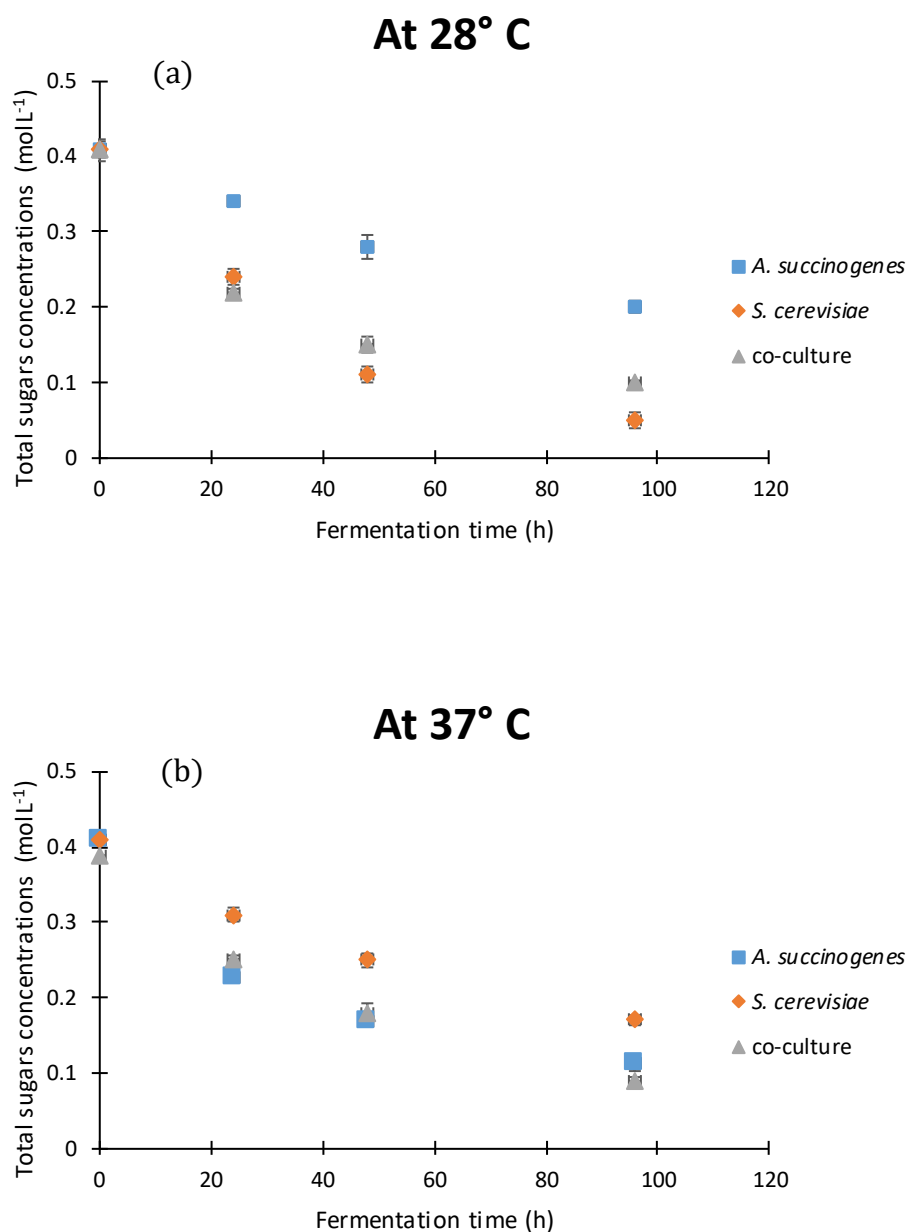


Figure 5. Fermentation parameters in term of sugars consumption during pure cultures (a) and co- cultures (b) at two different temperatures, 28°C and 37°C with a biomass % volume ratio of (0.5; 0.5) for *A. Succinogenes* - *S. cerevisiae* respectively. (-■-) for *A. succinogenes*, (-◆-) for *S. cerevisiae*, and (-▲-) for co- cultures.

Considering the sequential 48 h fermentations (Fig 6-a), there was no production of SA was observed before bacterial inoculation and the temperature shift at 37 °C; the final SA value was 0.27 mol L⁻¹ corresponding to 0.33 g L⁻¹ h⁻¹ productivity. Moreover, there was a general decrease in the profile of volatile compounds production with an increase of succinic acid; when the fermentations were performed at 37°C, with was a smaller variation for *S. cerevisiae* and *A. succinogenes* monocultures. Meanwhile, as compared to the respective controls along with the

highest SA production, there was a significant glucose consumption with a substantial decrease of glycerol might be attributed to the utilisation of the yeast/bacteria as carbon source which is described by many papers [23, 29, 30]. The percentage of Dissolved Oxygen (DO) in the medium Fig 6-b was found to have a considerable influence upon the OD value under conditions of rapid fermentation at 28° C. At T0, the DO content in the substrate was 100%, the ethanol concentration reached about 0.05 mol L⁻¹ in the substrates containing 43% DO after 48 h, and the glycerol concentration reached about 0.04 mol L⁻¹ after 48 h, then decrease to about 0.005 mol L⁻¹ after 120 h in the media containing 43% and 24 % DO in the fermentation in which the DO was maintained at about 24%, recording a maximum value of SA of 0.27 mol L⁻¹ and a pH value of 4.6 after 120h.

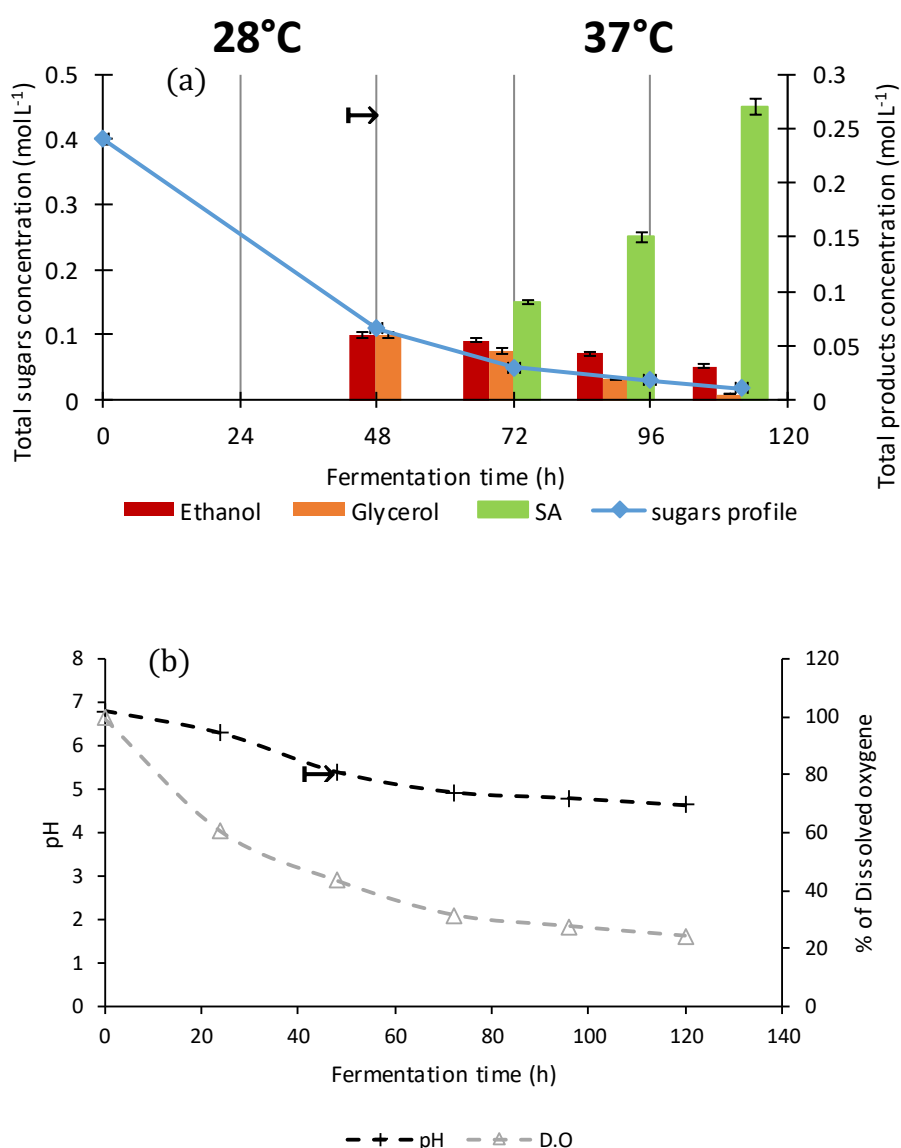


Figure 6. Sequential 48 h cultures sugars consumption and products formations (a) without MgCO_3 with pH and dissolved oxygen variation (b). The arrow (\rightarrow) represent the inoculation of *A. succinogenes* with the temperature shift from 28 °C to 37 °C.

Table 2. Final parameter values (96 h culture time) during single cultures and co-culture of *A. succinogenes* and *S. cerevisiae* at two different temperatures. Data are duplicates from two independent experiments represented as a mean \pm standard deviation.

T° C	28				37			
Inoculation Modality	Pure culture AS	Pure culture SC	Co-culture	Sequential 48 h co-culture	Pure culture AS	Pure culture SC	Co-culture	Sequential 48 h co-culture
OD	0.09 ± 0.002	1.2 \pm 0.2	0.38 ± 0.02	0.47 ± 0.03	0.13 ± 0.007	0.32 \pm 0.05	0.36 ± 0.04	0.42 \pm 0.03
pH	5.9 ± 0.2	4.3 \pm 0.4	5.2 ± 0.05	5.4 ± 0.02	5.7 ± 0.04	4.7 \pm 0.02	5.5 ± 0.09	4.8 \pm 0.4
Substrate (mole L⁻¹)								
Glucose	0.08 ± 0.005	0.02 ± 0.007	0.06 ± 0.004	0.08 ± 0.001	0.05 ± 0.004	0.07 ± 0.02	0.04 ± 0.009	0.006 ± 0.0001
Fructose	0.13 ± 0.003	0.03 ± 0.005	0.07 ± 0.006	0.13 ± 0.0002	0.06 ± 0.007	0.1 ± 0.001	0.06 ± 0.004	0.008 ± 0.0009
Product (mole L⁻¹)								
Ethanol	n.d	0.1 ± 0.01	0.08 ± 0.006	0.06 ± 0.004	n.d	0.04 ± 0.007	0.05 ± 0.01	0.06 ± 0.003
SA	0.07 ± 0.01	n.d	0.09 ± 0.003	n.d	0.3 ± 0.02	n.d	0.22 ± 0.01	0.27 ± 0.007
Glycerol	n.d	0.03 ± 0.002	0.06 ± 0.007	0.06 ± 0.001	n.d	0.02 ± 0.009	0.03 ± 0.005	0.005 ± 0.0008

AS: *A. Succinogenes*, SC: *S. cerevisiae*, n.d. non detected, pure culture in this experiment were performed in anaerobic fermentation.

IV. Discussion

Co-culture strategy is known longtime ago, however, for SA production; the concepts of co-cultures strategy are new. To our knowledge, co-culture of *A. succinogenes* and *S. cerevisiae* was not previously investigated. Here, in the present work, the co-culture was proposed as an alternative inoculation strategy to the traditional fermentation culture to produce succinic acid. It was previously checked that co-cultures of yeasts/bacteria pairs carried out in 250 mL bottles gave similar results as those obtained during pure cultures. Tremendous number of data have been published regarding the function relayed on bacteria or yeasts to produce chemicals alone, yet, reports on “if” or “how” they interact mutually together, start to be in the scope of interest recently. In both described co-cultures, *A. succinogenes* strain was able to grow on in the presence of *S. cerevisiae*, using glucose and fructose as substrate. As observed, the co-culture had only a little effect on the optical cell density compared to the pure cultures; however, *S. cerevisiae* led to a higher cell density than *A. succinogenes* over the whole culture (Fig 2). This is might be due to the fact that *S. cerevisiae* displayed a higher inoculum size which can

favourably impact the optical density [31]. The simultaneous co-occurrence of both strains had a smaller impact than the pure cultures in the fermentation systems. Thus, next to providing the co-substrate for the partner strain, the properties of *S. cerevisiae* strain make co-cultivation possible [32]

The study was realised in a synthetic medium to obtain a reproducible medium with a controlled and non-limiting composition. In microaerobic conditions, the advantage of the co-culture strategy over the monoculture could be due to the presence of the yeast from the beginning in a medium lacking MgCO_3 unlike the fermented medium used for the monocultures (Fig. 3 and Table 1). Some of the yeast inhibitory metabolites produced by *S. cerevisiae* strain, such as ethanol, were measured at the end of the co-culture (Fig 3, 4 and Table 1). These inhibitory compounds are known to limit bacterial growth and could reduce the ability of bacteria to synthesize SA at different levels depending on their concentrations and the medium composition and pH during co-culture [16, 33–35].

Even though these metabolites gradually occurred during the sequential culture, which lasted for 144 h of the co-culture, the bacteria had the time to grow and especially to produce SA (Fig. 5 and Table 2). The SA amounts were higher than obtained in simultaneous co-culture, which might be referred to the accumulation of CO_2 in the reactor which stimulates the production of succinic acid when applied directly the bacteria, in addition to the reactor form and volume utilised in a sequential culture which is different with that used in simultaneous co-culture.

It is generally recognised that glucose is consumed rapidly by both of *S. cerevisiae* and *A. succinogenes* [8, 36]. Accordingly, in sequential 48h fermentations with *S. cerevisiae* as a starter, it was observed that the residual fructose concentration was substantially greater, with higher discrepancy between glucose and fructose concentrations (Fig 5). From the collected data, we deduced that lower fructose was utilized during the sequential culture if compared to the fructose consumption in pure *S. cerevisiae* and simultaneous co-culture. As observed, the consumption of both sugars was less than those obtained during the sequential culture.

In our study, considering the simultaneous microaerobic co-culture, besides ethanol and glycerol production, a stimulatory effect of *S. cerevisiae* on *A. succinogenes* to produce SA was observed, as drawn from the comparison to *A. succinogenes* monoculture. Based on the physiological analysis, it was noticed that co-cultivation resulted in significant SA production by *A. succinogenes* during the whole culture.

Interestingly, no major changes were found regarding biomass amounts when comparing mono- and co-culture conditions, suggesting no changes take place on the genetic level. The product shift appears to be caused by changes in the thermodynamics of the system resulting in more CO₂ production as described by [37], inducing favorable conditions for succinate production as a result of *A. succinogenes* metabolism. A similar interaction also seems to have taken place in the case of the sequential 48 h co-culture, facilitating substrate and CO₂ conversion by the *A. succinogenes*.

For the sequential co-culture fermentation from substrate to succinic acid, the main challenges were the requirements of the involved microorganisms in terms of reactor set-up, medium composition and product synthesis. Optimizing product yield and productivity for a certain process usually addresses the needs of the organism involved. Since sequential co-culture fermentation uses at least two different organisms, the key aspect for sequential co-culture fermentation is either a medium compromise for both organisms or the compatibility of the first (optimized) medium for the second organism in terms of product synthesis. Furthermore, the second organism has to be able to use the product of the first process as a carbon source. The combination of both aspects must be fulfilled to achieve an optimal value added chain from sugars to succinic acid.

However, co-culture technology can display some drawbacks, which should be taken into consideration, such as the need to build various microbial pools, the requirement to survey and control microbial population and the prospects to engineer a transport model of metabolites between two upstream methods in one system, which on high- scale present a roadblock.

V. Conclusions and perspectives

Overall, the co-culture approach appears interesting from both an applied and fundamental point of view. We could successfully show that the production of succinic acid from sequential co-culture is possible by a temperature shift. The advantage of this kind of biotechnological process is the extension of the product portfolio of anaerobic fermentation. The work at hand demonstrates that *A. succinogenes* is able to use glycerol as a substrate for succinic acid formation. Moreover, it shows that it is possible to link aerobic fermentation to succinic acid production using sequential co-cultures of *S. cerevisiae* and *A. succinogenes*. Currently, production rates obtained in our study were about $0.3 \text{ g L}^{-1} \text{ h}^{-1}$ from a sequential 48 co-culture, which is promising in view of the production of SA at large scale, but still have to be improved to become commercially interesting. Yet, based on the reported co-culture, as compared with other strategies traditionally used in SA production research, co-culture can be done and give similar results from microbial pure cultures (co-culture strategy give the same results from a pure culture without the use of mineral carbon source such as MgCO_3 and the nitrogen sparging. Additionally, this research shows how important microbial interactions and thermodynamic interactions in a microbial-community are, besides the syntrophic systems described in the literature. Evaluation studies of efficiency, environmental impact and cost assessment will be also very interesting. The field still has several major obstacles to traverse before stable scale-up studies can be a reality.

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Supplementary table 1

Inoculation Modality						
Microaerobic				Anaerobic		
MgCO ₃ availability (mol L ⁻¹)	Without MgCO ₃	0.1 MgCO ₃	0.2 MgCO ₃	Without MgCO ₃	0.1 MgCO ₃	0.2 MgCO ₃
Products (mol L⁻¹) of % ratio (0.25 <i>A. succinogenes</i> :0.75 <i>S. cerevisiae</i>)						
SA	0.01	0.018	0.023	0.11	0.14	0.19
Glycerol	0.05	0.064	0.03	0.06	0.05	0.05
Ethanol	0.08	0.055	0.07	0.11	0.09	0.07
Products (mol L⁻¹) of % ratio (0.5 <i>A. succinogenes</i> :0.5 <i>S. cerevisiae</i>)						
SA	0.001	0.02	0.03	0.15	0.17	0.22
Glycerol	0.05	0.04	0.06	0.05	0.03	0.04
Ethanol	0.09	0.08	0.1	0.08	0.07	0.07
Products (mol L⁻¹) of % ratio (0.75 <i>A. succinogenes</i> :0.25 <i>S. cerevisiae</i>)						
SA	0.009	0.027	0.035	0.21	0.24	0.27
Glycerol	0.05	0.034	0.03	0.07	0.04	0.03
Ethanol	0.07	0.067	0.07	0.05	0.03	0.03

General conclusion and future work

General conclusion

In this thesis, we designed a process for bio-based and bio-electro based production of succinic acid using glucose and fructose as a feedstock. The major contributions resulting from these studies are summarized below.

In the first, focused on succinic acid (SA) production within a biorefinery concept and what other commodities can potentially be produced was investigated. Various sugars in different concentration were utilized, followed by optimisation parameter in succinic acid fermentation. Batch studies using *Actinobacillus succinogenes* with initial glucose and fructose concentrations of 0.05 mol L⁻¹ to 0.2 mol L⁻¹ were carried out, achieving succinic acid yields of (0.9 to 0.4 mol-C/mol-C) for glucose and (0.67 to 0.89 mol-C/mol-C) for fructose respectively in anaerobic conditions. The SA productivities seen after 96 h for the batch fermentations were 0.22 g L⁻¹ h⁻¹ and 0.27 g L⁻¹ h⁻¹ for glucose and fructose, respectively. The MgCO₃ addition was observed to be an effective inorganic carbon source and was capable of buffering the medium pH in the range of 6.4 – 6.8. The feasibility of SA production by combining glucose and fructose in different ratio amounts was evaluated. The highest SA production in co-fermentation was 0.22 mol L⁻¹, equivalents to yield of (0.94 mol-C/mol-C and a productivity of 0.27 g L⁻¹ h⁻¹) obtained from an equal ratio of glucose and fructose.

The dissociated forms of different succinic acid concentrations were also evaluated. Results indicates that succinic acid have an inhibitory effect of its production by the bacterium starting from a concentration of 0.4 mol L⁻¹. Reduction and oxidation between fumarate and succinate create a loop into the TCA cycle. According to this, batch fermentations using glucose and fructose in addition to fumaric acid as carbon source were evaluated, leading to a SA concentration of 0.5 mol L⁻¹ with a yield and productivity of 0.88 mol-C/mol-C, 0.48 g L⁻¹ h⁻¹ respectively.

Succinate production by *A. succinogenes* on carbon sources, using pure sources of the most prevalent monosaccharides in apple juice such as glucose, fructose, xylose and galactose and under the same conditions was evaluated. The succinic acid yield on glucose (0.8 mol-C/mol-C) was higher than those obtained for the other tested carbon sources; they were 0.77, 0.74 and 0.72 mol-C SA/mol-C. These results revealed the possibility to transpose the model on synthetic medium on a model medium apple to produce succinic acid.

Anaerobic fermentations of glucose and fructose were performed by *Actinobacillus succinogenes* 130Z in batch mode using three different volume bioreactors (0.25, 1 and 3 L). Work on the bench scale reactor (3L) in controlled pH showed the best results when compared

to 0.25 L anaerobic bottles recording a value of (0.88 mol C-SA/mol-C-sugars and 0.5 g L⁻¹ h⁻¹) of succinic acid yields and productivity after 96 h of fermentation, respectively.

A novel electrochemical system based on the utilisation of electrode in the fermentation of *Actinobacillus succinogenes* 130Z was developed. The system was shown to be capable of producing succinic acid in less time consuming with a high productivity (1.01 g L⁻¹ h⁻¹).

We set out a co-culture system of *A. succinogenes* (DSM 22257) and *S. cerevisiae* (CLIB 95) to for the production of SA in simultaneous and in sequential process. Succinic acid production using co-culture fermentation system was demonstrated, recording a maximum value of 0.27 mol L⁻¹ and 0.3 g L⁻¹ h⁻¹ for SA concentration and productivity respectively from sequential 48 co-culture. Results showed that MgCO₃ and N₂ sparging could be substituted by applying a sequential co-culture. Based on this thesis, we can conclude that bio-based and electro-based route for producing succinic acid has a lot of potential for future growth as it offers many advantages in addition to being environmentally friendly.

Research implications and Future outlooks

Succinic acid fermentation performances should be improved by shifting from batch to fed-batch and continuous process. New design parameters, such as the dilution rate, should then be considered together with more advanced experimental equipment. Moreover, process scaling-up into pilot plant should be done. Important environmental parameters such as, CO₂ availability in the broth, the reactor's agitation and nutrient availability should be re-estimated to find the optimum performance. Furthermore, inoculum preparation and sterilization methods should be also reconsidered and carefully planned. New processes using waste as feedstock also need to be explored

Biological processes affected by low productivity could overcome these constraints, by placing electrodes in the fermentation medium which is the working principle of electro-fermentation (EF). The first thing to do is to check our preliminary results by remaking electrolysis under the same conditions. From an electrochemical point of view, it will be necessary to test other working potentials or even current intensities, to study the influence of the electrode material. Then we need to study more in depth the influence of the current on microorganisms. Synergistic interaction between microbe and electric current applied to the system should be studied and well understood. The insertion of electrodes into the fermentation medium influences the microbial dynamics of the system, and this adds new opportunities to observe specific electroactive bacteria and their interspecies interactions with new metabolic functions. Techno-economic analysis and life cycle assessment based sustainability studies can be used for comparative evaluation of electro-fermentation process with conventional processes.

An important part of research studies must transit towards using co-culture or polycultures strategy to produce platform chemicals. Our results should be re-verified and rechecked, and then the use of other microorganisms should be also considered. Therefore, looking to nature's design strategies to inform our construction principles for synthetic co-culture or consortia may be a promising strategy.

In addition, efforts toward the development of genetically modified organisms (*E. coli*, *A. succinogenes*, *ect*) should be done. Definitely, prior to perform any genetic manipulations, cell metabolism should be extensively studied by bioinformatics tools. Then, metabolic engineering can be applied to *A. succinogenes* metabolism considering the conversion of feedstocks into succinic acid and illuminating which intracellular reactions need to be suppressed and/or supported to enhance succinic acid production.

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List of Abbreviations

5K4DG: 5dehydro-4-deoxy-D-glucarate

5KG: 5-ketogluconate

A6P: Ascorbate-6-phosphate

AcCoA: Acetyl-CoA

AcP: acetyl-phosphate

Ald: Aldehyde

Ara: Arabinose

DHAP: dihydroxyacetone phosphate

E4P: Erythrose-4-phosphate

F1,6P: Fructose-1,6-biphosphate

F1P: Fructose-1-phosphshate

F6P: Fructose-6-phosphate

G1P: Glucose-1-phosphate

Gal: Galactose

Gal1P: Galactose-1-phosphate

Galte: Galactarate

Glc: Glucose

Glcte: Glucarate

Gly: Glycerol

Gt6P: Gluconate-6-phosphate

Gte: Gluconate

Ido:Idonate

KDPG: 2-keto-3deoxy-6-phosphogluconate

Lac: Lactose

Mal: Maltose

Man6P: Mannose-6-phosphate

MOH1P: Mannitol-1-phosphate

Pec: Pectin

R5P: Ribose-5-phosphate

Rib: Ribose

Ribu: Ribulose

Ru5P: Ribulose-5-phosphate
S6P: Sucrose-6-phosphate
S7P: Sedoheptulose-7phosphate
SOH6P: Sorbitol-6-phosphate
SucCoA: Succinyl-CoA
Xu5P: Xylulose-5phosphate
Xyl: Xylose
Xylu: Xylulose
 β G6P: β -Glucoside-6-phosphate.
SA: succinic acid
GA: Glutamic acid
ORP: oxydoreduction potential
US DOE: The United State Department of energy
NREL: National Renewable Energy Laboratory reports
CEN: European Committee Standardized
HMF: hydroxymethylfurfural
FDCA: 2,5-Furandicarboxylic acid
ATP: Adenosine triphosphate
NAD⁺: Nicotinamide adenine dinucleotide
(PEP) phosphoenolpyruvate
TCA: Tricarboxylic cycle acid
LDH: lactose-dehydrogenase
PFL: pyruvate formate lyase
CSL: corn steep liquor
OPPP: oxidative pentose phosphate pathway
OAA: oxaloacetate

Scientific Contribution

Results extracted from this thesis have been published or are about to be published in several scientific journals and have been presented in a number of Local and International Conferences.

List of Journal Publications

Scientific paper issued from this thesis

- Alaa Salma, Hayet Djelal, Rawa Abdallah, Florence Fourcade, Abdeltif Amrane. Platform molecule from sustainable raw materials; case study succinic acid. Brazilian Journal of Chemical engineering (Under revision).

Scientific paper in preparation

- Alaa Salma, Hayet Djelal, Rawa Abdallah, Florence Fourcade, Abdeltif Amrane. Improvement the production of succinic acid; a well-known platform molecule (manuscript in preparation).
- Alaa Salma, Hayet Djelal, Rawa Abdallah, Florence Fourcade, Abdeltif Amrane. A new approach of co-culture system to produce succinic acid (manuscript in preparation).
- Alaa Salma, Hayet Djelal, Rawa Abdallah, Florence Fourcade, Abdeltif Amrane. Applications of continuous culture for the bio-production of a platform molecule; succinic acid using *A. succinogenes*; approach to use agro-industrial by-products (manuscript in preparation).

Scientific paper out of the thesis

- Alaa Salma, Yahya Salma, Saida Salman, Rawa Abdallah. Biosorption of acid red 14 found in industrial effluents using *Aspergillus fumigatus*. Asian Jr of Microbiol, Biotech. Env. Sc. 20(1): 120-127 (Published in press 2018).

List of Conference Proceedings

Oral communications

- Application of fermentation strategy for the production of succinic acid; a well-known platform molecule

PhD Journey, on 01 December 2020 at UniLasalle-EME in Bruz-FRANCE.

- Platform molecule bio-production; case study succinic acid its worldwide applications Apivale conference on 19 and 20 November 2019 in Rennes-FRANCE.
- Bio-Production of succinic acid, using *A. succinogenes* and optimization efforts. UniLasalle-EME, on 22 October 2019 in Bruz-FRANCE.

- Platform molecule production by fermentation -Bio-based platform molecule. ENSCR on 10 December 2019 in Rennes-FRANCE.
- Succinic acid bio-production by fermentation of *A. succinogenes* and optimization efforts. Doctoral school 3M Day, on 27 June 2019 in Nantes-FRANCE.
- Production of Bio-succinic acid, using *A. succinogenes* and its worldwide applications. PhD Journey of ISCR, on 13 June 2019 in Rennes-FRANCE.
- Platform molecules: production and applications, UniLasalle-EME, on 20 November 2018 in Bruz-FRANCE.
- Production of Bio-Platform molecule via fermentation, Bio-based Platform molecule, UniLasalle-EME, on 16 January 2018 in Bruz-FRANCE.

Poster communications

- Application of fermentation strategy for the production of succinic acid; a well-known platform molecule
PhD Journey, on 01 December 2020 at UniLasalle-EME in Bruz-FRANCE.
- Platform molecules: production and applications, case study, succinic acid Doctoral school 3M on 19 February 2019 in Brest- FRANCE.
- Platform molecule bio-production; case study succinic acid its worldwide applications Apivale conference on 19 and 20 November 2019 in Rennes-FRANCE.
- Ethanol production from hydrolysate of *Ulva rigida* a green macroalgae Apivale conference on 19 and 20 November 2019 in Rennes-FRANCE.

Teaching Assignment

- Practical work (32h): Impact of water activity on *Saccharomyces cerevisiae* culture. I3 student at UniLasalle-EME (2020).

Scientific dissemination

- Training in Quality Control Center LABORATORIES (QCCLAB) at Chamber of commerce, Industry & Agriculture North LEBANON during 4 months (2018).

Titre : Application d'une stratégie de fermentation pour la production d'acide succinique en utilisant *Actinobacillus succinogenus*

Mots clés : Acide succinique ; Fermentation ; Culture pure ; Co-culture ; Electro-fermentation ; *Actinobacillus succinogenus*.

Résumé : La production d'acide succinique en culture pure, en co-culture et par électro-fermentation a été examinée dans cette étude; ce qui implique la sélection des substrats, des micro-organismes et du potentiel d'électrodes. Dans ce processus, le glucose et le fructose ont été choisis comme substrat organique, tandis que le $MgCO_3$ a été utilisé comme co-substrat fournissant une source de carbone inorganique lors de la fermentation anaérobie en utilisant *Actinobacillus succinogenus*. Les résultats ont montré que pendant la fermentation en discontinue (250 mL), la concentration d'acide succinique la plus élevée était obtenue pour des quantités égales de glucose et de fructose. La concentration en acide succinique obtenue était alors de $0,22 \text{ mol L}^{-1}$ pour un rendement de $0,94 \text{ mol-C/ mol-C}$ et une productivité de $0,27 \text{ g L}^{-1} \text{ h}^{-1}$ après 96 h. Les fermentations ultérieures effectuées avec de l'acide fumarique utilisé comme amplificateur ont confirmé l'efficacité de ce procédé avec une concentration globale d'acide succinique de $0,5 \text{ mol L}^{-1}$ et une productivité de $0,48 \text{ g L}^{-1} \text{ h}^{-1}$.

En fermenteur de paillasse (3L), après 96 h de fermentation, un rendement de $0,88 \text{ mol-C / mol-C}$ et une productivité en acide succinique de $0,5 \text{ g L}^{-1} \text{ h}^{-1}$ ont été obtenus. Les résultats préliminaires en électrofermentation ont montré que l'acide succinique était produit en moins de temps avec une productivité élevée de $1,01 \text{ g L}^{-1} \text{ h}^{-1}$ après 48 h. La dernière partie de ce travail a été consacrée au processus de co-culture des deux micro-organismes sélectionnés dans des bouteilles de 250 mL. Il était basé sur l'utilisation du dioxyde de carbone produit par la levure *Saccharomyces cerevisiae* par les bactéries productrices de succinate (*Actinobacillus succinogenes*). Les résultats montrent que $MgCO_3$ et le barbotage d'azote pourrait être substitués en appliquant une co-culture séquentielle conduisant à la production de $0,27 \text{ mol L}^{-1}$ d'acide succinique et une productivité de $0,3 \text{ g L}^{-1} \text{ h}^{-1}$. Ces travaux ont démontré la possibilité de produire de l'acide succinique en utilisant le glucose et le fructose comme matière première en culture pure, co-culture et électro-fermentation.

Title: Application of a fermentation strategy to produce succinic acid using *Actinobacillus succinogenes*

Keywords : Succinic acid ; Fermentation ; Pure culture ; Co-culture ; Electro-fermentation ; *Actinobacillus succinogenes*.

Abstract: This PhD work integrates the succinic acid production, in a pioneer study using pure culture, co-culture and electro-fermentation that involves selection of substrates, microorganisms and electrode potential. In this process, glucose and fructose were chosen as organic substrates, while $MgCO_3$ was used as co-substrate providing inorganic carbon source in anaerobic fermentations using *Actinobacillus succinogenes*. The results showed that during anaerobic batch fermentation (250 mL), the highest succinic acid concentration was obtained from an equal ratio of glucose and fructose recording a succinic acid value of 0.22 mol L^{-1} equivalent to a yield of $0.94 \text{ mol-C/ mol-C}$ and a productivity of $0.27 \text{ g L}^{-1} \text{ h}^{-1}$ after 96 h. The subsequent fermentations carried out with fumaric acid as enhancer, confirmed the efficiency of the process with global succinic acid production of 0.5 mol L^{-1} and a productivity of $0.48 \text{ g L}^{-1} \text{ h}^{-1}$.

A lab scale-up studies showed the best results in bench scale reactor (3 L) after 96 h of fermentation recording a value of 0.88 mol-C/mol-C and $0.5 \text{ g L}^{-1} \text{ h}^{-1}$ for succinic acid yield and productivity, respectively. Preliminary results of bioelectro-based process showed high succinic acid productivity, $1.01 \text{ g L}^{-1} \text{ h}^{-1}$, after only 48 h. The last part of this work was dedicated to the co-culture fermentation process of two microorganisms in 250 mL bottles. It was based on the utilization of carbon dioxide produced from *Saccharomyces cerevisiae* in the fermentation by the succinate producing-bacteria (*Actinobacillus succinogenes*). Results showed that $MgCO_3$ and N_2 sparging could be substituted by applying a sequential co-culture leading to 0.27 mol L^{-1} of succinic acid and a productivity of $0.3 \text{ g L}^{-1} \text{ h}^{-1}$. This work proved the production of succinic acid by glucose and fructose as feedstock in pure culture, in co-culture and electro-fermentation in batch mode.