IMMOBILIZATION OF *ACTINOBACILLUS SUCCINOGENES* BY USING ALGINATE FOR SUCCINIC ACID PRODUCTION FROM DAIRY WASTES

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ABSTRACT

IMMOBILIZATION OF *ACTINOBACILLUS SUCCINOGENES* BY USING ALGINATE FOR SUCCINIC ACID PRODUCTION FROM DAIRY WASTES

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Succinic acid is a highly versatile building block that is used in a wide range of industrial applications. The biological production of succinic acid has emerged in the last years as an efficient alternative to the chemical production based on fossil fuels. However, in order to fully replace the competing petro-based chemical process from which it has been produced so far, some challenges remain to be surpassed. In particular, one main obstacle would be to reduce its production costs, mostly associated to the use of refined sugars.

The present work is focused on the development of a sustainable and cost-effective microbial production process based on cheap and renewable resources, such as dairy wastes. Hence, cheese whey, which is a by-product of cheese making process that mostly unutilized and disposed as a waste in industry, were identified as promising feedstock and used as inexpensive carbon sources for the bioproduction of succinic acid. *Actinobacillus succinogenes*, one of the best natural succinic acid producing strains, has employed in the fermentative production of succinic acid

from cheese whey. Aiming at obtaining high succinic acid yield and production rate, *Actinobacillus succinogenes* cells were immobilized in alginate beads. The production of succinic acid from cheese whey, was studied both with immobilized and free cells of *Actinobacillus succinogenes*. In order to compare the efficiency of the process, pure glucose and lactose were also used as substrates in fermentations. Process optimization was conducted by the trial of different neutralizing agents, indirect CO_2 sources and varied initial sugar concentrations in fermentations. To improve the system efficiency even further, durability of alginate beads was assessed and improved by using an alginate-whey mixture in immobilization procedure. In the study, fermentations were performed under three operation modes namely; batch, repeated-batch and continuous. Batch fermentations with free cells were conducted using cheese whey, lactose and glucose, highest succinic acid production was achieved with cheese whey (24.9 g L^{-1}) with 35 g L^{-1} starting lactose concentration. From immobilized batch fermentations, 74.9% yield and 1.09 g L^{-1} h^{-1} of productivity was achieved with cheese whey. Repeated-batch fermentations conducted with whey using the same beads in 5 repeated cycles (144 h) and 0.89 g L^{-1} h^{-1} of productivity was achieved. For continuous fermentations with whey and immobilized cells, maximum 0.84 g g^{-1} succinic acid yield was achieved in a 13 days of operation. Based on the results obtained from this research, it was suggested that immobilizing *A. succinogenes* with alginate-whey mixture is an effective technique and cheese whey is a valuable resource for the production of succinic acid.

Keywords: Succinic Acid, Immobilization, Alginate Beads, Cheese Whey, *Actinobacillus succinogenes*

ÖZ

ALJİNAT KULLANILARAK IMMOBİLİZE EDİLMİŞ *ACTINOBACILLUS SUCCINOGENES* İLE SÜT ENDÜSTRİSİ ATIKLARINDAN SÜKSİNİK ASİT ELDESİ

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Süksinik asit, çeşitli endüstriyel uygulamalarda kullanılan çok yönlü bir organik asittir. Süksinik asidin biyoteknolojik üretimi, son yıllarda fosil yakıtlara dayalı kimyasal üretime verimli bir alternatif olarak ortaya çıkmıştır. Bununla birlikte, günümüzde süksinik asit üretiminde petro bazlı kimyasal prosesi tamamen değiştirmek ve çevre dostu az maliyetli üretime geçebilmek için birtakım zorlukların giderilmesi gerekmektedir. Başlıca engellerden biri, çoğunlukla rafine şeker kullanımıyla bağlantılı olan üretim maliyetlerini azaltmaktır.

Mevcut çalışma, süt atıkları gibi ucuz ve yenilenebilir kaynaklara dayalı sürdürülebilir ve uygun maliyetli mikrobiyal üretim sürecinin geliştirilmesine odaklanmaktadır. Çalışmada endüstride büyük bölümü atık olarak kullanılan peynir yapım sürecinin bir yan ürünü peynir altı suyu, potansiyel bir fermentasyon hammaddesi olarak tanımlanmış ve süksinik asidin biyolojik üretimi için ucuz karbon kaynağı olarak kullanılmıştır. En verimli doğal süksinik asit üreten suşlardan biri olan *Actinobacillus succinogenes*, peynir altı suyundan süksinik asidin fermentatif üretiminde kullanılmıştır. Sistemin geliştirilerek yüksek süksinik asit verimi elde edilmesi ve üretim hızının arttırılması amaçlanarak *A. succinogenes*

hücreleri aljinat boncukları içerisinde immobilize edilmiştir. Peynir altı suyundan süksinik asit üretimi, *A. succinogenes*'in hem immobilize hem de serbest hücreleri ile çalışılmıştır. Prosesin verimliliğini karşılaştırmak için, fermentasyonlarda substrat olarak peynir altı suyu dışında saf glikoz ve laktoz kullanılmıştır. Proses optimizasyonu, farklı nötralize edici ajanların, dolaylı CO_2 kaynaklarının ve fermantasyonlarda çeşitli başlangıç şeker konsantrasyonlarının denenmesiyle gerçekleştirilmiştir. Sistem verimliliğini daha da artırmak için, aljinat boncuklarının dayanıklılığı, immobilizasyon prosedüründe aljinat-peynir altı suyu karışımı kullanılarak geliştirilmiştir. Çalışmada fermantasyonlar kesikli, tekrarlı kesikli ve sürekli olmak üzere üç farklı çalışma modunda gerçekleştirilmiştir. Peynir altı suyu, laktoz ve glikoz kullanılarak gerçekleştirilen serbest hücreli kesikli fermantasyonlarda en yüksek süksinik asit üretimi peynir altı suyundan (24.9 g L^{-1}) 35 g L^{-1} başlangıç laktoz konsantrasyonu ile elde edilmiştir. İmmobilize kesikli fermentasyonlardan peynir altı suyu ile %74.9 verim ve 1.09 g L^{-1} s^{-1} üretim hızı elde edilmiştir. Peynir altı suyu ile aynı immobilize boncuklar kullanılarak 5 tekrarlı döngüde (144 saat) gerçekleştirilen tekrarlı kesikli fermantasyonlar sonucunda 0.89 g L^{-1} h^{-1} üretim hızı sağlanmıştır. Peynir altı suyu ve immobilize hücrelerle sürekli fermantasyonlar ile 13 günlük proses sonucu maksimum 0.84 g g^{-1} süksinik asit verimi elde edilmiştir. Bu araştırmadan elde edilen sonuçlara dayanarak, *A. succinogenes*'in aljinat-peynir altı suyu karışımı ile immobilize edilmesinin etkili bir teknik olduğu ve peynir altı suyunun süksinik asit üretimi için değerli bir kaynak olduğu gösterilmiştir.

Anahtar Kelimeler: Süksinik Asit, İmmobilizasyon, Aljinat, Peynir Altı Suyu, *Actinobacillus succinogenes*

Dedicated to my parents;

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ABBREVIATIONS

α-La	α-lactalbulmin
AA	Acetic acid
ACE	Angiotensin I-converting enzyme
β-Lg	β-lactoglobulin
BDO	1,4-butanediol
BOD	Biochemical oxygen demand
BSA	Bovine serum albumin
C	Carbon
°C	Degree celcius
$C_4H_6O_4$	Succinic acid
Ca	Calcium
CAGR	Compound annual growth rate
CO_2	Carbon dioxide
COD	Chemical oxygen demand
CSL	Corn steep liquor
CSTR	Continuously stirred tank reactor
EMP	Embden–Meyerhof–Parnas pathway
EPS	Exopolysaccharides

FA	Formic acid
FBR	Fluidized bed reactor
FBS	Fetal bovine serum
g	Gram
GMP	Glycomacropeptide
GRAS	Generally recognized as safe
h	Hour
H_2	Hydrogen
HMF	Hydroxymethylfurfural
HMP	Hexose-monophosphate route
HPLC	High-performance liquid chromatography
IG	Imminoglobulin
kg	Kilogram
L	Liter
LA	Lactic acid
LAB	Lactic acid bacteria
m	Meter
M	Molar
mg	Milligram
$MgCO_3$	Magnesium carbonate
mL	Milliliter
mm	Millimeter

MT	Megatonne
nm	Nanometer
OAA	Oxaloacetate
OPPP	Oxidative pentose phosphate pathway
p	p-value
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PHA	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrates
PBS	Polybutylene succinate
PLA	Polylactic acid polymer
PBR	Packed bed reactor
rpm	Rounds per minute
s	Second
SA	Succinic acid
SCP	Single cell protein
TCA	Tricarboxylic acid
TOC	Total organic content
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UN	United Nations
v	Volume

VFA	Volatile fatty acid
w	Weight
WPC	Whey protein concentrate
WPH	Whey protein hydrolysate
WPI	Whey protein isolate

CHAPTER 1

INTRODUCTION

It is predicted that by 2025, bio-based products would account for more than 15% of the $3 trillion worldwide chemical markets, according to industry estimates (Vijayendran, 2010). In order to assure long-term supply in the face of fossil fuel depletion and climate change, a transition to renewable resources are being implemented (Luo et al., 2010). To meet this demand, greater emphasis has been dedicated in recent years to succinic acid synthesis by fermentation utilizing renewable feedstocks, which is becoming more popular. Since its vast range of uses in a variety of industries, succinic acid ($C_4H_6O_4$), a dicarboxylic acid also known as amber acid or butanedioic acid, is considered as a platform chemical. Succinic acid has been identified by the United States Department of Energy as one of the top 12 most promising value-added bio-based compounds (Bozell and Petersen, 2010; Hermann and Patel, 2007). For a bio-based molecule to be considered as a platform chemical, the researchers specified six dimensions of needs (Bomtempo et al., 2017). Interestingly enough, succinic acid meets the majority of the parameters that have been presented.

In general, there are four extant markets for succinic acid, which are detergent/surfactant/foaming agent/extender, food industry, ion chelator, and health sector (Zeikus et al., 1999). In addition, due to its linear building and the high capability of the two functional carboxylic groups to undergo reactions, succinic acid can be used as a precursor for the production of a variety of industrial chemical products, including 1,4-butanediol, adipic acid, gamma-butyrolactone, n-methylpyrrolidone, tetrahydrofuran, biodegradable polymers (butylene succinic acid) (PBS) and linear aliphatic esters (Pateraki et al., 2016; Song and Lee, 2006;

Zeikus et al., 1999). By 2022, the worldwide succinic acid market, is expected to reach $1.1 billion, stated by the Global Industry Analysts, Inc.. Among the most important aspects contributing to the rise in popularity of biosuccinic acid are technological advances in manufacturing and processing, as well as a growing preference for biosuccinic acid over petrochemical-based options due to the advantages such as reduced price volatility and cost efficiency, as well as a minimal carbon footprint (Dessie et al., 2018).

As an intermediary in the tricarboxylic acid (TCA) cycle, succinic acid is a frequent metabolic product in many species. As a result, it is reasonable to believe that practically all organisms are capable of producing succinic acid. Despite the fact that the selection of production hosts is quite wide, the natural production hosts that have been considered thus far are capnophilic rumen bacteria. The strains *Actinobacillus succinogenes*, *Corynebacterium crenatum*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, *Bacteroides fragile*, *C. glutamicum*, *Escherichia coli*, *Yarrowia lipolytica* and *Saccharomyces cerevisiae* are the most commonly used strains in the production of succinic acid (Beauprez et al., 2010; Ferone et al., 2019a; Li et al., 2018; Okino et al., 2008; D. Yan et al., 2014). Among these, *A. succinogenes*, has been identified as one of the most efficacious strains for usage in the manufacture of succinic acid (Ercole et al., 2021).

A. succinogenes is a ruminal, osmotolerant, facultative anaerobic gram-negative bacterium (Guettler et al., 1999). It is for a variety of reasons that *A. succinogenes* has regarded as one of the most promising succinic acid generating strains. This includes the following characteristics; capability to produce succinic acid in relatively significant quantities (Carvalho et al., 2016), capability to utilize wide variety of carbon sources (Chapter 2), applicability on scalable biorefineries (Bradfield et al., 2015), high resistance to substrate inhibition (Song and Lee, 2006), high tolerance to end product (succinic acid) inhibition (Van Der Werf et al., 1997), ability to work under high CO_2 presence, fixation and consumption of

CO_2 (McKinlay et al., 2005; Van Der Werf et al., 1997), non-pathogenicity (McKinlay et al., 2007a), and the ability to withstand chemicals found in hydrolytes, such as furfural and HMF (Diaz et al., 2018).

Although, while the dairy sector is one of the vital industries for human life, it is also one of the most water-intensive and polluting industries due to the high organic content that results in a COD (chemical oxygen demand) of 0.1–100 g L^{-1} (Prazeres et al., 2012). Cheese whey is one of the most common effluents generated as a by-product of the cheese-making process. In terms of composition, it comprises lactose (4.5–6% w/v), lipids (0.4–1%), proteins (0.8–1.0% w/v), and mineral salts (8-10% of dried extract) (Louasté and Eloutassi, 2020). Cheese whey has a high concentration of biodegradable materials, which makes it a promising bioresource for the biotechnological synthesis of value added chemicals. This has been researched extensively in several studies (Chapter 2). In this study, main focus was the production of succinic acid by using cheese whey as main substrate in fermentations and *Actinobacillus succinogenes* as fermentation agent.

One of the two goals of this research was to present the efficiency of bio-based succinic acid synthesis from renewable feedstock cheese whey. Furthermore, fermentation system was improved by using immobilization technique by immobilizing *A. succinogenes* cells with alginate. In order to investigate the fermentation efficiency of cheese whey, fermentations were conducted in three different modes (batch, repeated-batch and continuous) with both immobilized and free cells of *A. succinogenes* and in order to compare the results, experiments were also performed with pure glucose and lactose as carbon sources (Chapter 4).

As second goal, durability of immobilization matrix (Ca-alginate beads) was investigated. By using an alginate-whey mixture to immobilize cells, a solution to poor stability of immobilization support was proposed in a cheap and efficient way for the first time and applied successfully.

This study was aimed to be the first in literature that investigates the fermentative production of succinic acid with immobilized *A. succinogenes* using cheese whey as carbon source.

CHAPTER 2

LITERATURE REVIEW

2.1 Organic Waste and Biomass Valorization

Consumption of nonrenewable fuel sources have become a major problem, not only because of resource depletion, but also the pollution they are causing and the production of CO_2 gas, both of which contribute to global warming. Currently, fossil based fuels provide 88% of the world's energy needs, and researches predict that need of energy will likely to increase by 50% in the coming 50 years (Giwa et al., 2018; Wang and Yin, 2018). Moreover, present rate of consumption would likely to lead depletion of fossil fuel reservoirs by the year 2150 (Mandley et al., 2020; Quilcaille et al., 2018). Thus, there is an ever-increasing global need for energy. Renewable energy sources, rather than fossil fuels, and biological building blocks, are thus of importance. One of the most promising sustainable choices right now is converting waste into energy carriers and useful products, particularly since waste disposal takes either space (landfills) or energy (incineration).

Organic waste consist of food and fiber processing by-products, vegetable waste, fruit, sewage sludge, garbage, feedstock animal manure and/or industrial waste (Table 2.2) (Murto et al., 2004). The challenges regarding the management of these wastes have earned noticeable recognition in current times from both public and politics. As a result, reducing the waste creation through waste management is strongly advised for long-term sustainability. Various waste management applications are in use today, these are; (1) composting, (2) incineration, (3) landfilling, (4) recycling, (5) dumping (in the open and water streams) and (6) anaerobic digestion (Karak et al., 2012; Patel et al., 2021; Slorach et al., 2019; Z. Wang et al., 2018). These methods could be used separately or in combined for efficient waste management and all of them showed some benefits over each other.

Some benefits and percentage of global usage of these management methods are presented in Table 2.1. Widely used two techniques, landfilling and dumping are contributing 80% of the total waste management globally (Levaggi et al., 2020). Although, these applications have some benefits (Table 2.1) their contribution to environmental pollution cannot be ignored. In contrast, anaerobic digestion and recycling methods are more eco-friendly and provides renewable energy and bio-products for further use. Using advanced biotechnological routes to produce platform chemicals and biofuels are considered as the most promising applicable technologies for sustainable development.

Table 2.1 Management methods for disposal or valorization of wastes (Patel et al., 2021)

Process	**Contribution (%)**	**Benefits**
Composting	5.05	Allow microorganisms to produce nutrient-filled materials Enrich soil and prevent plant infections or pests Decrease methane emissions Decrease chemical fertilizers requirement
Incineration	6.45	Efficiently reduce waste quantity Energy generation Prevents methane generation and can be operated in any weather Significantly reduce harmful microorganisms and chemicals
Landfilling	37.4	If operated with eco-friendly advanced systems, an excellent energy source Easy method to keep living areas clean All kinds of waste can be managed Economical
Dumping	32.2	Simplest method Very economical Convenient
Recycling	12.6	Reduce quantity of waste management by other methods Environmental Save energy Conserve natural resources
Anaerobic digestion	6.30	Provide renewable energy (biogas) and/or valuable bio-products (organic acids, biopolymers, proteins) Reduce pollution Conservation of energy and agricultural land Generate fertilizers

Table 2.2 Major food products and processing wastes

Food Products	Processing wastes/by-products
Oil (palm)	Palm empty fruit bunch (lignocellulose)
Oil (soybean)	Soybean meal (protein, carbohydrate)
Oil (rapeseed)	Rapeseed meal (protein, carbohydrate)
Rice	Rice hull (lignocellulose, ash)
Wheat	Bran (arabinoxylan, cellulose, protein)
Potatoes	Potato peel and other processing waste (lignocellulose, starch)
Banana	Rejected banana (lignocellulose, pectin, starch)
Apple	Rejected apples (fucogalactoxyloglucan, lignocellulose, glucose, fructose)
Raw sugar	Molasses (sucrose, glucose and fructose)
Beer (barley)	Brewery waste (carbohydrate, protein, organic acids, high COD)
Wine	Brewery waste (carbohydrate, organic acids, high COD)
Cheese	Whey (lactose, protein, high COD)
Beef	Slaughter waste (animal fat, protein)
Pork	Animal fats, protein

2.2 Valorization of Dairy Wastes and Whey

Constant development of the dairy industry leads to huge amounts of by-product production which in need to be properly managed. Whey is the main by-product coming from dairy processes. It is an organic discharge that can be considered as hazardous for the environment when disposed to land or water streams because of its high chemical (50-80 g L^{-1} COD) and biochemical (40-60 g L^{-1} BOD) oxygen

demands (Chatzipaschali and Stamatis, 2012). Most of the organic loads in the whey are lactose, fat and proteins. In today's practices, most of the whey is disposed as wastewater without preprocessed and this application can cause serious environmental problems (Macwan et al., 2016). Since whey holds significant amounts of nutrients which are potential candidates for utilization to produce energy and value-added products, it is important to manage whey disposal applications properly and develop sustainable routes to valorize whey into valuable products.

Since the 60s and 70s, public environmentalist groups, regulatory agencies and dairy processors have noticed and pointed out the environmental impact of unprocessed whey disposal (Smithers, 2015). Dumping of untreated whey in water streams is nowadays regulated in most of the countries by strict environmental laws (Ryan and Walsh, 2016). UN Agenda 2030 for Sustainable Development which was proposed at the UN Summit on Sustainable Development in 2015 provided a basis for an approach to today's challenges concerning waste management and environmental problems. The agenda constitutes of 17 sustainable development goals and these goals are further examined in detail under 169 sub-groups which are associated with sustainable waste management including reduction of the emissions to the environment, and also reduction of generated waste by increasing the recycle and reuse applications (UN General Assembly, 2015). So that whey is no longer perceived as a waste but rather as an extremely valuable renewable dairy by-product, substantial research has been performed to develop efficient, environmentally acceptable whey valorization processes that may be used to replace the disposal of whey in the field (Valta et al., 2017). In 2018, almost 50 % of whey produced from dairy industry has recycled through chemical and food industries in order to produce value-added products instead of disposed as waste (Panghal et al., 2018).

Traditionally whey has been used as a health promoter in human and animal nutrition, it has been considered as a source of bioactive and functional

compounds, particularly peptides and proteins. However, a big portion of whey produced globally still not valorized. This is due to the lack of necessary equipment and process integration investments of small and medium size dairy industries (Sommella et al., 2016). While new advancements on ultra and nanofiltration technologies increased the yield of whey utilization into high-value nutritional products, several biotechnological approaches developed with new application technologies also emerged for bioprocessing of whey into valuable bioproducts as a possible candidate for valorization. Establishment of new biorefinery concepts which constitutes integrative application of several process units to produce valuable products from whey can be a sustainable and an economical alternative while also reducing the environmental impact of whey disposal (Lappa et al., 2019).

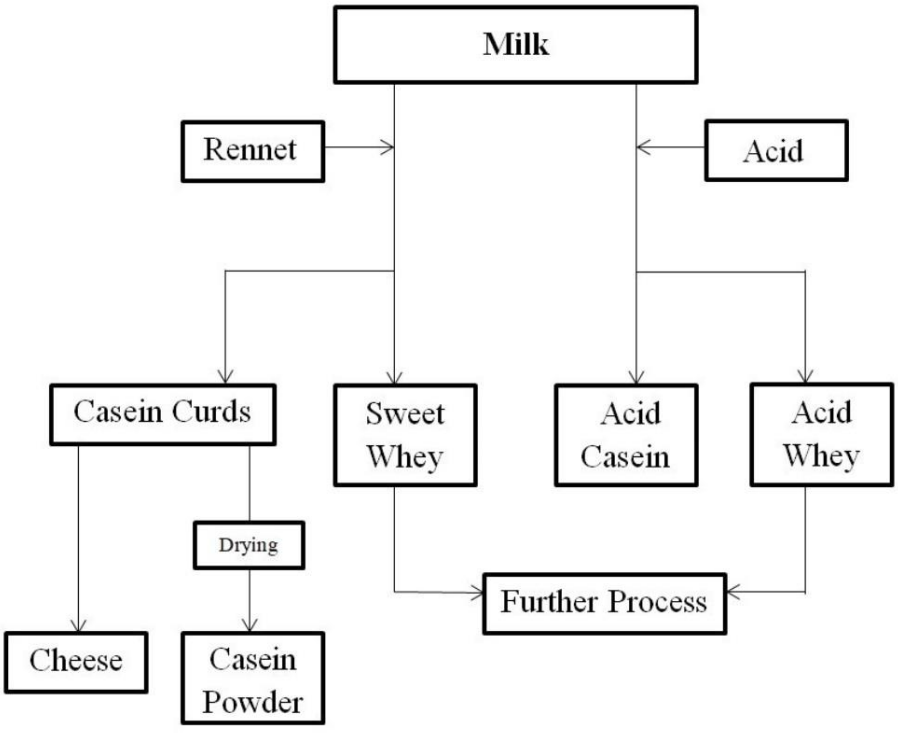


Figure 2.1 Flowchart of cheese whey production

Whey is a constituent of milk serum which is obtained by separation of curd during cheese making (Figure 2.1), and it makes up 85-90% of milk. Fifty five percent of

nutrients in milk come from whey. Generally, it contains 70% lactose -the amount may change according to the acidity-, 14% proteins, 4% fats, 9% minerals and 3% other organic compounds mainly lactic acid (Ryan and Walsh, 2016). Composition of obtained whey may vary for different products by the result of many parameters such as different process conditions and produced cheese type. According to the milk protein coagulation method, whey can be categorized in two parts as sour and sweet whey. Sour whey is a by-product of fermentation or coagulation of casein by acidification. It has a pH value of 5.0 or lower. Fresh cheese production can be given as an example of acidification of casein in which sour whey can be obtained. On the other hand, sweet whey is formed by the coagulation of casein by the use of rennet. Rennet is an industrially used coagulant, and it includes either chymosin or enzymes that coagulate casein. Levels of the components exhibit differences in sweet and sour whey. For instance, fat, lactose and protein percentages are higher in sweet whey whereas sour whey is richer in lactic acid, phosphorus and calcium (Pires et al., 2021). Sour whey is also treated with plant's water which makes it even more diluted in lactose. However, it still contains COD and total organic carbon (TOC) and direct release of sour whey as an effluent causes environmental concerns. Thus, purification process is required before it is discharged. High nutritional value and ease of digestion and assimilation are just some of the advantages of whey in general. Other nutrients found in it include functional proteins, minerals (such as calcium, phosphates and potassium), and lactose. It is also regarded as an excellent source of vitamins B (Papademas and Kotsaki, 2020).

A large quantity of whey is processed into whey powders because of the exceptional functional and nutritional properties of solids present in the whey. The remaining whey is used to produce sweet whey powder, demineralized whey, delactosized whey, whey protein isolate (WPI), whey protein concentrate (WPC), or lactose (Zandona et al., 2021). Along with these applications, extensive research has been conducted to exploit nutrients of whey to produce biochemical products such as organic acids and ethanol. In the following subchapters common usage areas of whey will be summarized.

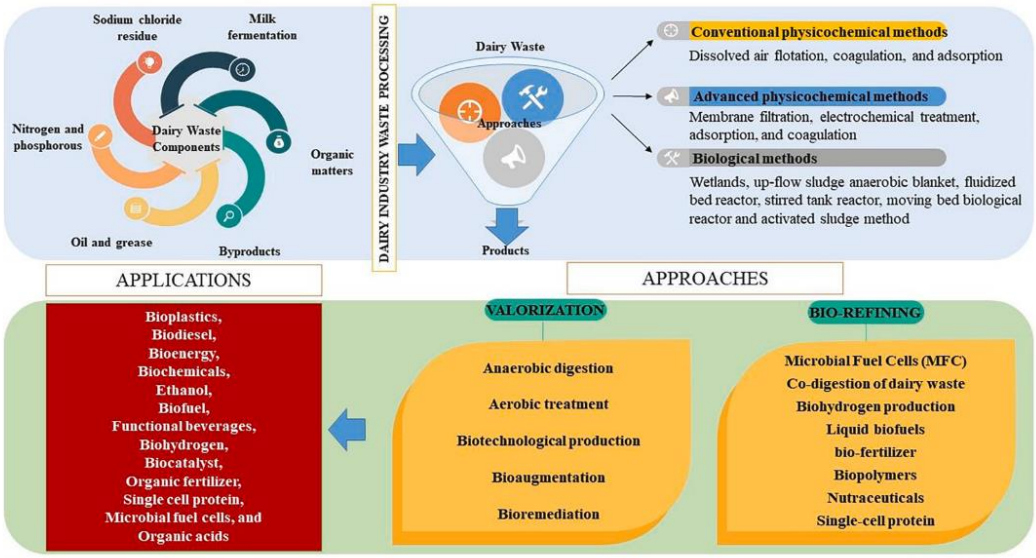


Figure 2.2 Overall scheme of dairy waste valorization applications (Kumar Awasthi et al., 2022)

2.2.1 Traditional Uses of Whey

Unaltered whey was traditionally utilized as an livestock feed for lambs, cattle, and pigs or as a fertilizer on farmland (Schingoethe, 1976; Watson et al., 1977). Whey is an excellent source of high-quality protein and lactose, as well as phosphorus, calcium, water-soluble vitamins, and sulphur, as a direct animal feed. However, high amounts of minerals and lactose contained in whey might cause problems for farm animals, necessitating a limit in the use of unprocessed whey as an animal feed (Macwan et al., 2016). Also, large amounts of whey applied to the ground produce excessive salty deposits in the soil, reducing its fertility (Kosikowski, 1979). For the quantity of whey being produced today, both usages have issues with volume and significant transportation costs that make both options infeasible.

2.2.2 Whey Powders

Production of whey powders from liquid whey is a common application for valorization of cheese whey. New technologies have made it possible to explore alternative ways of transforming whey into key value-added products, even though drying process holds for 70% of its yearly processing expanses (Papademas and Kotsaki, 2020). Evaporation, clarifying, separation of cream, and pasteurization are all common steps in the process of making whey powders. Drying of whey and lactose crystallization are other common steps. It should be noted that, if the lactose crystallization operation is not performed properly, the concentrated fraction of the resulting bulk powder is applicable solely for use as a livestock feed as a source of high-quality carbohydrates and proteins at an economical cost (Zandona et al., 2021). There are no residues that need to be dealt with individually, and the whey powder's quality is maintained during shipping or subsequent manipulation thanks to different drying procedures. However, it is costly to acquire the essential equipment, and it uses a lot of energy when it's being produced, so it is not ideal. In addition, selling price of whey powders are lesser compared to whey protein concentrates (WPC), which is a drawback. Whey powder products are commonly used in a variety of food and beverage industries, including processed meats, infant formula, soups, refreshments, toppings, sauces, condensed milk, squeezed nuts, nut coatings, snack foods, cheese-based sauces, savoury baked goods, savoury flavourings, special baked foods such as biscuits, pizza and pasta (Božanić et al., 2014). Whey powder may be utilized as a fat and oil adsorbent and binder as well (Zandona et al., 2021). Ultrafiltration or diafiltration are the most common methods of membrane separation used in the production of higher-quality whey protein powders (Blazic et al., 2018). It is likely that foods that are produced with the inclusion of whey powder will have a superior flavor and physical features than those made without it (acid stability or foaming). However, the texture, flavor, and presence of these foods can be similar to cheese-containing products (Zandona et al., 2021). Both whey and whey-derived products (WPH, WPI, and WPC) are

being used as the primary or predominant component in a wide range of drinks. Sports drinks and nutrition drinks for the undernourished commonly contain these components (Lappa et al., 2019).

2.2.3 Foods and Beverages

Whey may be used to manufacture various human food products, including whey cheese and drinks. Fruit juices that have been combined with whey are the most often encountered whey drinks (some famous brands are Djoez and Taksi produced in Netherlands and Nature's Wander from Sweden) (Holsinger et al., 1974; Kosikowski, 1968; Shraddha RC and Nalawade T, 2015). Among the many carbonated soft drinks available today one other example is Rivella, which has been produced and distributed in Switzerland since the early 1950s. This beverage is prepared from carbonated whey permeate that has been flavor-infused with herbal extracts. These items, on the other hand, have not succeeded in gaining popularity outside of their respective home markets (Ryan and Walsh, 2016). Although whey derived beverages can be a good approach to use of whey in human nutrition there are several drawbacks about both their taste and manufacturing processes. A pretreatment of whey is a must before processing beverages because of the susceptibility of whey to microbial contamination because of the high water and organic content of whey. Thermal treatment, which is the most common sterilization method, is not desired because of the sensitivity of whey proteins to temperatures above 60 °C. After the typical heat treatment (at 72 °C for 15-20 s) of whey, the majority of the whey proteins denature and precipitate (Macwan et al., 2016). Extensive research has being done to find ways to include non-thermal techniques into the manufacture of whey drinks, such as high-intensity ultrasound or membrane separation. Another goal is to develop supercritical carbon dioxide technology to produce whey beverages. Using non-thermal sterilization and extraction technologies in the manufacturing of whey drinks allows for the elimination of the challenges outlined above, as well as an improvement in the

qualities of the goods now available (Amaral et al., 2018; Barukčić et al., 2015a, 2015b; Režek Jambrak et al., 2018).

It is also possible to make alcoholic beverages from whey, including low-alcohol beverages (<1% alcohol content), whey beer, whey wine, and whey champagne (Macwan et al., 2016). It is necessary to add specific additions, such as sucrose and malt, to the mixture before fermenting it with yeasts such as *Saccharomyces lactis* or *Kluyveromyces fragilis* in order to produce these products (Holsinger et al., 1974).

It may also possible to produce whey cheeses from whey, of which there are two primary types: Ricotta and Mysost. When making Ricotta cheese, the whey is heated above to 80 °C, causing the proteins present in whey to become denatured. Afterwards, the denatured proteins cluster together (sometimes with the addition of citric acid) to create whey protein curds, with these curds cheese making process performed (Pintado et al., 2001). Cheeses of the Mysost kind are made by condensing whey under specified settings. Whey condensate is then collected and heated to 95 °C, after this cheese is processed (Jelen and Buchheim, 1976). In addition to whey cheese, another possible product that can be produced from whey is whey butter. Whey butter is separated from whey following its processing for cheese production and before its treatment for protein extraction or spray drying. While whey butter has a saltier taste and texture than ordinary butter, researchers have determined that it is rather softer (Jinjarak et al., 2006). Despite their attractive features, these products have had limited market share and they do not provide a viable solution for dealing with the vast quantities of whey waste that are generated.

As water binders/gelling agents, emulsifiers, whipping agents/foaming agents, and other food additives, whey proteins have also found use in the food sector, where their physical properties allow them to perform a variety of functions in the production of food items. Their presence may be recognized in a wide range of

products, such as salad dressings, soups, dairy products, processed meats, and baked goods (Walsh, 2014).

2.2.4 Whey Proteins and Peptides

Since all necessary amino acids are present in high amounts in whey protein and its biological value is 15% higher than the original standard egg protein, it is regarded to be an excellent source of high-quality protein for the body (Kassem, 2015).

Whey proteins are heat sensitive (Kassem, 2015). Bovine serum albumin (BSA), immunoglobulins (IGs), α-lactalbulmin (α-La), and β-lactoglobulin (β-Lg) are the four major whey proteins (Table 2.3). In cow milk, BSA has been found in low concentrations, and it is thought that the molecule moved from blood serum into the milk. To protect the growing mammalian immune system, IGs are antibodies that are generated in response to particular antigens. IGs are big glycoproteins that are heat labile when other whey proteins are present (Walstra et al., 2005). Lactoferrin, oxidoreductases, lysozyme, lactoperoxidase, phosphatases, proteinases, lipolytic enzymes are some of the other whey proteins that can be listed (Table 2.3; Walstra et al., 2005).

When casein curds are produced with the aid of rennet, a portion of the k-casein molecule known as glycomacropeptide (GMP) is produced, and this peptide is found in the whey. Although the GMP is present in sweet, rennet-based wheys (where it accounts for roughly 15 % of the whey protein composition), acid whey does not contain this protein (Lim et al., 2007; Neelima et al., 2013).

By enzymatic breakdown of whey proteins, enzymes chymotrypsin and trypsin can be used to produce bioactive peptides. There are a number of sources for these enzymes, such as bovine and porcine proteases (Madureira et al., 2010) as well as yeast, bacterial and fungal protease (Morais et al., 2014). Microbial fermentation of whey protein can also provide certain bioactive peptides. For example, an angiotensin I-converting enzyme inhibitory (ACE) peptide can be produced via the

fermentation of whey by the microorganism *Lactobacillus helveticus* (Yamamoto et al., 1999). Table 2.3 lists several whey-derived peptides.

The presence of growth factors in whey has also been discovered, such as fibroblast growth factor, insulin-like growth factor, platelet-derived growth factor, and transforming growth factor (Pouliot and Gauthier, 2006). It is possible that these growth factors will be employed as a dependable alternative for, or as a complement to, fetal bovine serum (FBS) (Smithers et al., 1996). FBS is utilized in the production of vaccines and biopharmaceuticals, as well as in the development of mammalian cell cultures. Because it is not in risk of contamination from bovine spongiform encephalopathy, mycoplasma, or viruses, the use of a whey derived growth factor medium would be a less expensive and safer alternative to FBS (Keenan et al., 2006).

Table 2.3 Primary protein components of whey and their potential activities, along with peptide derivatives

Protein	Composition (%)	Peptide derivatives	Potential functions	References
β-Lactoglobulin	50-55	*β*-Lactophorin *β*-Lactotensin	Angiotensin I converting enzyme (ACE) inhibition to lower blood pressure Appetite enhance Appetite suppressant	(Welderufael et al., 2012)
α-Lactalbumin	20-25	α-Lactophorin	Infant formula ingredient	(Sandström et al., 2008)
			Opioid agonist	(Pihlanto-Leppälä, 2000)
			Increase the levels of tryptophan to improve sleep quality and mood under stress	(Markus et al., 2005, 2002, 2000)
			Lowers blood pressure	(Nurminen et al., 2000)
Immunoglobulins	10		Assistance to protection against oral and intestinal microbial infections	(Hurley and Theil, 2011)

Table 2.3 (continued)

Serum albumin	5-10	Albutensin Serophorin	Cancer prevention	(Merlot et al., 2014)
			Suppressor of appetite	(Ohinata et al., 2002)
			Opioid agonist	(Madureira et al., 2010)
Proteose peptone 3	12			
Lactoferrin	1-2	Lactoferricin	Anti-microbial, Anti-viral	(Beaulieu et al., 2006)
			Bone growth promoter	(Cornish et al., 2004)
			Anti-cancer function	(Kozu et al., 2009; Tsuda et al., 2000)
Lactoperoxidase	0.5		Prevention against microbial infection Prevention of dental caries Preservation of raw milk	(Beaulieu et al., 2006)
Glycomacropeptide (a casein peptide)	10-15 (in renneted whey only)		Anti-thrombotic functions	(Requena et al., 2010)
			As additive in infant formula	(Lim et al., 2007)

2.2.5 Lactose

Through the crystallization of whey permeate or cheese whey, the lactose sugar of milk can be extracted from the whey (Paterson, 2009).

Because of its moderate sweetness, which corresponds to 16% that of sucrose, lactose is widely used in the food and confectionery sectors (Joesten et al., 2006)? By activating the Maillard reaction, it is utilized in the production of bakery goods to increase crust browning and crisping. It is also used in the manufacturing of infant formula, where it can be mixed with bovine milk (4.5–5.3 % in cow milk compared to 7.1 % in human milk). Excipients, such as lactose, are employed in the pharmaceuticals to protect the integrity of the drug (Paterson, 2009). Since the 1940s, there has been a significant rise in the quantity of lactose generated from whey (Sienkiewicz and Riedel, 1990).

Many additional chemicals can be made directly from lactose. Lactulose, for instance, is a lactose derivative with a various possible uses. Lactulose can be employed as a sweeter. It has a sweetness of 48-62% of sucrose and it is relatively more soluble compared to lactose making it easier to be utilized in food industry (Parrish et al., 1979). For diabetics, lactulose can be utilized as a replacement for sucrose as a sweetener in the production of confectionery goods. Also it can be used as an additive in milk and dairy products, and in various foods (liquid or dry) that are specifically produced for elder consumption(Mayer et al., 2004). Aside from these, it may be used as a laxative in the treatment of chronic and acute constipation (Tramonte et al., 1997), as well as the treatment of hyperammonemia (high ammonia in the blood) and chronic hepatic encephalopathy (impairment of brain function due to liver failure) (Cammà et al., 1993). Another use of lactulose is as a prebiotic (De Souza Oliveira et al., 2011). In most cases, it is produced by the alkaline isomerization of lactose; nevertheless, studies have been conducted to see if it can be produced through enzymatic synthesis (Aider and Halleux, 2007; Tang et al., 2011).

Another substance that can be derived from lactose is lactitol. It is generated by the chemical hydrogenation of lactose. Lactitol is a sugar alcohol that can replace sucrose as a bulk sweetener in slimming products, low-calorie foods, and foods specifically formulated for diabetics thanks to its sweetness that 40% of sucrose (Zacharis, 2012). It has been examined as a possible prebiotic since it can be metabolized by the intestinal bacteria into short chain fatty acids, which are beneficial to the body (Dills, 1989). Much like with lactulose, lactitol may help to relieve constipation and chronic hepatic encephalopathy in certain people (Maydeo, 2010; Miller et al., 2014).

Another possible product is lactosylurea. Lactosylurea is a derivative of lactose which can be used in ruminant feeding playing a role as a non-protein source. When compared to the other non-protein sources like urea, lactosylurea has some

advantages. For example, slow degradation of the compound does not result in the production of dangerous ammonia levels (Suyama et al., 2011).

Hydrolyzing lactose found in whey permeate into glucose and galactose by an immobilized enzyme β-galactosidase may be another possible application. This process allows sweetening capability to enhance due to the resulting compounds [glucose has 80% of the relative sweetness of sucrose, whereas galactose has 60% (Joesten et al., 2006)]. Therefore, several industries such as confectionary, ice cream, and soft drink may use hydrolyzed lactose solutions instead of saccharides and HFCS (Gänzle et al., 2008). Further enhancement of the sweetness of lactose solutions can be achieved by the use of glucose isomerase which allows the conversion of remaining glucose to fructose.

Lactose derived from whey can also be utilized to make Galactooligosaccharides. Galactooligosaccharides are prebiotics that can benefit human health by promoting probiotic bacteria development inside intestine. Although, they can be synthesized chemically, they are typically synthesized from lactose via a process called transglycosylation, which is catalyzed by β-galactosidase (Torres et al., 2010; Golowczyc et al., 2013).

2.2.6 Biological Treatment and Utilization of Whey

All across the world, there is a considerable deal of attention in finding new ways of using wasted whey in the bioconversion process. It is possible to utilize lactose, which is the main carbohydrate component present in whey, as a substrate for growth and product generation in several biotechnological processes. Whey can be utilized by using variety of applications, these can be listed as; anaerobic digestion, aerobic treatment, bioaugmentation, bioremediation, and biotechnological production (Figure 2.3).

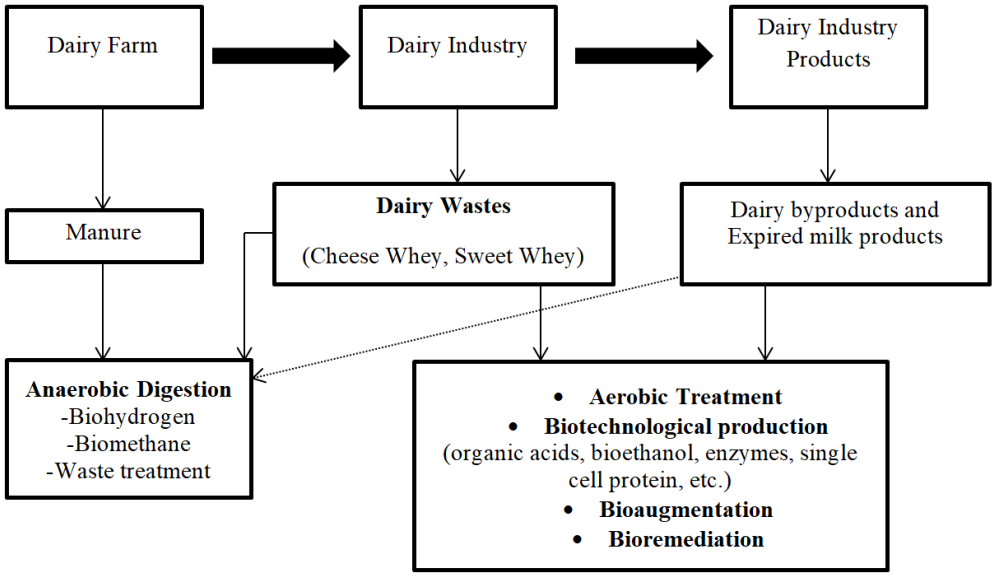


Figure 2.3 Biological application routes for dairy wastes [adapted from (Kumar Awasthi et al., 2022)]

2.2.6.1 Anaerobic Digestion

Whey is a compound that has a high COD value because it is rich in organic content. High organic content present in whey, on the other hand, can be utilized. It can be defined as a practical source of biohydrogen which allows its use in anaerobic digestion in terms of energy saving (Nuri Azbar et al., 2009; Wainaina et al., 2020). On the other hand, because of its high COD content, low bicarbonate alkalinity and very quick acidification propensity, anaerobic treatment of whey may present some difficulties to perform. Thus, there has been ongoing research in order to establish an efficient system. Some mesophilic and thermophilic bacteria species can be utilized in anaerobic digestion process of dairy wastes and produce biohydrogen as a final product. Kargi et al., claimed that although the thermophilic bacteria have longer lag phase compared to mesophilic bacteria, they produce hydrogen more efficiently (Kargi et al., 2012). On the contrary, according to the findings of Azbar et al., mesophilic bacteria was more efficient in terms of hydrogen generation (N. Azbar et al., 2009). Apart from those, there are several

factors like pH, configuration of reactors, inoculum type and incubation time that are taken into consideration in order to increase the efficacy of hydrogen production from whey. According to the findings in literature, whey can be employed in hydrogen production practices (Table 2.4). Several compounds other than hydrogen can also be detected during the process of hydrogen generation. Variety of volatile fatty acids including iso-butyric acid, butyric acid, acetic acid, lactic acid, formic acid, and propionic acid can be given as an example to these compounds (Davila-Vazquez et al., 2008; Pandey et al., 2019; Rosa et al., 2014). Additionally, it has been reported by Egas et al. that production of other key metabolites like ethanol is possible during process (Egas et al., 2019). Production of biomethane can also be achieved through the combination of dairy industry waste with other substances like cow manure and vinasse (Escalante et al., 2018; Náthia-Neves et al., 2018; Sar et al., 2021). With the development of the most optimal reactor design and incubation conditions for the production of methane and hydrogen from whey, a large quantity of COD (up to 95%) can be removed from the process (Zhang et al., 2021). It can be stated that, anaerobic digestion of whey has the potential to be a cost-effective technology for both the production of methane/hydrogen and treatment of wasted effluent (Choudhury et al., 2020; Wainaina et al., 2020).

Table 2.4 Production of hydrogen via anaerobic digestion from dairy wastes

Substrate	**Microorganisms**	**Yield**	**References**
Cheese whey	Thermophilic bacteria	8.1 mmol H_2/gCOD	(N. Azbar et al., 2009; Mahboubi et al., 2017a)
Cheese whey	*Clostridium* and *Klebsiella*	1.1 mol H_2/mol lactose	(Asunis et al., 2019; Perna et al., 2013)
Cheese whey	Mesophilic bacteria	9.2 mmol H_2/gCOD	(N. Azbar et al., 2009; Mahboubi et al., 2017b)
Cheese whey	*Clostridium* genus	2.8 mol H_2/mol lactose	(Davila-Vazquez et al., 2009; H. Liu et al., 2021)

Table 2.4 (continued)

Crude cheese whey	*Clostridium saccharoperbutylacetonicum*	7.9 mmol g/L lactose	(Asunis et al., 2020; Suman et al., 2017)
Cheese whey	*Thermoanaerobacteriaceae bacterium*	4.7 mmol H_2/gCOD	(Asunis et al., 2020; N. Azbar et al., 2009)
Cheese whey	Anaerobic Granular Sludge	2 L H_2 L^{-1} d^{-1}	(Das et al., 2016)
Cheese whey	*Clostridium* and *Thermoanaerobacterium*	52-56 mol H_2 m^{-3} d^{-1}	(Hou et al., 2021)
Cheese whey powder (15 g)	Mixed	3.1 mol H_2/mol lactose	(Davila-Vazquez et al., 2008; Qin et al., 2021a)

2.2.6.2 Aerobic Treatment

To improve the quality of the waste by degrading lipids and other compounds, as well as reducing the odor of the waste by fermenting it with different microorganisms, aerobic treatment of food processing wastes can be performed. When applied at high temperatures (over 45 °C), thermal aerobic therapy can also effectively remove harmful pathogens. Using this technology, it is possible to successfully reduce the amounts of BOD, COD, odor, and ammonium present in animal wastes and different food wastes including dairy wastes such as whey (C. Liu et al., 2021; H. Liu et al., 2021). Thermal aerobic treatment preserves the nitrogen content of the final product since there is no nitrification or denitrification occurring throughout the process. Because of this, the finished product might be regarded as a biofertilizer (Mahboubi et al., 2017b). The aerobic treatment of whey, on the other hand, is not always possible for most processes. As a consequence, to perform the whey treatment, an aerobic-anaerobic treatment procedure is necessary in two stages (Karki et al., 2021; Liu et al., 2018).

2.2.6.3 Bioaugmentation

Bioaugmentation can be used as a remediation method for reducing COD levels in organic wastes and organic matter (proteins, carbohydrates, and lipids). Bioaugmentation technique may be used to purify whey using anaerobic sludge cultures or a fungi community (*Galactomyces geotrichum*, *Mucor hiemalis*, and *Aspergillus niger*) (Djelal and Amrane, 2013; Schneider and Topalova, 2011). It is also possible to enhance both the removing capacity and the yield of biobased chemicals (e.g. acetic acid, ethanol, and lactic acid) in this approach by boosting the activity of specific bacteria such as LAB in the process (Luongo et al., 2019; Policastro et al., 2021).

2.2.6.4 Bioremediation

As an alternative to other biological applications like aerobic or anaerobic fermentations, bioremediation procedure may be carried out with the use of non-pathogenic microorganisms at a minimal cost, as well. The effluent from the dairy sector may be effectively isolated to obtain a wide range of bacteria including *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas fluorescens*, *Enterobacter*, *Streptococcus faecalis* and also yeasts like *Cryptococcus*, *Candida*, and *Saccharomyces* (H. Liu et al., 2021). A significant reduction in organic waste load and an improvement in its physicochemical attributes can be achieved by cultivating microorganisms isolated from dairy wastewater, such as *B. subtilis*, and *P. aeruginosa*, as well as *Staphylococcus aureus*, *Lactobacillus delbrueckii*, *Alternaria* species, *Enterococcus hirae*, *Aspergillus* species, and *Fusarium* species (Al-Wasify et al., 2017; Jain et al., 2022).

2.2.6.5 Biotechnological Valorization

Bioconversion is accomplished by the employment of biotechnological processes such as aerobic and anaerobic fermentation, fuel cell and anaerobic digestion. Furthermore, for the creation of value-added products, it is possible to employ a combination of biotechnological and physiochemical processes. Table 2.5 summarizes the most common biotechnological approaches utilized in dairy industry wastes.

Lactose-consuming microorganisms may be utilized for direct fermentation of whey with the goal of converting it into bioactive substances. Whey and whey permeate may be utilized to make a broad variety of goods with the aid of fermentation techniques. It is possible to utilize waste in a variety of ways, resulting in a various high-value products (Panesar et al., 2013). In the following subparts most common products that can be obtained from utilization of dairy wastes, particularly cheese whey, will be investigated briefly.

Biomass Production

The microorganism *Acutodesmus dimorphus* was shown to be highly efficient in consuming nutrients in raw dairy effluent (Table 2.5). Four days of cultivation was shown to be the most efficient method for reducing the level of pollution while also producing the most amount of biomass. After four days of culture, the level of COD had decreased by more than 90 percent, whilst the amount of ammoniacal nitrogen had been used completely after six days of cultivation. The researchers discovered that this biomass included 30% carbohydrates and 25% lipids, and it was claimed that it may be utilized for farther transformation into bioethanol and biodiesel. After conducting their research, the authors came to the conclusion that the culture of *Acutodesmus dimorphus* in dairy waste absent of any prior treatment is beneficial to waste discharge and produces a significant amount of biomass (Chokshi et al., 2016).

Geotrichum candidum is a fungus which produces lignolytic enzymes and may be capable of clearing industrial effluent. This microorganism was utilized in processing olive oil production wastes with a combination of whey. The recovery of olive oil yields 20% oil and 80% waste, referred to as the oil press. The oil press comprises 50% water and is made of olive soft tissues, plant material separates and water. The feasibility of growing *Geotrichum candidum* and producing biomass on a combination of milk whey and oil press water (20 % oil press water and 80% whey) was investigated (Aouidi et al., 2010). The authors cultured the fungus for five days at 30 °C in anaerobic environment and found that the combination may well be the most economic and most productive platform for biomass production, without the requirement for water dilution or nutrient addition.

Bioplastics

When it comes to biodegradable materials, bioplastics, particularly polyhydroxyalkanoates (PHA), have proven to be an excellent substitute for plastics generated from extremely polluting fossil-fuel sources. Several researches have been conducted to investigate the generation of PHA utilizing whey (Pakalapati et al., 2018). Using *Ralstonia eutropha DSM545*, Marangoni et al. synthesized PHA from disintegrated whey permeate. Interestingly, the scientists observed that the final concentration of the polymer had 38% of 3HV, in which quite intriguing from an economic standpoint, according to their findings (Marangoni et al., 2002).

Bacillus megaterium SRKP-3 may produce polyhydroxybutyrates (PHB), which are the most frequent kind of PHA. As a substrate, it is possible to utilize dairy industry waste, with the highest possible generation output of PHB (11.32 g L^{-1}) achieved after 36 hours of culture in an optimal medium (Ram Kumar Pandian et al., 2010). PHB is also produced by the bacteria *Brevibacterium casei*, which also has the potential to use wasted whey as a substrate, resulting in the creation of PHB. This bacterium can generate PHB (2.94 g L^{-1}) in 48 hours at 37 °C when grown in a submerged fermentation (Ram Kumar Pandian et al., 2010). Also

discovered was that *Pseudomonas hydrogenovora* was capable of co-metabolizing galactose and glucose from lactose-hydrolyzed whey permeates and producing PHB as a result (Koller et al., 2008). PHB is safe for the environment and has a significant impact on the medical area, among other things. Besides that, it may be utilized for the manufacture of nanoparticles, which are intended to enhance the administration of medications into the body (Ram Kumar Pandian et al., 2010).

Biofertilizer

It was discovered that the sludge created by the dairy sector to be beneficial in aiding the growth of fast-growing rhizobia. When comparing the findings of the study to the results of the conventional media (Yeast Extract Mannitol Broth) used for *Rhizobium*, it was discovered that 60% dairy sludge is a favorable growth condition that produces superior results. The study came to the conclusion that exploiting dairy industry waste as a substrate for biofertilizer synthesis will lessen the price of producing biofertilizers (Singh et al., 2013).

Biofuels

As contrast to burning fossil fuels, the combustion of biofuels emits far less pollutants into the atmosphere (Bhatia et al., 2018). Several yeasts, including *Kluyveromyces fragilis* (Parrondo et al., 2000), *Candida inconspicua* W16 (Dahiya and Vij, 2012), and *Kluyveromyces marxianus* DSMZ-7239 (Ozmihci and Kargi, 2007), can be utilized to produce ethanol from dairy by-products. By hydrolyzing the lactose in cheese whey (20%), *Kluyveromyces fragilis* generates 35.2 g L^{-1} of ethanol, which may be utilized to make alcoholic drinks or for other purposes. The process took place at 200 rpm, 30 °C, and for 24 hours, after which anaerobic fermentation took place for 34 hours (Parrondo et al., 2000). Dahiya and Vij's research involved immobilizing *Candida inconspicua* in Ca-alginate matrix for 72 hours, after which they were able to obtain large concentrations of bioethanol (30.3 g L^{-1}) using whey as a substrate. In the study, the yield of ethanol obtained from the experiments conducted with non-immobilized microorganism was 19.2 g L^{-1}, from

these data authors concluded that the immobilization process could result in a cost saving due to the higher yield obtained by immobilization (Dahiya and Vij, 2012).

Whey powder can also be utilized to produce bioethanol. By using *Kluyveromyces marxianus* DSMZ-7239 as biological agent and whey powder as substrate Ozmihci and Kargi obtained 0.54 grams of ethanol from per gram of lactose in whey powder (Ozmihci and Kargi, 2007). Spray or drum drying may produce whey powder for as little as 20 to 40 cents per kilogram of whey powder, which makes up for the distillation expenses of making pure ethanol from diluted cheese whey fermentations. Using whey powder instead of ultrafiltration to increase lactose concentration prior to fermentation may provide some other benefits such as stability over longer periods, a greater concentration of nutrients (lactose and others), and compact volume.

Clostridium acetobutylicum DSM 792 might be used to produce butanol from cheese whey having lactose within the range of 4.5 to 5.0%. Foda et al. studied the batch fermentation of whey and achieved the final titer of 1.5 g L^{-1} butanol after 5 days (Foda et al., 2010).

Green microalga *Chlorella pyrenoidosa* has the capacity to use nutrients from wastewater. An integrated strategy was employed to investigate the applicability of this microalga for the treatment of dairy effluent as well as the generation of biofuel (biodiesel). The conversion of biomass into biofuel was a multi-step process that required several steps. The extraction of oil from powdered algal biomass (after drying) was performed with the use of methanol and n-hexane. In the dairy wastewater, it was discovered that the phosphorus and nitrogen content decreased by 80–85% and 60–80%, respectively, with the generation of biofuels (6.7 mL) and biomass (18.8 g L^{-1}) after 15 days of incubation. It was concluded that *C. pyrenoidosa* is an effective agent for pollution load reduction and that it might be exploited as a possible agent for biofuel production. The integrated approach has the potential to bring down the cost of the process because the use of algal biomass for wastewater treatment from industries can aid in the reduction of

pollution load on the environmental sector, while the creation of fuel source can aid in the reduction of pollution load on the energy sector (Kothari et al., 2012).

It has been claimed that the culture of *Chlorella sp.* utilizing dairy effluent has a considerable potential for nutrient removal and biodiesel generation (Lu et al., 2015). Despite the fact that, Passero et al. as has demonstrated, more research, development, and implementation of algal production systems are required in order to produce economically viable algal biofuel, recent work has demonstrated significant progress in this field (Ding et al., 2015; Dong et al., 2016; Lu et al., 2015; Passero et al., 2014). The production of biofuel from microalgae *Acutodesmus dimorphus* was successful using dairy effluent as the primary substrate and without pretreatment. In four days of microalgae *Acutodesmus dimorphus* production, the findings of carbohydrate (29.64 ± 0.99%) and lipid (25.05 ± 0.77%) resulted in biofuel yields of up to 273 grams for kg of biomass (78 g bioethanol and 195 g biodiesel) which were indicate high scaling up prospects (Chokshi et al., 2016).

Another option is the creation of hydrogen by dark fermentation. Dark fermentation, which is a subtype of anaerobic digestion, is a common method of fermentation. It is the hydrolytic breakdown of rich carbohydrate feed into respective monomeric sugar molecules, followed by transformation into lactic acid, which is then converted into bio-hydrogen and other volatile fatty acids (Kargi et al., 2012). Several microorganisms are capable of producing hydrogen, such as *Enterobacter* sp., *Clostridium* sp., *Bacillus* sp., *Lactobacillus* sp., *Citrobacter* sp., and *Klebsiella* sp. are known to undergo the hydrolysis and acidogenesis processes (Kavitha et al., 2019; Qin et al., 2021a, 2021b). Researchers have obtained bio-hydrogen production yields of 160–170 L H_2 kg^{-1} TOC of whey. There are several criteria that must be closely followed, including the inoculation, bioreactor type, organic loading rate, substrate/feed content, residence duration, reaction temperature, acidity of the solution, and pre-treatment procedures that must be used. Given that pure strains like *Escherichia coli* and *Clostridium*

saccharoperbutyacetonicum produced 2.7 mol H_2/mol lactose (158 L H_2/kg COD lactose), it is worth noting that a combined mixture of both pure strain and dairy sludge bacteria could be utilized for best possible dark fermentation output. Dairy waste, particularly raw untreated whey feed, is the optimum feedstock for fermentation since the bacteria able to consume all of the nutrients present in the dairy by-product (Li et al., 2021). Using dairy cow manure and cheese whey in conjunction with sufficient ash and protein present in both waste streams appears to be the most beneficial, with yield quantities of 3 mol H_2/mol lactose and acetate/iso-butyrate concentrations above 5 g L^{-1} being the most favorable results (Policastro et al., 2021). However, even while pre-treatment can convert waste stock into a more acceptable substrate for dark fermentation by inhibiting methanogenesis, it incurs additional expenditures as a result of the treatment procedure. The highest capacity of load retention and the lowest mechanical energy consumption have been demonstrated in studies to be found in units such as sequencing batch reactors and fluidized bed reactors, which are the most suited types for dairy waste dark fermentation (Awasthi et al., 2020; Rosa et al., 2014). In the dark fermentation stage, the pH is an important aspect to consider since it impacts the generation of additional VFAs during the acidogenesis process. In a detailed research on the usage of cheese whey to produce bio-hydrogen, it was discovered that an optimal pH in the range of 5.5 to 6.5 was required for a mesophilic consortium to produce bio-hydrogen (Asunis et al., 2019; Qu et al., 2021).

Microbial fuel cell technology, which could also utilize dairy waste, has been investigated as an alternate and renewable source of electric energy (Bhatia et al., 2018). For two days, *Saccharomyces cerevisiae* PTCC 5269 cells generated a constant voltage, 470 μA of energy with a maximum current of 50 μW from sugar obtained from whey (Chandra et al., 2018). Another source of electricity might be the fatty waste generated by the flotation process in the dairy industry's wastewater treatment facility. These fatty wastes include a considerable concentration of organic content, mostly proteins and lipids. Due to their properties and the high

potential conversion yields of fat to methane, as a sustainable source of energy anaerobic biodegradation of fatty residues may be an intriguing method of creating biomethane (Hamawand et al., 2016). Wastewaters with high fat content, however, have drawbacks: lipids are difficult for microbes to access, and long-chain fatty acids exhibit inhibitory effects. Thus, numerous pretreatment methods such as enzymatic hydrolysis (mostly lipase), acid treatment (adding HCl to achieve a pH of 2–2.5), saponification, or thermal hydrolysis may be used to promote microbial degradation of fatty wastes (Carrere et al., 2012).

Single Cell Proteins

Single cell proteins (SCP) have a high protein content, which ranges from 40 to 80% of dry mass calculated upon crude protein. This is the most important property of single cell proteins. Furthermore, it is more similar to animal protein than to plant protein (Kasmi, 2018). When it comes to the creation of SCP, there are a variety of microorganisms that have been deemed "Generally Recognized as Safe" (GRAS) (Spalvins et al., 2018). Yeasts, on the other hand, are favoured since they are relatively inexpensive, can quickly grow, and can be recovered more easily than bacteria since they are bigger in size than bacteria (Kasmi, 2018).

The capacity of an organism to digest whey lactose is a major factor in its selection for use in SCP manufacturing. Biomass production is primarily restricted to lactose-consuming bacteria. There are a number of different yeast species that are utilized because of their capacity to break down lactose: Candida (*C. pseudotropicalis*), *Trichosporon* and *Kluyveromyces* (*K. lactis* and *K. marxianus*) (Kasmi, 2018). *K. marxianus* is considered as the best of the bunch, and it is also the most commonly employed for commercial purposes. For optimal development, microorganisms require simple inorganic or organic sources of nitrogen (for example yeast extract and urea) that are easily digested. A temperature range of 25–35 °C was shown to be ideal for the development of yeast and the generation of SCP. However, some strains of yeast may differ such as *C. utilis*. Most bacteria grow best at temperatures about 30 °C, although certain strains of bifidobacteria,

lactobacilli, and *Streptococcus thermophilus* (thermophilic) grow best at temperatures between 37 and 47 °C (Kasmi, 2018).

Bioactive Peptides

Precursor proteins include inactive amino acid sequences called "bioactive peptides", which can be located in the center of the protein. When released by enzymatic or chemical hydrolysis, they can have biological effects. Because of the variety of precursor protein resources and enzymes, the settings under which active peptides are produced vary. Using these bioactive peptides as nutritional supplements is possible due of their structure, quick absorption, and prompt exclusion. These chemicals are most often found in milk, whey, and colostrum (De Jesus et al., 2015).

Biotechnology has been used in several investigations to produce bioactive molecules. Pomiferin was extracted from the latex of Maclura pomifera fruits and used as a milk clotting agent to produce a hard and stable clot, suggesting that it might be used in place of chymosin in the production of milk clots. The bioactive peptides produced in the separated whey have antihypertensive and antioxidant effects. The researchers concluded that the resulting whey may be used in multifunctional foods by industry (Corrons et al., 2012).

Polysaccharides

It is possible to create polysaccharides such as xanthan gum (heteropolysaccharide) from whey by incubating it with *Xanthomonas campestris*. As a substrate for the fermentation process, hydrolyzed lactose derived from whey permeate is utilized in this approach (Mesomo et al., 2009). EPS (exopolysaccharides) are also produced using milk sugar, which is a byproduct of the dairy industry. In order to create exopolysaccharides such as dextrans, a strain of *Leuconostoc mesenteroides* can be used. Microbes are able to cling to surfaces thanks to EPS compounds, which also aid in the production of biofilms and provide protection to organisms from harmful

environmental agents. These chemicals contain functional features that can be used in the food and pharmaceutical sectors, as well as in other fields. These features include emulsifiers, viscosifiers, texturizers, stabilizers, detoxifiers, and anti-neoplastics, to name a few of their characteristics (De Jesus et al., 2015). Researchers found by utilizing *Streptococcus thermophilus* in a culture medium including deproteinized whey, it is possible to achieve an EPS production of 152 mg L^{-1} by fermentation (Ricciardi et al., 2002).

Biosurfactants

Biosurfactants are amphipathic compounds with chemical groups that are both hydrophilic and hydrophobic in nature, and they have the ability to lower the surface tension of liquids. Their synthesis might be carried out by bacteria, yeast, and fungi and they could be employed in a variety of industries, including food , agricultural, cosmetics, and pharmaceutical (De Jesus et al., 2015). It has been demonstrated that *Candida bombicola* is able to produce sophorolipids from synthetic dairy effluent. The authors hypothesized that real-world industrial effluent may be used for both the synthesis of sophorolipids and the pretreatment of effluent for use in other applications (Daverey and Pakshirajan, 2011). In a related work, the scientists investigated the viability of using dairy wastewater for the creation of sophorolipids by *C. bombicola*, with the twin goals of decreasing the organic pollutants in the effluent and lowering the cost of sophorolipid production (Daverey and Pakshirajan, 2011). An increase in the yield of sophorolipids from the process was observed when the yeast was grown in batch shake flasks on dairy wastewater enhanced with yeast extract, glucose, and soybean oil. The chemical oxygen demand measurement of the wastewater generated during the yeast fermentation indicated that the wastewater was cleared with an efficiency of greater than 80%. As a result, the researchers concluded that the system has a high potential for pretreatment of such wastewaters and the production of important bioproducts (Daverey and Pakshirajan, 2011).

If sophorolipids are to be employed in high-volume commercial applications like oil recovery and industrial cleaning, they must compete with surfactants of petrochemical origin on production capacity and cost. In other circumstances, such as in low-scale niche industries such as healthcare and cosmetics, high production expenses can be accepted because of the limited volume of product being produced. Sophorolipids, on the other hand, will require low-cost feedstock, such as whey, in order to be economically viable in low-cost applications (Felse et al., 2007). Ashby et al. proposed a process economic model for the fermentative production of sophorolipids utilizing existing system simulation software and actual equipment, reagent, and supplier costs, all while adhering to current manufacturing procedures. Glucose and either oleic acid or high oleic sunflower oil were employed as feedstocks in this experiment. The expense of large-scale sophorolipid manufacture through fermentation from glucose:high oleic sunflower oil was determined to be US$ 2.95 kg^{-1} while the cost of sophorolipid production from glucose:oleic acid was calculated to be US$ 2.54 kg^{-1}. Sophorolipids, according to the authors, are not economically feasible compared to their petroleum-based alternatives that are more commonly used; however, as the emphasis continues to shift toward eco-friendly products and the implementation of "green" components across many application fields, biobased materials like as sophorolipids might well proceed to become more appealing (Ashby et al., 2013). Rodrigues and Teixeira stated that studies should be concentrated on the integration of low-cost techniques for the manufacturing of biosurfactants. Some of the aspects that can have an impact on costs are the microorganisms that are chosen or modified, the method that is established, the choice of fermentation medium, the by-products of the operation, and the collection of the final product. It is also critical to consider the downstream processing that will be required to recover the obtained product (Rodrigues and Teixeira, 2008).

It has been demonstrated that *Bacillus licheniformis* has the ability of synthesizing a biosurfactant lipopeptide when being grown in whey. This lipopeptide has antibacterial efficacy against gram-positive bacteria as well as pathogenic

enterobacteria. As a result of their research, the authors came to the conclusion that the biosurfactant that was generated had significant potential for use in biotechnological and biopharmaceutical applications (Gomaa, 2013).

Enzymes

Biotechnology has been used to commercially synthesis a diverse spectrum of enzymes from microorganisms that have been screened and chosen. These chosen microbes have been optimized, characterized, and purposefully engineered in order to provide high-quality enzymes for pharmaceutical applications. Certain catalysts have a wide range of industrial uses in a variety of different bioprocessing applications (Jaganmai and Jinka, 2017). Certain strains, including *Pseudomonas sp*, *A. niger*, and *Streptomyces sp*, have found to be effective and shown to be capable of degrading organic molecules with relative simplicity. These microorganisms are capable of degrading fats and lipids contained in wastewater, and they are further separated for the production of lipase, which has a wide range of applications in industry. Lipases are used in a variety of applications, including fat modification, special organic synthesis, chemical analysis, racemic mixture resolution, and taste improvement in food applications (Jaganmai and Jinka, 2017).

Another example of enzyme that was effectively recovered from crude whey is lactoperoxidase. Lactoperoxidase can be extracted using an anionic surfactant and a liquid emulsion membrane (LEM) (Priyanka and Rastogi, 2018). The researchers also indicated that LEM with reverse micelle might be used in extraction and purification of lactoperoxidase as an alternate method.

Organic Acid Production

Industries such as the food, beverage, and pharmaceutical have a high need for a variety of chemical substances, which are typically utilized as additives in the production of various goods. Organic acids have the capacity to function as stabilizers, taste enhancers, preservatives, or acidifying agents, among other things.

Organic acids can be produced from dairy whey. Succinic acid, lactic acid, propionic acid and citric acid can be listed as the most common additives that are being used in industries (De Jesus et al., 2015).

Lactic acid might be produced utilizing yoghurt whey. In the study of Alonso et al., the strain *Lactobacillus casei* was used, and the yoghurt by-products was converted into lactic acid under regulated pH conditions. During the first 34 hours, the highest output of 25.9 g L^{-1} was achieved. The authors came to the conclusion that the suggested approach is a sustainable and intriguing bioprocess for the production of a value-added essential commodity from a feedstock that is often considered to be useless (Alonso et al., 2010). Lactic acid can be used in the manufacture of polylactic acid polymer (PLA), propylene oxide, acrylic fibers, and, propylene glycol.

Propionibacterium was shown to be capable of producing propionic acid from cheese whey. *Propionibacterium* strains might be called probiotic cultures since they produce vitamin B12 compounds and generate biomass. Propionic acid is routinely utilized as a preservative in the manufacture of vitamin B12 compounds and the formation of biomass (De Jesus et al., 2015).

Succinic acid is another valuable chemical that can be produced by using dairy wastes, particularly cheese whey, with biotechnological route. Cheese whey and *Actinobacillus succinogenes* might be used to create succinic acid. The first ever research performed to produce succinic acid from cheese whey with *Actinobacillus succinogenes* was conducted by Wan et al. (Wan et al., 2008). Accumulated succinic acid output of 0.57 g g^{-1} lactose was achieved under optimum process conditions. *A. succinogenes* 130Z was shown to be capable of converting whey to succinic acid, ultimately results in a fermentative process that was both efficient and affordable, according to the research (Wan et al., 2008).

Table 2.5 Compilation of selected studies on products produced with a biotechnological route by using dairy wastes and by-products as substrate

Products	Substrate	Microorganisms	Product	References
Food products	Whey and whey permeate	Lactose fermenting microorganisms	Whey-derived products	(Panesar et al., 2013)
Biomass	Whey	*Geotrichum candidum*	Biomass	(Aouidi et al., 2010)
	Wastewater	*Acutodesmus dimorphus*	Biomass (further processed to biodiesel and bioethanol)	(Chokshi et al., 2016)
Bioplastics	Whey	*Ralstonia eutorpha* DSM545, *Pseudomonas hydrogenovora*	PHA	(Koller et al., 2008; Marangoni et al., 2002)
	Dairy industry waste	*Bacillus megaterium* SRKP-3, *Brevibacterium casei* SRKP2	PHB	(Ram Kumar Pandian et al., 2009; RamKumar Pandian et al., 2010)
Biofertilizers	Dairy sludge	*Rhizobium*	Low cost biofertilizers	(Singh et al., 2013)
Biofuels	Whey	*Kluyveromyces fragilis*	Ethanol	(Parrondo et al., 2000)
	Whey powder	*Kluyveromyces fragilis*	Ethanol	(Ozmihci and Kargi, 2007)
	Whey	*Candida inconspicua* W16	Ethanol	(Dahiya and Vij, 2012)
	Whey	*Clostridium acetobutylicum*	Biobutanol	(Foda et al., 2010)
	Wastewater	*Chlorella pyrenoidosa*	Biomass and biofuels	(Kothari et al., 2012; Lu et al., 2015)
Bioenergy	Wastewater	*Acutodesmus dimorphus*	Biodiesel and bioethanol	(Chokshi et al., 2016)
	Fatty waste	Mixed	Biomethane	(Hamawand et al., 2016)
	Dairy waste	Mixed	Biohydrogen and methane	(Chandra et al., 2018; Venkata Mohan et al., 2010)
Bioactive peptides	Whey	-	Bioactive peptides	(Corrons et al., 2012)
Enzymes	Wastewater	*A. niger*, *Pseudomonas sp*, *Streptomyces sp*	Lipase	(Jaganmai and Jinka, 2017)
Biosurfactants	Wastewater	*Candida bombicola* ATCC22214	Biosurfactants	(Daverey and Pakshirajan, 2011)
Single cell protein	Whey	Lactose fermenting yeasts	Single cell protein	(Spalvins et al., 2018)
Polysaccharides	Whey permeate	*Xanthomonas campestris*	Xantham gum	(Mesomo et al., 2009)
	Deproteinized whey	*Streptococcus thermophilus*	Exopolysaccharides	(Ricciardi et al., 2002)
Organic acids	Whey	*Aspergillus niger* ATCC9642	Citric acid	(El-Holi and Al-Delaimy, 2003)
	Whey	*Actinobacillus succinogenes* 130Z	Succinic acid	(Wan et al., 2008)
	Sweet whey	*Propionibacterium shermanii*	Propionic acid	(De Jesus et al., 2015)
	Yoghurt whey	*Lactobacillus casei* ATCC393	Lactic acid	(Alonso et al., 2010)

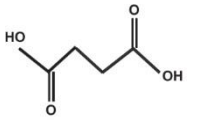
2.3 Bio-based Succinic Acid

Global warming, the greenhouse effect, and our reliance on fossil carbon, as well as heightened environmental laws, have prompted scientists to research ways for the production of industrially significant compounds from renewable sources. Currently, various nations throughout the world are engaged in the production and evaluation of bio-based compounds to determine their potential. Succinic acid, one of the most significant critical molecules that can be created biologically, was recognized as one of the most crucial of them (Davis et al., 1976; Dishisha et al., 2013; Q. Li et al., 2010b; Paster et al., 2004; Patel et al., 2006; Song and Lee, 2006). The relevance of this acid as a crucial ingredient in the synthesis of more than 30 economically relevant goods contributes to its applicability as a platform chemical (Bechthold et al., 2008; Isar et al., 2006; Jiang et al., 2014; Zeikus et al., 1999).

Succinic acid is a four-carbon dicarboxylic acid that is created as an intermediary in the tricarboxylic acid (TCA) cycle and is among the most significant fermentation products in the process of energy metabolism (Bretz, 2015; Yun et al., 2004; Zeikus et al., 1999). Succinic acid is also frequently identified in samples of atmospheric aerosol particles (Grosjean et al., 1978; Song et al., 2007). Pure succinic acid is solid at room temperature, and because of its poor solubility, it has a deliquescence point that is near to, or at, 100% relative humidity. It will, however, remain as an aqueous solution once it has deliquesced if the relative humidity is less than 60% (Peng et al., 2001; Riipinen et al., 2006). A summary of the chemical and physical characteristics of succinic acid has provided in the Table 2.6.

Table 2.6 Physical and Chemical Properties of Succinic Acid (Saxena et al., 2016)

Succinic acid also known as	amber acid; butanedioic acid; dihydrofumaric acid; asuccin; bernsteinsaure; kyselina jantarova
IUPAC name	Butanedioic acid
Molecular formula	$C_4H_6O_4$ ($HOOCCH_2CH_2COOH$)
Physical state	Colorless, odorless white crystals
Melting point	185-187 °C
Boiling point	235 °C
Solubility in solvents	Poor solubility in ethanol, ether, acetone, and glycerin; insoluble in benzene, carbon sulfide, carbon tetrachloride, and oil ether
Solubility in water	Soluble
Molar mass	118.09
Specific gravity	1.552
Flash point	206 °C
Density	1.56 g cm^{-3}
Vapor density	3.04
Acidity (pK_a)	pK_{a1} = 4.2, pK_{a2} = 5.6
Stability	Stable under normal conditions
Occurrence	Occurs naturally in plant and animal tissues
Common applications	Agriculture, food products, pharmaceuticals, and other industrial applications



Structural Formula of Succinic Acid

Succinic acid is also referred to as "amber acid" in some contexts. Amber has been utilized as a natural antimicrobial and general curative throughout Europe for hundreds of years as a result of its medicinal properties. At the time, Europeans were unaware that amber contains a high concentration of succinic acid, which has been shown to be a therapeutic agent.

Georgius Agricola, a mineralogist and physician, discovered how to purify succinic acid via dry distillation in 1546. Process of dry distillation separated amber into three components: acid, resin, and oil, each of which is extremely valuable and beneficial (Smyth et al., 1951). Robert Koch, a Nobel Prize laureate in bacteriology, studied succinic acid over three centuries later (1886). This study of Koch's turned out to be his master's thesis. He validated its beneficial effects and determined that, even after introducing significant amounts into the body, there is no risk of a buildup of excess succinic acid in the human organism (Petrie, 1943). Since then, succinic acid has found a variety of applications in various industries, such as food, agricultural, and pharmaceutical (Song and Lee, 2006; Zeikus et al., 1999).

Today, succinic acid is utilized in a variety of applications and is a crucial component in the production of many industrially essential goods, such as flavors and perfumes, surfactants and detergents, food additives, biodegradable polymers (clothing fibers), herbicides and, fungicides. It is also utilized as a precursor for a variety of compounds, such as N-methylpyrrolidinone, adipic acid, succinate salts, 2-pyrrolidinone, maleic anhydride, 1,4-butanediol, g-butyrolactone and, tetrahydrofuran, most of these are useful in various sectors but mainly they utilized in pharmaceutical industry. Succinic acid is an essential organic acid that is also applied as a plant growth regulator (Agarwal et al., 2007; Corona-Gonzalez et al., 2010; Lee et al., 2002, 2000; Song and Lee, 2006; Zeikus et al., 1999).

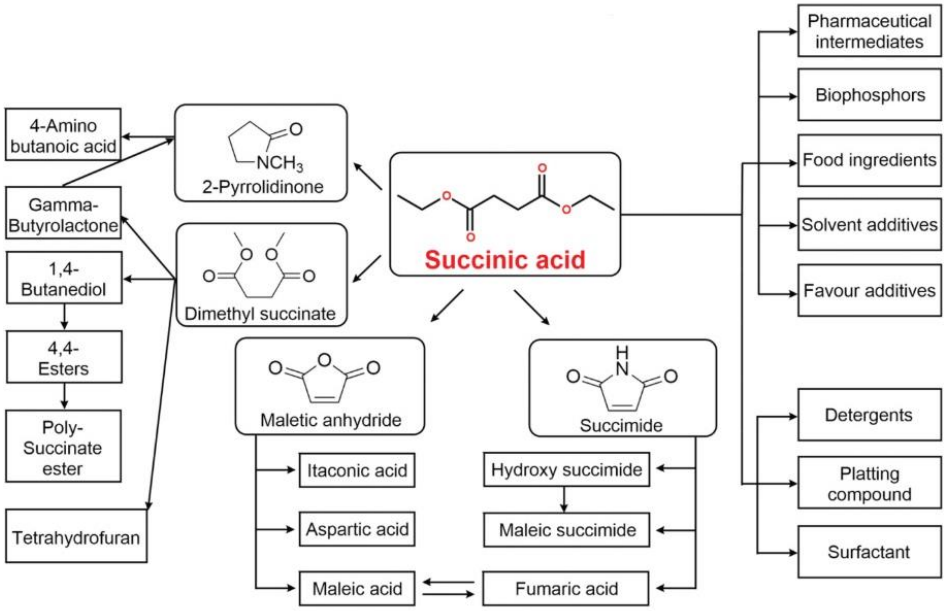


Figure 2.4 Summary of products and chemicals produced by using succinic acid

2.3.1 Market and Commercial Production of Succinic Acid

In accordance with the United States Department of Energy (DOE), succinic acid is one of the top 12 highest added value biobased chemicals and one of the most competitive biochemicals on the market today. Succinic acid, which is used as an intermediate in a variety of chemical processes, is also used as a platform chemical in the manufacturing of a variety of specialized compounds. During the 2013–2014 period, the market for bio-based succinic acid was estimated to be 38,000 tonnes per year, with a value of $2.90 per kilogram (Alexandri et al., 2019; E4tech et al., 2015). The global market for succinic acid is expected to increase at a compound annual growth rate (CAGR) of 24 % and reach 700,000 tonnes year^{-1} by 2020, according to industry predictions (Choi et al., 2015; Jansen and van Gulik, 2014; McKinlay et al., 2007b; Zeikus et al., 1999). Indeed, the succinic acid industry was valued at USD 131.7 million in 2018; the market is expected to develop at a rate of 6.8% per year until 2023, reaching a market size of USD 182.8 million by that time (Markets and Markets, 2019). This will be due to the growing number of uses for it

as well as the shift in the chemical industry toward environmentally friendly compounds derived from biological sources. The high costs of processing, as well as intense market competitiveness are, nonetheless, two of the most significant obstacles (Li and Mupondwa, 2021).

In comparison to petroleum-based succinic acid, biosuccinic acid is used mostly as deicer solutions, solvents and lubricants and in food, cosmetics, and pharmaceuticals. Others include the use of 1,4-butanediol (BDO), polyester polyols, plasticizers, and polybutyrate succinate (PBS) (Jansen and van Gulik, 2014).

Because of the bleak outlook for fossil energy accessibility, the dangers of petroleum resource depletion, and strict environmental regulations, the governments are becoming increasingly interested in investing in ecofriendly chemical compounds, which will almost certainly lead to an increase in the market's growth in the future. However, the high cost of biosuccinic acid, as well as the time-consuming and complicated downstream procedures, are the two most significant hurdles impeding market expansion (Li and Mupondwa, 2021).

According to reports, the most revenue was generated during the year 2013 in the pigment, coating and resin industries. However, it is anticipated that in 7 years, BDO would surpass all other application sectors in terms of market share. This is mostly due to the increased use of biosuccinic acid in the manufacture of BDO, as opposed to maleic anhydride. It is preferable to use 1.2 MT of biosuccinic acid to replace one MT of maleic anhydride (Law et al., 2019; Song and Lee, 2006).

The worldwide succinic acid market is categorized based on the applications for which it is used. There are several uses for succinic acid, including industrial applications (58%), pharmaceuticals (15%), food and beverage applications (14%), and others (13%). All of these applications have tremendous development potential, which is subsequently fueling the expansion of the succinic acid market in general. BDO has the greatest market share in the industrial applications

segment. Industry analysts believe that the key driver of increased demand for succinic acid in the chemical industry is the growing need for ecologically friendly chemicals (Research and Merkets, 2021).

A total of four businesses, Reverdia, Myriant, Succinity and BioAmber, built commercial-scale succinic acid facilities between 2012 and 2015. BioAmber and Reverdia developed and marketed a yeast-based method for succinic acid production, while Succinity and Myriant created a bacterial fermentation process, as well as a downstream process involving precipitation schemes, for succinic acid manufacturing (Sosa-Fernández and Velizarov, 2018). According to Reverdia, they have fundamentally created a process setup that manufactures succinic acid directly from maize starch in fermentation at a low pH (maximum 3.0) with *S. cerevisiae* (Jansen and van Gulik, 2014). This method leads to lower downstream yield losses and operational expenses due to the fact that it includes less unit operations. However, in order for this technology approach to be economically viable, a process design must be developed (Jansen and van Gulik, 2014). The technology employed by Myriant in its 14,000 tonnes $year^{-1}$ capacity facility was based on a platform that includes non-genetically modified *E. coli* strains in the fermentation stage (Jansen and van Gulik, 2014). Sorghum grain and other industrial sugars are among the renewable feedstock options available and used as substrates at this facility, which also has the potential to process other renewable feedstock options. Succinity used *Basfia succiniciproducenes* as fermentation agent, and its renewable feedstock sources include glycerol, sucrose, and glucose (Jansen and van Gulik, 2014; Sosa-Fernández and Velizarov, 2018). Despite their commercialization attempts, these firms found difficulties in entering new markets and maintaining sustainable growth only a few years after starting out. BioAmber, a Canadian succinic acid company, filed bankruptcy in 2018, and Myriant and Succinity have ceased operations, while Reverdia, potentially the last commercial succinic acid factory on the market, was dissolved in 2019 (McCoy, 2019). There was a confluence of causes that led to the downfall of these organizations. It has generally been acknowledged that certain technologies have proven to be too costly

and/or not yet suitable for commercialization in their current form. Today, few companies are claiming the continuation of production of biosuccinic acid and marketing bio-based succinic acid products, these are; Koninklijke DSM NV, BASF SE, LCY Chemical Corp., Mitsui & Co. Ltd., Shandong LanDian Biological Technology Co. Ltd., Mitsubishi Chemical Holdings Corp., Roquette Freres SA, PTT Global and Chemical Public Co. Ltd.. However, there are no official reports published in literature on procedures and applications used in the production process of these companies.

Table 2.7 Companies that have commercially involved in the production of bio-based succinic acid (Saxena et al., 2016)

Name	**Organism**	**Substrate**	**Capacity**	**Country**	**Year of Commercialization**
BioAmber-DNP Green Technology	*Escherichia coli*	Wheat-derived glucose	2000 tonnes/year	France	2010
Royal DSM NV-Roquette	*Escherichia coli*	Glucose/starch	10,000-20,000 tonnes/year	France	2011
BASF-CCM	*Basfia succiniproducens*	Glycerin or glucose	Unknown	Spain	2010
Roquette-Rice University	Genetically engineered *E. coli*	Glucose from corn and wheat	Unknown	France	2011
Reverdia	*Saccharomyces cerevisiae*	Starch derivatives	10,000 tonnes/year	Italy	2012
Myriant-BioEnergy International	*Escherichia coli*	Starch	15,000 tonnes/year	USA	2013
Succinity	*Basfia succiniproducens*	Glycerol, sucrose, glucose	10,000 tonnes/year	Spain	2013

2.3.2 Synthesis of Succinic Acid

Succinic acid was first discovered in nature in all plant and animal tissues. Molasses, beer, beef, peat, eggs, fruit, coal, urine, and honey are some of the examples that contains succinic acid (Vaghela et al., 2002). It has a substantial impact on their intermediate metabolism as well as the Krebs cycle. Despite their efforts, scientists were unable to discover a technique to extract succinic acid out from animal and plant tissues in its original form. Today, there are two ways that succinic acid can be created: chemically or biologically.

2.3.2.1 Chemical Synthesis

In terms of chemical production, succinic acid is produced via hydrogenation of benzene or n-butane to form maleic anhydride, which is then hydrated to form maleic acid. Maleic acid then hydrogenated to form succinic acid (Figure 2.5). As a result of the hydrogenation of maleic acid, obtained succinic acid is generally considered as not pure and requires further significant and often sophisticated purifying operations in order to produce a commercially viable product. A number of businesses have established hydrogenation technology for the production of succinic acid by converting maleic anhydride into succinic acid (Zeikus et al., 1999).

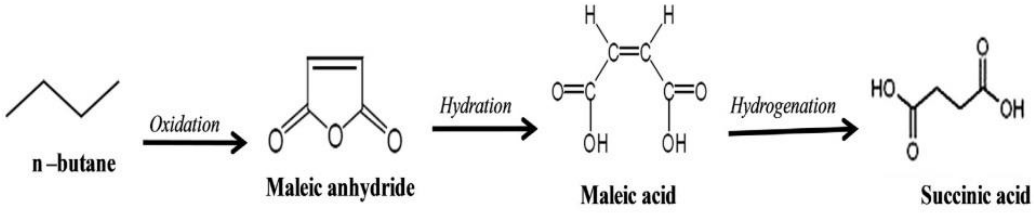


Figure 2.5 Chemical synthesis of succinic acid

2.3.2.2 Biological Synthesis

There are three biological mechanisms by which succinic acid can be created. The first is called the TCA cycle (Krebs cycle or the citric acid cycle), the second is called the glyoxalate cycle, and the third is called the reductive TCA cycle. However, succinate does not accumulate in cells during the Krebs cycle or the glyoxalate cycle and will further transform into other forms; as a result, these cycles cannot be used in the generation of succinic acid (Guettler et al., 1999).

Succinic acid is generated anaerobically by the reductive arm of the TCA cycle, in which PEP is transformed into oxaloacetate (OAA), which results in the synthesis of the end products succinic acid and propionic acid (Figure 2.6). In order to accomplish this, the assimilation of four electrons and one mol of CO_2 is required (Isar et al., 2006).

Processing of glucose into the four-carbon succinic acid product by either the oxidative arm of the TCA cycle or by the glyoxylate bypass pathway conserves only four of the six carbons originally present in glucose. However, the reductive arm of the TCA cycle generates two four-carbon acids for every glucose molecule digested by glycolysis in combination with pyruvate carboxylase, resulting in a net production of two four-carbon acids. Therefore, succinic acid-producing microbes favor anaerobic metabolism to aerobic metabolism (Van Der Werf et al., 1997). Most anaerobes are devoid of any enzymes that might further convert succinate. As a result, succinate is only accumulated in the cell when the cell is in anaerobic conditions.

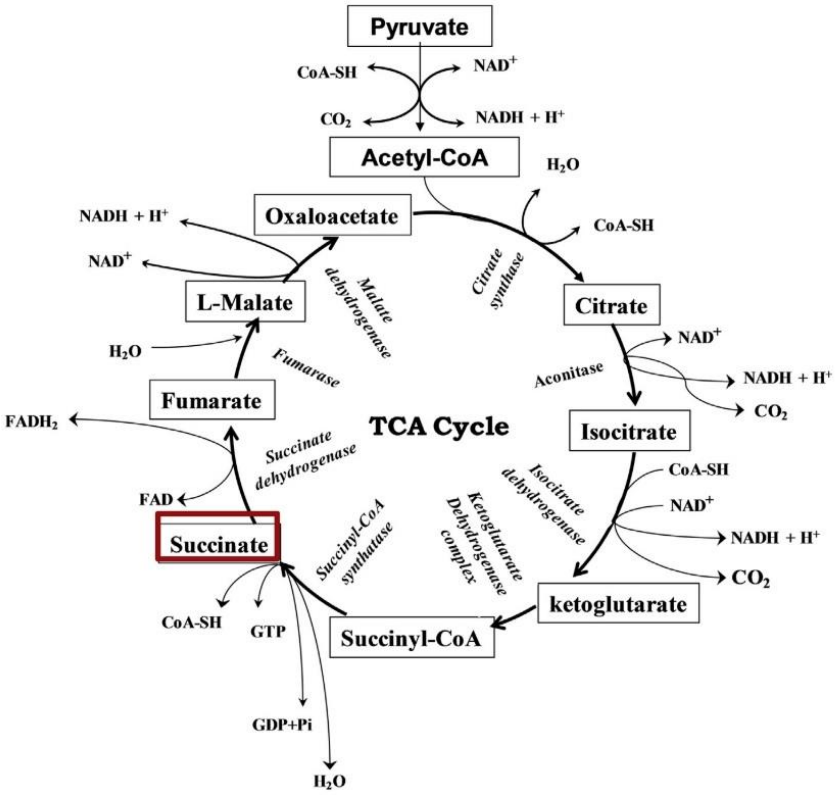


Figure 2.6 Biological synthesis of succinic acid (TCA cycle)

2.3.3 Succinic Acid Producing Microorganisms

A number of microorganisms have been reported that are capable of bioproducing succinic acid from carbohydrate substrates, ranging from naturally occurring species to genetically modified organisms (Table 2.8). However, while searching for prospective – industrially usable – biocatalysts for succinic acid synthesis, it is critical to take into account the microorganism's ability to create succinic acid in high yields, titers, and productivity. The yield of a biocatalyst is a measure of its capacity to channel the majority of the carbon source into the targeted product molecule, and it has an impact on the variable costs associated with the process. It is the concentration of product in the fermentation medium that dictates the ease with which it can be separated in downstream processes. This has an impact on both the capital investment required for separation equipment as well as the

variable expenses of the process. Finally, productivity shows the effectiveness with which production capacity is utilized, which has an impact on the process's capital expenses (capital expenditures). A thorough assessment of the fermentative synthesis of succinic acid has been carried out, encompassing topics such as market analysis and microbial hosts for biobased succinic acid production (Cao et al., 2013; Jansen and van Gulik, 2014; Song and Lee, 2006).

Naturally occurring producers of succinic acid, the microbial strains *Mannheimia succiniciproducens*, *Basfia succiniciproducens*, and *Actinobacillus succinogenes* have all demonstrated excellent performance, with the capability of degrading a diverse range of carbon sources under anaerobic conditions, resulting in efficient succinic fermentative production (Pateraki et al., 2016; Song and Lee, 2006). Each of these strains were isolated from the bovine rumen, in which they were most likely responsible for the accumulation of succinic acid, which serves as a precursor for the production of propionate, which accounts for 30% (w/w) of the volatile fatty acids in ruminal (Song and Lee, 2006). Natural producers, on the other hand, frequently face difficulties due to their non-fastidious characteristics, which necessitates the use of expensive and complicated growing medium. Fermentative succinic acid production capacity has been proven in two genetically engineered microorganisms: *Escherichia coli* AFP111/pTrc99Apyc and *Corynebacterium glutamicum* ldhApCRA717, both of which are capable of producing succinate in high titers and productivities (Okino et al., 2008; Vemuri et al., 2002). An important downside of genetically engineered microbes is their proclivity to return to the metabolism of their original environment when subjected to long-term fermentation (Christensen et al., 1995). When evaluating the commercialization of the method, this is a key risk issue. In order to improve performance, strains that naturally generate succinic acid at high titres and rates may serve as suitable starting points for optimization via metabolic engineering (Stephanopoulos, 1999). It would be advantageous to use these strains since they would require less genetic tinkering and would maintain stable phenotypes throughout the course of a lengthy fermentation.

The rumen bacterium *Actinobacillus succinogenes* is considered as the most promising amog competitive wild-type producers of succinic acid. Some of the most significant benefits of *A. succinogenes* in succinic acid production include its ability to utilize a wide range of carbon sources, its high tolerance to inhibitors, and its high fermentation efficiency, even when using raw renewable resources as substrates. Among the most significant drawbacks are *A. succinogenes*' particular need to vitamins (for example, biotin) and nitrogen sources (for example, yeast extract), the necessity of neutral pH for optimal growth, and the restricted genetic engineering tools available for genetic modification. The use of *A. succinogenes* to produce succinic acid in integrated biorefineries that use diverse renewable resources would provide nutrient-complete fermentation conditions at a lower cost than commercial nutrient sources. It is important to note that the usage of neutralizers, as well as the neutral pH of the fermentation broth produced by *A. succinogenes* cells, has an impact on both fermentation and subsequent separation expenses. Because the pH value of the fermentation broth impacts the dissociation level of succinic acid (pKa1 = 4.16 and pKa2 = 5.6), lower pH values of the fermentation medium result in lower downstream separation costs (Jansen and van Gulik, 2014).

Table 2.8 Succinic acid producing microorganisms

Type	Microorganism	Growth	References
Bacteria	*Enterococcus faecalis RKY1*	Facultative anaerobe	(Wee et al., 2002)
	Enterococcus flavescens	Facultative anaerobe	(Agarwal et al., 2007)
	Bacteroides fragilis	Obligate anaerobe	(Isar et al., 2006)
	Klebsiella pneumoniae	Facultative anaerobe	(Cheng et al., 2013)
	Succinivibrio dextrinosolvens	Obligate anaerobe	(O'Herrin and Kenealy, 1993)
	Basfia succiniciproducens	Facultative anaerobe	(Scholten et al., 2009)
	Mannheimia succiniciproducens	Facultative anaerobe	(Lee et al., 2002)
	Anaerobiospirillum succiniciproducens	Obligate anaerobe	(Samuelov et al., 1999)
	Actinobacillus succinogenes	Facultative anaerobe	(Guettler et al., 1999)
	Fibrobacter succinogenes	Obligate anaerobe	(Gokarn et al., 1997)
	Recombinant Escherichia coli	Facultative anaerobe	(Vemuri et al., 2002)
	Recombinant *Corynebacterium gluctamicum*	Facultative anaerobe	(Okino et al., 2008)
Fungi	*Aspergillus niger*	Facultative anaerobe	(David et al., 2003)
	Paecilomyces variotii	Facultative anaerobe	(Ling et al., 1978)
	Penicillium simplicissimum	Facultative anaerobe	(Gallmetzer et al., 2002)
Yeasts	Recombinant *Saccharomyces cerevisiae*	Facultative anaerobe	(Otero et al., 2013)
	Recombinant *Yarrowia lipolytica*		(Cui et al., 2017)

Table 2.9 Compilation of succinic acid production by different organisms from glucose

Strain	Process mode	Titer ($g\ L^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)	By-products	References
A. succinogenes						
$GXAS_{137}$	Anaerobic; batch	54.6	0.18	0.78	AA; FA;PA;PPA	(Shen et al., 2016)
130Z	Anaerobic; continuous	32.5	10.8	0.90	AA; FA; PA	(Maharaj et al., 2014)
DSM 22257	Anaerobic; continuous	26.5	0.36	0.75	AA; FA; PA	(Ferone et al., 2017)
CCTCC M2012036	Anaerobic; batch	38.3	0.97	0.77	AA; FA	(P. C. Chen et al., 2017)
CCTCC M2012036	Anaerobic; fed-batch	41.8	1.15	0.87	AA; FA	(P. C. Chen et al., 2017)
A. succiniciproducens						
ATCC 5348	Anaerobic; continuous	39.1	2.03	0.85	AA;FA	(Q. Yan et al., 2014b)
ATCC 5348	Anaerobic; batch	34.4	1.80	0.45	AA	(Q. Yan et al., 2014b)
ATCC 29305	Anaerobic; continuous	15.1	5.5	0.81	AA	(Lee et al., 2009)
M. succiniciproducens						
LPK7	Anaerobic; batch	52.4	1.80	0.76	AA; LA; PA; MA	(Lee et al., 2006)
E. coli						
AFP111	Anaerobic; dual phase	101.2	1.30	1.10	AA; EtOH	(J. Wang et al., 2018)
NZN111	Anaerobic; dual phase	6.93	0.70	0.62	AA; PA	(Jian et al., 2017)
HL27659K	Anaerobic; continuous	7.0	0.70	0.95	AA; PA	(Thakker et al., 2013)
AFP111-*lld*	Anaerobic; dual phase	30.1	0.70	0.77	AA; EtOH	(J. Wang et al., 2018)
C. glutamicum						
NC-3	Microaerobic; fed-batch with cell recycling	81.0	1.68	0.73	AA; PA	(Xu et al., 2016)
NC-3-1	Microaerobic; fed-batch with cell recycling	113.0	2.30	0.94	AA; PA	(Xu et al., 2016)

Table 2.9 (continued)

Y. lipolytica	fed-batch	30.2	0.40	0.50	CA; ICA	(Cui et al., 2017)
S. cerevisiae	fed-batch	57.8	1.10	0.58	CA; ICA; PA	(Wahl et al., 2017)

2.3.4 *Actinobacillus succinogenes*

Actinobacillus succinogenes is a gram-negative, facultative anaerobic, non-spore forming, non-motile, capnophilic rod that was isolated from the rumen of cattle and taxonomically put in the *Pasteurellaceae* family (Guettler et al., 1999). Based on the 16S rRNA gene amplification, *A. succinogenes* was classified as a Pasteurellaceae bacterium. The following is the taxonomical hierarchy that has been established: Bacteria, Proteobacteria, Gammaproteobacteria, Pasteurellales, *Pasteurellaceae*, and *Actinobacillus succinogenes*. *A. succinogenes* is a mesophilic organism that thrives at 37–39 °C in chemically defined media. *A. succinogenes* has the ability to utilize a wide variety of C5 and C6 sugars, along with numerous disaccharides and other carbon sources, such as glucose, arabinose, xylose, galactose, mannose, sucrose, fructose, cellobiose, lactose, maltose, glycerol and, mannitol (Carvalho et al., 2014; Jiang et al., 2014, 2013; Q. Li et al., 2010b; Wan et al., 2008; Zheng et al., 2009). Reduced carbon sources other than glucose, such as glycerol, mannitol, and sorbitol, result in greater succinic acid output. The use of C5 sugars, such as arabinose and xylose, on the other hand, leads in decreased succinate yields (J. Li et al., 2010a).

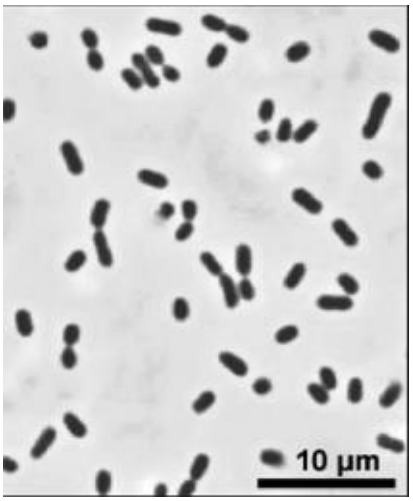


Figure 2.7 Microscopic image of *A. succinogenes* (Image source: James McKinlay, Michigan State University)

2.3.4.1 Metabolic Pathway of *Actinobacillus succinogenes*

Amongst the most important contributions to our understanding of the metabolism of *A. succinogenes* was provided by McKinlay et al. (McKinlay et al., 2007a) with the conduction of genome sequencing (McKinlay et al., 2010) and a ^{13}C metabolic analysis (McKinlay et al., 2005; McKinlay and Vieille, 2008). In the central metabolism of *A. succinogenes*, glucose is carried into the cell by a permease. Not only does glucose undergo phosphoenolpyruvate-dependent phosphotransferase activity, but it also undergoes hexokinase activity (Guettler, 1996; McKinlay et al., 2007a). Additionally, glucose-6-phosphate is catabolized via the glycolytic route to phosphoenolpyruvate (PEP), but the oxidative pentose phosphate pathway (OPPP) plays a minor role in the catabolism of glucose. Thus, NADPH is created from NADH by transhydrogenase and/or a conjunction of NADH-oxidizing malate dehydrogenase and NADP-reducing malic enzyme activity, whereas NADH is made via pyruvate and formate dehydrogenase activity (McKinlay et al., 2007a). Additionally, NADPH needs had an effect on the flow distribution between the C3 and C4 pathways, owing to the activities of pyruvate dehydrogenase and formate dehydrogenase. The Entner-Doudoroff and glyoxylate pathways are absent from *A.*

succinogenes fermentative metabolism. The four major flux nodes in *A. succinogenes* for succinate generation are pyruvate, phospoenolpyruvate (PEP), malate, and oxaloacetate (OAA) (Figure 2.8) (McKinlay et al., 2005).

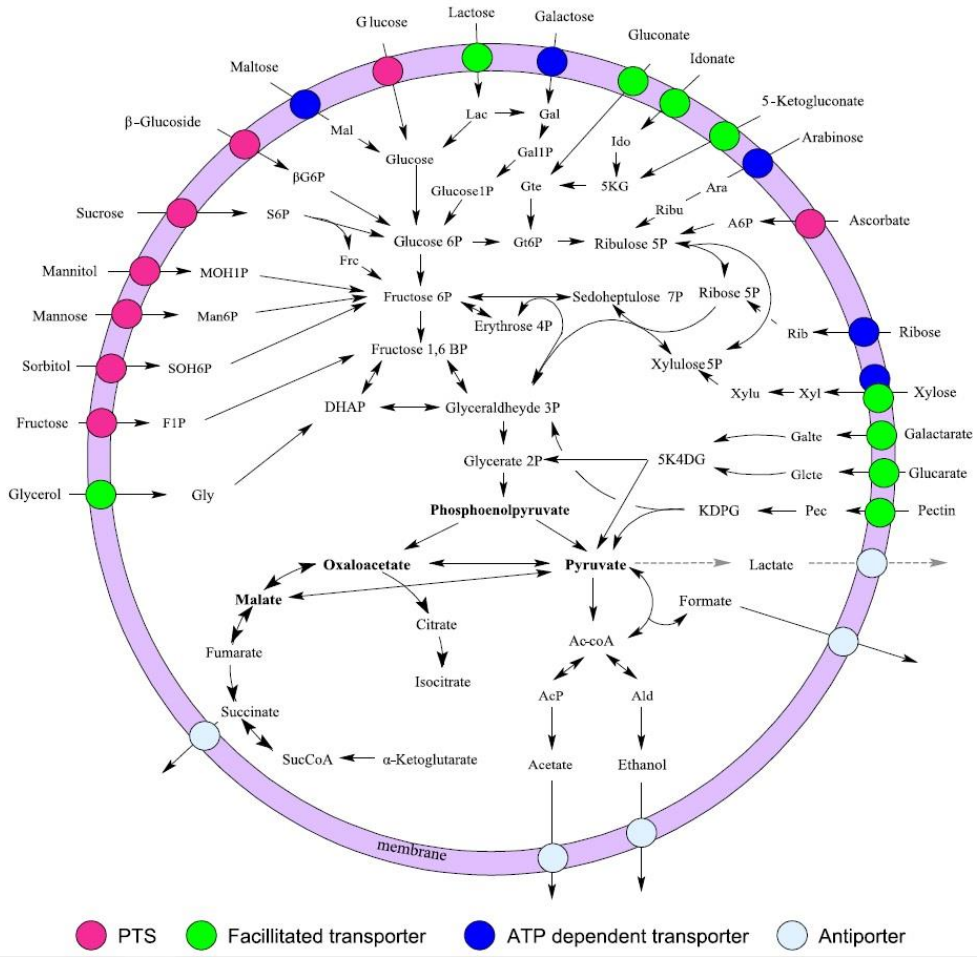


Figure 2.8 Metabolic pathways and possible sugar transporters of *Actinobacillus succinogenes*. (5K4DG: 5-dehydro-4-deoxy-D-glucarate; 5KG: 5-ketogluconate; A6P: Ascorbate-6-phosphate; AcCoA: Acetyl-CoA; AcP: acetyl-phosphate; Ald: Aldehyde; Ara: Arabinose; DHAP: dihydroxyacetone phosphate; E4P: Erythrose-4-phosphate; F1,6P: Fructose-1,6-biphosphate; F1P: Fructose-1-phospshate; 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-3-deoxy-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-7-phosphate; SOH6P: Sorbitol-6-phosphate; SucCoA: Succinyl-CoA; Xu5P: Xylulose-5-phosphate; Xyl: Xylose; Xylu: Xylulose; βG6P: β-Glucoside-6-phosphate.) (Pateraki et al., 2016)

The fixation of 1 mol of CO_2 and the input of 0.5 mol of glucose, 0.6 mol of xylose, or 1 mol of glycerol can theoretically result in the synthesis of 1 mol of succinic acid. Additionally, during the conversion of OAA to malate and fumarate to succinate, 2 moles of NADH are oxidized along the reductive route of the TCA cycle to form 2 moles of NADH. Therefore, the increased reducing capacity (NADH) required by the C4 pathway should be provided by other areas of the system (e.g. C3 pathway, glycolysis). The maximal glucose to succinic acid conversion yield is 1.12 g/g, which can be calculated using the stoichiometric equation shown below (McKinlay and Vieille, 2008).

$$CH_2O + 1/7CO_2 \longrightarrow 8/7CH_{3/2}O + 1/3ATP$$

Because of biomass and by-product generation, the predicted yield cannot be realized. By-product production is required for the synthesis of NAD(P)H and ATP. In order to produce 1 mol of acetic acid, 1 mol of ATP must also be produced in the same reaction. By using pyruvate-formate lyase, one mole of formate is created along with one mol of acetyl-CoA, which is a precursor to structural molecules and, eventually, biomass is formed. As a result, formate may be transformed into H^+ and CO_2, which is catalyzed by formate dehydrogenase, which can then be used as an electron donor for the formation of succinic acid. In addition, the C3 route leads in the creation of NADH, which is required for the generation of succinic acid via the C4 pathway. As a result, the potential conversion yields of glucose to succinic acid vary based on the metabolic pathway used (McKinlay et al., 2007b, 2007a, 2005). The reduction of by-product generation is the most important factor in increasing carbon source to succinic acid conversion yields. Genomic modification or evolutionary adaptation to produce superior strains of *A. succinogenes*, such as the FZ6 and FZ53 mutants, can be used to increase the efficiency of homo-fermentative succinic acid production (Guettler, 1996). In addition, the advancement of continuous fermentations with cell immobilization may result in less by-product production and greater yields and/or productivities than fed-batch or batch processes (Bradfield and Nicol, 2014; Uysal

and Hamamcı, 2021; Q. Yan et al., 2014b). During continuous cultures, *A. succinogenes* cells develop biofilms, reducing the amount of acetyl-CoA required for biomass creation and, as a result, reducing the amount of acetic acid and formic acid present in the media (Bradfield and Nicol, 2014).

2.3.4.2 Succinic Acid Production with *Actinobacillus succinogenes*

C5 and C6 sugars, disaccharides, and other carbon sources such as glycerol and mannitol, whey, cane molasses and corn-stover hydrolytes are some of the examples which can all be metabolized by *Actinobacillus succinogenes*. In biotechnological procedures, the cost of the substrate typically accounts for around half of the total expenses. In order to develop a cost-effective bioprocess, the use of low-cost renewable sources for succinic acid synthesis is a key consideration.

Often the most favored substrate for bio-based chemical synthesis is glucose. *Actinobacillus succinogenes* is capable of rapidly converting glucose to succinic acid. For example, *A. succinogenes* 130Z could produce 35.3 g L^{-1} succinic acid from 60 g L^{-1} glucose with a yield of 0.59 g g^{-1} (Kim et al., 2009). Similarly, 28.4 g L^{-1} succinic acid was produced from 40 g L^{-1} glucose with a yield of 0.71 g g^{-1} (van Heerden and Nicol, 2013). The metabolic kinetic study revealed that *A. succinogenes* 130Z could continuously create 32.5 g L^{-1} succinic acid from 50 g L^{-1} glucose (Maharaj et al., 2014).

Studies on the generation of succinic acid by *A. succinogenes* from various carbon sources have also been conducted recently and have been thoroughly examined. Due to the fact that xylose, the most prevalent pentose found in lignocellulosic hydrolyte, cannot be utilized as a substrate by most microorganisms, the economics of employing lignocellulosic hydrolyte as a substrate have been severely restricted. It was fortunate that *A. succinogenes* has the ability to naturally convert xylose into succinic acid. Succinic acid yields of 0.55–0.68 g g^{-1} were obtained when xylose was utilized as the substrate in a customized biofilm reactor. The production ranged

from 10.9 to 29.4 g L^{-1} of succinic acid (Bradfield et al., 2015). Furthermore, it was shown that xylose and glucose may be used at the same time without causing carbon catabolic suppression (Bradfield et al., 2015; Maharaj et al., 2014). *A. succinogenes* is capable of using disaccharides as well as monosaccharides. In serum bottle fermentation, *A. succinogenes* NJ113 was able to consume 80 g L^{-1} of sucrose in 36 hours, demonstrating that it is an efficient consumer of sucrose. Afterwards, a significant production rate of succinic acid (57.1 g L^{-1}) was achieved, with a yield of 0.72 g g^{-1}. It was also observed that, when sucrose was used, more metabolic routes were involved in production compared to glucose (Jiang et al., 2014). Jiang et al. was also investigated the potential of *A. succinogenes* NJ113 to utilize cellobiose and discovered that from 45 g L^{-1} of cellobiose, *A. succinogenes* was able to produce 30.3 g L^{-1} of succinic acid with a yield of 0.68 g g^{-1} in batch fermentation.

Succinic acid can be produced from a variety of industrial by-product and waste streams, some of the extensively studied examples are; sugar cane molasses, crude glycerol from biodiesel production, by-products of wheat milling, cheese whey, sake lees, agricultural residues such as corncob, corn fiber, bio-waste cotton, and sugarcane bagasse. Succinic acid production requires considerable amounts of nitrogen sources, such as yeast extract. The use of low-cost nitrogen supplies derived from renewable resources such as corn steep liquor (CSL) or the same renewable resource that supplies the carbon source such as waste bread, cheese whey or wheat milling by-products is potential candidates that can be utilized to lower the cost of succinic acid manufacturing costs. In the presence of yeast extract, whey as a carbon source gave succinic acid yields of 0.72 g SA/g lactose, which were only marginally decreased to 0.71 g SA/g lactose in the addition of CSL. Other nutrients, including vitamins and minerals, may also be provided via the exploitation of agri-industrial by-product and waste streams (Lee et al., 2003).

2.3.4.3 Parameters Effecting Succinic Acid Production by *A. succinogenes*

Substrate and Product Inhibition Effects

Product and substrate inhibition impedes the generation of succinic acid during fermentation. Homeostasis regulation and membrane breakdown owing to osmotic stress are possible mechanisms for growth inhibition caused by metabolic product production. Acids that are lipophile and undissociated can enter cells at neutral pH. H^+-ATPase, which expels protons from the cytoplasm (proton motive force) against concentration gradients, maintains pH homeostasis at high acid concentrations by the production of excessive ATP and the control of H^+-ATPase. Stress caused by excessive sugar and/or mineral concentrations causes cell shrinkage, which leads to membrane disintegration and eventual cell death (Pateraki et al., 2016).

A. succinogenes fermentation with sugar cane molasses and glucose as carbon sources was evaluated for substrate inhibition, with results revealing that substrate inhibition began at 50–60 g L^{-1} of concentration of glucose and 65 g L^{-1} of total sugar concentration, respectively (Liu et al., 2008a, 2008b). At initial glucose concentrations more than 158 g L^{-1}, the viability of the *A. succinogenes* 130Z strain was entirely stopped, whereas the yield of succinic acid was lowered and the lag phase was lengthened at greater than 100 g/L glucose concentrations (Lin et al., 2008). The inhibitory levels of formic acid, acetic acid, ethanol, pyruvic acid, and succinic acid were observed as 16.0, 46.0, 42.0, 74.0, and 104.0 g L^{-1}, respectively (Lin et al., 2008).

Effects of Different pH Controlling Agents

Acidic conditions are known to affect cell maintenance and control of intracellular enzyme activity throughout the succinic acid fermentation process. Succinic acid synthesis is directly linked to changes in the pH of a bacterial culture, which affects bacterial metabolism as well as CO_2 dissolution levels and the dissociation

equilibrium of HCO_3^- and CO_3^{2-}. Therefore, it may be concluded that optimum pH is a critical parameter for optimization. Succinic acid and by-product accumulation throughout the fermentation process resulting in a fall in pH of the system. Large quantities of neutralizing chemicals are therefore required to control pH levels (Yang et al., 2020).

Several studies have focused on the use of *A. succinogenes* to produce succinic acid using various neutralizing agents, such as $MgCO_3$, $CaCO_3$, Na_2CO_3, NaOH, and $NH_3.H_2O$. Complex interactions between nutrients and physicochemical variables led to the discovery that cells might be flocculated under certain circumstances (Yang et al., 2020). Due to the extreme cell flocculation, the addition of NaOH or KOH resulted in a reduction in succinic acid. Neutralizing agents $NH_3.H_2O$ or $Ca(OH)_2$ were also shown to inhibit bacterial growth (Uysal and Hamamcı, 2021). Cell flocculation can be prevented by employing the combination of 40 g L^{-1} of $MgCO_3$ and 5 M NaOH rather than using NaOH as the only neutralizing agent, resulted in a 28% increase in final concentration of succinic acid (59.2 g L^{-1}) (Wang et al., 2012). $MgCO_3$ supplementation not only halted the flocculation of *A. succinogenes* CGMCC1593, but it also extended the stationary phase and increased succinic acid synthesis (Liu et al., 2008a). In the study of Liu et al, the addition of solid $MgCO_3$ at pH 7.2increase the succinic acid production rate significantly (Liu et al., 2008a).

$MgCO_3$ was shown to be the most efficient pH regulator for the synthesis of succinate by *A. succinogenes* because it provides both CO_2 and Mg^{2+}, which are co-factors for PEP carboxykinase (Chen et al., 2016). To put it another way, $MgCO_3$ is the best alkaline neutralizer in terms of succinic acid production , sugar consumption, and bacterial growth. Despite its intriguing properties, $MgCO_3$ cannot be used on a big scale due to its prohibitive costs. Using a 1:1 combination of $Mg(OH)_2$ and NaOH in place of the more costly $MgCO_3$ might produce succinic acid in a similar manner (Li et al., 2011). If mixed alkali is used as the pH regulator in an industrial fermentation system, the overall cost of fermentation was reduced

by 54.9% (Li et al., 2011). However, researchers are currently looking for a more efficient and cost-effective pH adjuster for bio-succinate production.

Effect of CO_2 Source

Succinic acid synthesis in *A. succinogenes* is a CO_2 fixating process. Thus, it is important to optimize CO_2 supply for the production process. Bicarbonate (HCO_3^-) and CO_3^{2-} are in balance with the present dissolved CO_2 in the fermentation medium. It is important to note that, however, when gaseous CO_2 is used to feed system, the concentration of dissolved CO_2 during fermentation is affected by various parameters such as temperature, agitation, CO_2 flow rate, and partial pressure of CO_2. *A. succinogenes* fermentation can be facilitated by the addition of carbonate or bicarbonate salts (e.g. $MgCO_3$), irrespective of gaseous CO_2 supply (Zou et al., 2011). Carbonates and bicarbonates are converted into CO_2 during fermentation as a means of maintaining the reaction balance. Energy is required for the movement of carbonates and bicarbonates through cell membranes (Xi et al., 2011). However, from an industrial standpoint, delivering significant concentrations of insoluble carbonates is not feasible. According to Samuelov et al., the enzymes responsible for CO_2 fixation have a low affinity for CO_2 which means that succinic acid synthesis requires high CO_2 partial pressures in order to redirect metabolic flow towards succinic acid synthesis (Samuelov et al., 1991).

Using *A. succinogenes* ATCC 55618 as a model, Zou et al. studied the effects of gaseous CO_2 partial pressure, dissolved CO_2 concentration, and $MgCO_3$ supplementation on the synthesis of succinic acid. When just gaseous CO_2 was provided, the concentration of dissolved CO_2 was insufficient to optimize succinic acid synthesis, and the process failed. With the addition of 40 g L^{-1} of $MgCO_3$ and 101.33 kPa of CO_2 partial pressure (attained by pumping gas with 100% CO_2 content), the maximum succinic acid titer (60.4 g L^{-1}), productivity (0.84 g L^{-1} h^{-1}), and yield (0.58 g SA/g glucose) were achieved (Zou et al., 2011). According to Xi et al., when *A. succinogenes* NJ113 was grown with a CO_2 partial pressure of 0.1 MPa combined with 150 mM $NaHCO_3$, the best possible CO_2 fixation rate of 0.57

g L^{-1} h^{-1} could be obtained, resulting in a succinic acid production of 51.6 g L^{-1} and a yield of 0.76 g g^{-1} (Xi et al., 2011).

Rather than just capturing and storing CO_2, anthropogenic energy-related CO_2 emissions might be utilized to produce succinic acid, which focuses on CO_2 sequestration and recycling (Quadrelli et al., 2011). As the market price of CO_2 ranges between $60 and $450 per tonne, the amount of CO_2 that can be captured and stored each year might expand significantly (Quadrelli et al., 2011). Transportation costs for CO_2 should be kept to a minimum since they can contribute significantly to the total expense when the capture location is a long distance from the storage/recycling facility. As an alternative, the precipitation of CO_2 with Mg^{2+} solution for the creation of $MgCO_3$ which later might be employed in the synthesis of bio-succinate could be considered (Quadrelli et al., 2011).

Redox Potential Effect

The redox potential is a physicochemical measure that indicates whether a medium is being oxidized or reduced. The redox potential is a function of the pH, the equilibrium constant, the redox potential of the chemicals in solution and the amount of dissolved oxygen present in the system (Husson et al., 2006). The redox systems of all living species play critical roles in the processes of their respective lives. Redox potential ranges differ across microorganisms and between different species. In addition, extracellular redox potential has been found to influence the production or stability of specific enzymes, which has the potential to alter metabolic fluxes and/or ATP output (L. J. Chen et al., 2017).

There have been several attempts to explore the influence of redox potential on the generation of succinate by *A. succinogenes*. Researchers looked at the impacts of feeding H_2 and carbohydrate sources, as well as the effects of feeding electrons utilizing electricity (in a microbial fuel cell) on the synthesis of succinic acid (Schindler et al., 2014). Another study examined the flow distribution in *A. succinogenes* 130Z cultivated with and without H_2 (McKinlay and Vieille, 2008).

Although the mechanism of redox potential regulation in microbial physiology has been extensively described, it has not been thoroughly investigated to date. In the study of Li et al., the redox potential effect on succinic acid synthesis by *A. succinogenes* was studied and it was discovered that the flow of the hexose-monophosphate route (HMP) and the Embden–Meyerhof–Parnas (EMP) pathways were considerably altered following redox potential regulation and increased succinic acid yields and productivities were obtained (J. Li et al., 2010b).

In its role as an electron donor, H_2 has the ability to lower intracellular redox potential and improve NADPH recycles, which might lead to increased biomass creation and succinic acid yield owing to the incorporation of electrons acquired from H_2 (Carvalho et al., 2014). If 5% H_2 flow is used along with 95% CO_2 instead of 100% CO_2 flow, succinic acid production and productivity are raised by 5.8% (from 0.86 to 0.91g g^{-1}) and 80% (from 1.0 to 1.8 g L^{-1} h^{-1}) (Carvalho et al., 2014). A similar pattern of results was demonstrated once dimethylsulfoxide was employed to regulate the redox imbalance as an external electron acceptor, which was found to considerably increase succinic acid synthesis by *A. succinogenes*.

2.3.4.4 Immobilization of *Actinobacillus succinogenes*

Fermentation efficiency may be improved by using immobilized cultures, which are an alternate technology that can be used to improve process performance. Some of the features that make immobilization of microorganisms desirable for industrial biotechnological applications and an important subject for scientific study include increased fermentation yield, the suitability for continuous operation, the ability to reuse the biocatalyst, and an increase in microbial tolerance to inhibitors. In numerous techniques, such as trapping and encapsulation, electrostatic/covalent bonds, or absorption, microorganisms may be immobilized in a variety of different materials (Kourkoutas et al., 2004).

The synthesis of succinic acid by immobilized *A. succinogenes* cultures has been extensively investigated. Researchers studied the distribution of metabolites at steady state at various rates of sugar consumption, the rate and yields of succinic acid production when using immobilized and suspended cells (Bradfield and Nicol, 2014; Brink and Nicol, 2014; Maharaj et al., 2014; Urbance et al., 2004), and the fermentations of various carbohydrates and hydrolysates of lignocellulosic biomass in various types of reactors (Alexandri et al., 2017; Bradfield et al., 2015; Brink and Nicol, 2014; Corona-González et al., 2014; Ferone et al., 2018, 2019b; Salvachúa et al., 2016; Urbance et al., 2004; Zheng et al., 2009).

In the work done by Urbance et al. it was discovered that *A. succinogenes* immobilized on plastic substrates was capable of producing succinic acid (50% polypropylene). It was found that continuous fermentations, which were performed by using a 20 g L^{-1} glucose carrying stream fed at a rate of 0.02 h^{-1} dilution rate, resulted in a succinate productivity of 2.1 g L^{-1} h^{-1} and a yield of 0.72 g g^{-1} (Urbance et al., 2004). Fermentation studies using 40 g L^{-1} glucose solution as substrate were conducted in a repeated-batch mode, with the ultimate succinic acid titer in the second batch reaching 35.1 g L^{-1} (Urbance et al., 2004) . In the study of Yan et al., *A. succinogenes* cells were immobilized in a cotton fabric and fermentation experiments were performed using a continuous fiber bed bioreactor. The highest results were 55.3 g L^{-1} of succinic acid, 2.44 g L^{-1} h^{-1} of productivity, and 0.8 g g^{-1} of succinic acid output (Q. Yan et al., 2014a). Longanesi et al. evaluated five available biofilm carriers and determined that Glaxstone® – a sintered glass porous substance – was the most effective option in this case (Longanesi et al., 2018). Succinic acid synthesis by connected cells was unaffected by the feedstock used, which included pure lactose solution and cheese whey. It was also presented that in repeated-batch fermentations to synthesize succinic acid by biofilms of *A. succinogenes* in a packed bed bioreactor with a two-phase process. A succinic acid productivity of 0.72 g L^{-1} h^{-1} and a biofilm concentration of approximately 4 g L^{-1} were achieved (Longanesi et al., 2018). *A. succinogenes* entrapment in a continuous reactor to generate succinic acid from a non-detoxified,

xylose-rich maize stover hydrolysate stream was reported by Bradfield et al. (Bradfield et al., 2015). In this study, the greatest titer of succinic acid, the maximum yield, and the maximum productivity were reported as 39.6 g L^{-1}, 0.78 g g $^{-1}$, and 1.77 g L^{-1} h^{-1}, respectively.

Concerning immobilization through trapping in alginate or agar beads, various researches have been published demonstrating the method's efficacy. Corona-Gonzalez et al., for example, investigated the synthesis of succinate by immobilized *A. succinogenes* cells adhered/entrapped on a variety of materials, including zeolite, agar, and polyacrylamide hydrogel (Corona-González et al., 2014). The greatest results were obtained by beginning with a glucose containing medium and encapsulating cells in agar beads led to a final succinic acid titer of 43 g L^{-1}. Immobilization by entrapment, on the other hand, would offer more favorable conditions for the organisms to generate succinic acid in comparison to the adhesion technique (Corona-González et al., 2014). Alexandri et al. evaluated *B. succiniciproducens* and *A. succinogenes* cultures immobilized in alginate beads. The fermenting medium was spent sulfite liquor. Succinic acid was generated by *B. succiniciproducens and A. succinogenes* at final concentrations and yields of 45.0-35.4 g L^{-1} and 0.66-0.61 g g^{-1}, respectively. *B. succiniciproducens* cells immobilized in alginate beads were utilized in four subsequent fed-batch fermentations. Alginate beads were shown to retain cells and allow for adequate nutrient absorption, resulting in higher succinic acid concentrations than free cell fermentations. Additionally, the reuse of the same biocatalyst in multiple batch systems provided evidence that entrapment in alginate might be employed to produce succinic acid on a wide scale (Alexandri et al., 2017).

Biofilms and immobilized cells, as has been well documented in the literature, may be employed in a variety of bioreactor configurations, including CSTRs, fluidized bed reactors (FBRs), airlift reactors, packed bed reactors (PBRs) (Qureshi et al., 2005). The study of Alexandri et al. found that the size of (Ca-alginate) beads at the start of the experiment influences the set of consecutive batches that can be

performed by reusing the beads (Alexandri et al., 2017). This is consistent with previous observations that the size of the beads at the start of a fermentation influences the performance of cells entrapped in permeable beads (Urbance et al., 2003). It was discovered that the size of the beads grew with each fermentation cycle as a result of cell development, and that after multiple cycles, beads ruptured as a result of both erosions resulted from calcium sequestration and cell growth. In the study of Mishra et al., the influence of bead size on ethanol generation via fermentation was investigated, and it was discovered that medium and large size beads (diameter of 4–5 mm) might be utilized for more fermentation cycles than smaller size beads (3.0 mm) (Mishra et al., 2016). It was also asserted that the loss of smaller beads is more rapid since the increased specific surface area of the beads causes them to tend to connect to one another. Furthermore, calcium release happens quickly as a consequence of the increased mass transfer from the beads to the solution.

Studies in the literature that have investigated the succinic acid production with immobilized *A. succinogenes* are listed in Table 4.6.

2.4 Aim of the Study

This study aims to become the first in the literature for the fermentative production of succinic acid with immobilized *Actinobacillus succinogenes* cells using cheese whey as carbon source. Furthermore, stability and strength of immobilization support (Ca-alginate beads) was investigated. Improvement in Ca-alginate bead strength was achieved by using alginate-whey mixture to immobilize cells. Applied procedure in cell immobilization using combination of fermented whey directly with alginate was studied for the first time. To meet these goals, the following objectives were achieved:

- *A. succinogenes* cells were grown in both defined media containing glucose and lactose and also cheese whey containing media.

- Successful fermentations were conducted and succinic acid was produced by using all three fermentation media in shake flasks and a bench-top bioreactor with free cells of *A. succinogenes*.
- *A. succinogenes* cells were immobilized using alginate and the system was further improved by using alginate-whey mixture as immobilization support.
- Fermentations using immobilized cells were performed in a bioreactor using cheese whey, glucose and lactose containing media in batch form.
- Continuous and repeated-batch fermentations were performed using immobilized cells in cheese whey containing media.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and Chemicals

The cheese whey used in the experiments was kindly provided by Açkar Dairy Products Inc., directly supplied from the cheese production facility located in Kazan, Ankara, Turkey.

The analytical grade of all of the substances used in the studies was carefully selected. A list of chemicals and the commercial manufacturers of those substances are presented in the Table A.1.

3.1.1 Microorganism

Actinobacillus succinogenes 130Z (CCUG 43843T) was obtained from the Culture Collection University of Gothenburg within the scope of a project supported by the Scientific and Technological Research Council of Turkey (TUBITAK) (Project No: 115Y826). Obtained cultures were shared with all project associates after they were cultivated, analyzed and stocked for transportation in the laboratories of the project partner Genetics and Bioengineering Department, Yeditepe University.

3.2 Methods

3.2.1 Genetic Analyses and Stock Conditions of *Actinobacillus succinogenes*

Obtained lyophilized cell samples of *A. succinogenes* were cultivated on blood agar, chocolate agar and TSA (Tryptic Soy Agar) solid media in sterile conditions, grown in an anaerobic jar, then individual colonies were grown in TSB (Tryptic Soy Medium) liquid medium and their optical densities were determined. When it comes to around 1.0 (approximately 18-24 hours), they are taken into glycerol stock (50% v/v) and kept at -80 °C.

DNA isolation was made from cells taken from liquid culture grown by inoculation from selected colonies; 16S region of the organism was amplified by Polymerase Chain Reaction (PCR) using degenerate primers and sequenced with Sanger technique. In this way, it has been confirmed that the organism used is *A. succinogenes* (against the danger of contamination etc., since the organism has no known antibiotic resistance or auxotrophy). Genetic analyses were conducted by the project team from Yeditepe University in the Department of Genetics and Bioengineering, Yeditepe University.

3.2.2 Inoculum Preparation and Biomass Determination

From glycerol stocks kept at -80 °C, *A. succinogenes* cells were taken by using a sterilized loop and inoculated on TSA aseptically with spread plate method. Inoculated TSA solid media were incubated for 24 hours at 37 °C. From incubated agar single colonies of *A. succinogenes* cells were inoculated and activated in 5 mL sealed screw-capped tubes containing TSB. Inoculated tubes containing 5 mL of TSB were incubated at 150 rpm and 37 °C for 18-24 hours. Explained procedure was repeated two times in fresh culture activation before all experiments to obtain a mature preculture.

In order to determine inoculation time, activated cells in 5 mL TSB were cultivated in 250 mL Erlenmeyer flasks containing sterilized TSB at 37 °C. Samples were taken every two hours under aseptic conditions and optical density at 600 nm was recorded by spectrophotometer (UV 1202, Shimadzu, Japan). In order to establish a correlation between optical density and dry mass of cells, taken samples were precipitated by centrifugation after optical density measurement. Precipitated cells were washed several times with distilled water and left to dry at 100 °C. After all liquid evaporates dry weight of cells determined. According to the results obtained, *A. succinogenes* cells with an optical density of 1.00 at 600 nm correspond to a value of approximately 0.48 g Dry Biomass/L.

3.2.3 Growth and Fermentation Conditions

3.2.3.1 Synthetic Media

In the experiments, as synthetic growth media two different pure carbon sources were tested. Glucose and lactose containing synthetic media compositions are presented in Table 3.1.

Table 3.1 Synthetic media compositions

Compound	**Concentration (g L^{-1})**
Glucose or Lactose	35-60
Yeast Extract	7.0
K_2HPO_4	2.7
KH_2PO_4	2.4
NaCl	1.0
$CaCl_2$	0.3
$MgCl_2$	0.3

Batch fermentation and shake flask tests were carried out with two alternative glucose and lactose concentrations (60 g $^{-1}$ and 35 g L^{-1}). In all of the experiments, magnesium carbonate ($MgCO_3$) was employed as an indirect CO_2 source within the system (40 g L^{-1}). This is preferable to continuous CO_2 gas sparging. In the study of Zou et al., it was claimed that when $MgCO_3$ was utilized alone as a CO_2 donor, no significant changes were seen as compared to gas sparging in both succinic acid synthesis and cell growth of *A. succinogenes*, leading the determination that sparging of gas CO_2 was not required (Zou et al., 2011).

3.2.3.2 Cheese Whey Containing Media

Açkar Dairy Products Inc. provided the cheese whey for this project, which came from their production facility in Ankara, Turkey. The precise initial lactose and lactic acid amounts may differ from sample to sample. Each batch of cheese whey acquired was tested using high-performance liquid chromatography (HPLC) to ascertain its lactose and lactic acid concentrations before being included in the study. The amount of protein found in cheese whey can also vary. It was necessary to apply the Lowry protein test to evaluate the protein composition in cheese whey. Protein concentrations were found to be between 2.0-2.5% in all of the samples tested. All cheese whey samples were autoclaved upon arrival and stored at 4 °C to reduce the buildup of lactic acid caused by starter cultures that were remained from the cheese making process (Rao et al., 2004).

Concentration of lactose present in cheese whey samples were determined by using HPLC and found approximately 60 g L^{-1}. The mineral and yeast extract solution was used to dilute the cheese whey to a final lactose concentration of 35 g L^{-1}. Concentrations of minerals and yeast extract in the final mixture were adjusted to same compositions with the synthetic media containing lactose or glucose.

Table 3.2 Cheese whey containing media compositions

Compound	**Concentration (g L^{-1})**
Whey	35 (concentration of lactose in the final mixture)
Yeast Extract	7.0
K_2HPO_4	2.7
KH_2PO_4	2.4
NaCl	1.0
$CaCl_2$	0.3
$MgCl_2$	0.3

3.2.4 Free Cell Experiments

Free cell experiments were conducted by using synthetic media with different glucose-lactose concentrations and also with cheese whey containing media. Batch fermentations were tested in shake flasks and in a bioreactor.

3.2.4.1 Shake Flask Fermentation Experiments with Free Cells

Aluminum sealed shake flasks with 250 mL of total volume were used in shake flask experiments. Glucose, lactose and cheese whey containing media were inoculated with previously prepared precultures by 10 % (v/v). Working volume was chosen as 200 mL in all experiments. In order to approach anaerobic conditions as much as possible, high working volume preference was made since flushing the flasks with CO_2 gas was not applicable.

The fermentation experiments were carried out in a shaker incubator (Infors HT, Switzerland) at a constant temperature of 37.2 °C and 150 rpm stirring speed in

order to achieve homogeneous mixing for 24 hours. In preparation for the aseptic inoculation, the fermentation media were autoclaved at 121 °C for 15 minutes.

Incubated shake flask growth medium cultures were analyzed and later these cultures were used as inoculum for bioreactor fermentation experiments.

3.2.4.2 Batch Fermentation Experiments in Bioreactor with Free Cells

Batch fermentation tests were carried out utilizing previously incubated growth medium cultures as the inoculum. For the fermentations, a 3 L jacketed bench top fermenter was used with a 2 L of working volume. Lactose concentration in cheese whey was determined to be 60 g L^{-1}. The final lactose content in the solution was 35 g L^{-1} after the cheese whey was diluted with the mineral and yeast extract solution. The final mixture's yeast extract and mineral concentrations were adjusted to match those given in Table 3.1. With this working volume (2 L), 35 g L^{-1} lactose or glucose in defined fermentation medium and 1 L of cheese whey was used in whey fermentation media (corresponding lactose concentration to be 35 g L^{-1}) were used for the comparison of findings. The undesirable Maillard reactions were prevented by autoclaving cheese whey, lactose, and glucose individually for 10 minutes at 121°C in all trials. Separately autoclaved minerals and salts (15 minutes at 121 °C) were also combined with carbon source aseptically to eliminate undesired precipitations. To avoid bioreactor line obstruction and to maintain the pH probe moist, the fermenter was autoclaved for 15 minutes at 121 °C with deionized water loaded instead of fermentation medium. Deionized water was pushed out of the fermenter with high pressure CO_2 gas before the process began, which was filtered through a 0.22 μm PTFE filter. After that, using CO_2 pressure, the inoculated fermentation medium was reloaded into the bioreactor. This method was chosen over using peristaltic pumps to discharge/load media because it maintains an anaerobic environment while also reducing the danger of contamination. The bioreactor was once again flushed with CO_2 prior to the commencement of the procedure to ensure anaerobic conditions. Samples were

collected every 6 hours and after each sample collection reactor was flushed with CO_2 gas for 30 minutes to ensure anaerobic environment. A 5M KOH solution was used to maintain a pH of 6.80±0.1 by using automated controller. Temperature, agitation speed and pH were controlled and monitored automatically kept constant at 37.2 °C, 150 rpm and 6.80, respectively.

3.2.4.3 Parameters Evaluated in Free Cell Bioreactor Experiments

In batch fermentation experiments with bioreactor, effect of 4 parameters on fermentation results were evaluated while all other conditions were kept constant. These are; effect of different carbon sources, initial carbon source concentration, different neutralizing agents and indirect CO_2 source.

Effect of Initial Sugar Concentration

Two different initial lactose and glucose concentrations were tested to observe the effect of initial substrate content on fermentation. Explained procedures in chapter 3.2.4.2 were applied with starting concentrations of 35 and 60 g L^{-1} for both lactose and glucose containing synthetic media.

Effect of Neutralizing Agent

For pH control in bioreactor fermentation experiments three different neutralizing agents (KOH, $Ca(OH)_2$, NaOH) were tested to observe their effects. For NaOH and KOH, solutions with same molarities were used (5 M). For $Ca(OH)_2$; 100 g L^{-1} of $Ca(OH)_2$ solution was used. Solution of $Ca(OH)_2$ was prepared in 1 L Erlenmayer flask with stirring magnet in it. Since $Ca(OH)_2$ is poorly soluble in water, in order to maintain homogeneous flow of solution into the bioreactor, flask is kept on a magnetic stirrer and constantly stirred at 200 rpm.

Effect of CO_2 Source

Two different bicarbonate salts ($NaHCO_3$ and $MgCO_3$) were tested as indirect CO_2 sources in fermentation experiments. Solutions were added into fermentation medium at the start of the process with a concentration of 40 g L^{-1}.

3.2.5 Immobilized Cell Experiments

3.2.5.1 Immobilization of *Actinobacillus succinogenes*

As immobilizing agent alginate was employed in this study. The approach used earlier by Ercole et al. for bead preparation was followed in immobilization procedure (Ercole et al., 2019). Na-alginate solution (2% w/v) was prepared and autoclaved (15 minutes at 121 °C) to ensure that it was completely sterilized. Cell/polymer suspension was obtained by mixing the alginate solution with previously incubated (24 h) TSB preculture by 20% (v/v) (Ercole et al., 2019). It was decided to conduct the Ca-alginate bead production directly in the fermenter in order to eliminate contamination risk while transporting beads into bioreactor. After autoclaving the fermenter, it was filled with 500 mL sterilized 2% v/v $CaCl_2$ solution. In the fermenter one of the inlet lines was fitted with a syringe, prepared 20% (v/v) alginate-cell culture mixture was introduced to $CaCl_2$ solution drop wise through syringed line while the solution in bioreactor was being stirred at 35 rpm. The beads created were left in the $CaCl_2$ solution for 2 hours before being removed from the solution. A final wash with fresh 2% $CaCl_2$ solution was performed on the remaining beads, which had a diameter of around 4 mm before the addition of fermentation medium into the bioreactor. In order to discharge any liquid solution or media from fermenter while keeping beads inside, a fermenter outlet line with small diameter (1 mm) was fitted on bioreactor.

3.2.5.2 Immobilization of *A. succinogenes* by Using Alginate-Whey Mixture

In order to obtain beads with higher durability, a mixture of whey and alginate solution was used while applying the immobilization procedure. First, the fermentation medium containing whey was autoclaved and then inoculated with preculture. A stirrer magnet was present in the fermentation medium during autoclaving. This prepared inoculated medium was incubated at 150 rpm and 37 °C for 24 hours in the incubator. After all the lactose in the medium was consumed by the cells, the resulting medium was mixed with a 2% alginate solution at a rate of 40% by volume. The resulting mixture was fed to the fermenter by continuous mixing on a magnetic stirrer so that it could be fed to the fermenter in a homogenized manner. Feeding to the fermenter was done drop wise, as described previously, via a fermenter line with a syringe at the end. Other than that, all procedures were performed as described in the previous section.

3.2.5.3 Batch Fermentation Experiments with Immobilized Cells

The batch fermentation procedures described earlier were also applied in the immobilized system. Fermentation media containing 35 g L^{-1} glucose, lactose and whey (lactose concentration) were tested separately using immobilized cells. Concentration of yeast extract, minerals and salts in fermentation media were kept same as the batch fermentation experiments conducted with free cells.

Batch fermentation experiments were performed using both beads obtained with whey-alginate solution and beads produced with only alginate solution, separately. In order to test, optimize and compare the durability of the beads, the system was tested by changing parameters such as stirring speed, pH control agent and working volume (Table 3.3). Optimization of system was conducted by using lactose and glucose containing synthetic media. After the determination of best options for working volume, neutralizing agent and stirring speed all tests were performed again with optimized conditions with cheese whey containing media. During

fermentations, samples were collected from the system every 6 hours and analyzed. After each sampling, the system was pumped with carbon dioxide gas, trying to preserve the anaerobic environment as much as possible. In all immobilized batch experiments 40 g L^{-1} $MgCO_3$ was employed as an indirect CO_2 source. Temperature of the system was kept constant at 37.2 °C in all experiments.

Table 3.3 Operating conditions of immobilized cell fermentation experiments

	Stirring Speed (rpm)	Neutralizing Agent	Working Volume (L)
1	150	NaOH	2.0
2	150	KOH	2.0
3	150	$Ca(OH)_2$	2.0
4	100	KOH	2.0
5	75	KOH	2.0
6	150	KOH	2.5
7	150	KOH	1.0

3.2.5.4 Repeated Batch Fermentation Experiments with Immobilized Cells

Repeated batch fermentation experiments were performed under optimized conditions that were tried and determined previously during batch fermentations with immobilized cells. Cheese whey containing fermentation medium (Table 3.2) and the beads prepared by using alginate-whey mixture were used in the repeated batch fermentations. Stirring speed, temperature, and pH were controlled automatically and kept at 150 rpm, 37.2 °C, and 6.80±0.10, respectively. Working volume was chosen as 2 L and 250 g of immobilize beads were used with this working volume. Bead preparation procedure was applied as same as in the batch

fermentation experiments. As neutralizing agent 5 M KOH solution was used. $MgCO_3$ was introduced as indirect CO_2 source.

Samples were taken every 6 hours and each cycle of fermentation was terminated when all lactose in the medium were consumed completely or stood unchanged for 24 hours. After each cycle, fermentation medium in the bioreactor was discharged with the help of CO_2 gas pressure. For consecutive fermentation cycle, before the addition of fresh sterilized medium into the bioreactor, remaining beads in the fermenter were washed with sterilized 2% $CaCl_2$ solution and lastly washed with sterilized deionized water to ensure that no free cells or residues were remained in the system. For the first 2 hours of fermentations in each cycle, system was gassed with CO_2 gas through a 0.22 μm filter to remove all dissolved/free oxygen in the system and ensure anaerobic conditions.

3.2.5.5 Continuous Fermentation Experiments with Immobilized Cells

Continuous fermentations were carried out by using whey containing medium with a starting working volume of 2 L. A schematic diagram of continuous system used in the experiments was presented in Figure 3.1. Two Erlenmeyer flasks with 2.5 L total volume were used as inlet and outlet vessel with inlet flask containing 2 L sterilized whey medium. Fresh medium was fed into the bioreactor with a dilution rate of 0.05 h^{-1} and outlet stream discharge medium from the bioreactor with the same rate. Inlet vessel continuously stirred with the help of a magnetic stirrer throughout all process in order to maintain a homogenous inlet stream. Both inlet and outlet flasks were replaced with sterilized new flasks when all medium in the inlet vessel used (approximately 20 hours).

During the whole fermentation process, the fermentation conditions were controlled and kept constant as during the batch fermentation experiments. Temperature, pH and mixing speed are automatically controlled and, held at 37.2 °C, 6.8±0.1 and 150 rpm, respectively. KOH solution (5.0 M) was used for pH

control. Anaerobic conditions were maintained as much as possible by pumping CO_2 gas into the system through 0.22 μm filter every 24 hours and after the each collection of samples.

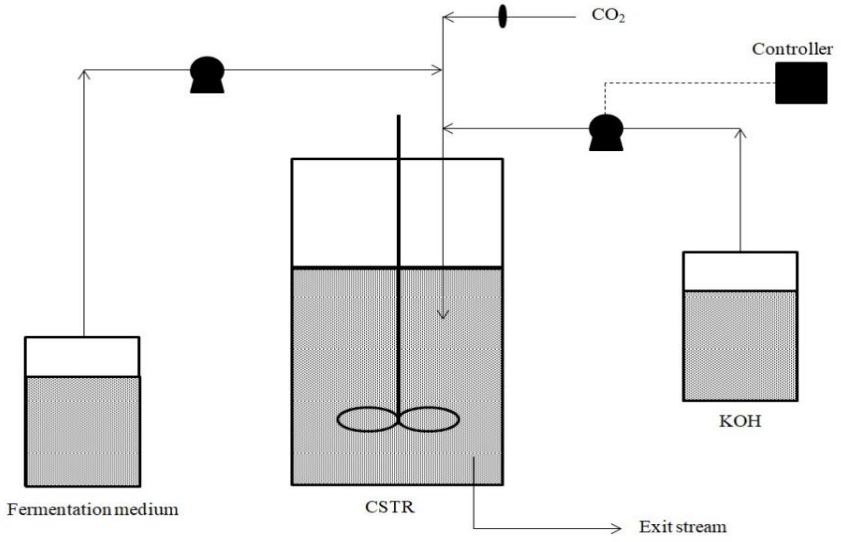


Figure 3.1 Continuous fermentation system schematic diagram

3.3 Analytical Methods

Organic acid and sugar content of samples were analyzed and determined by using high-performance liquid chromatography (HPLC) system (Agilent Technologies, USA) equipped with Rezex ROA-Organic Acid H+ (8%) column, 300×7.8 mm (Phenomenex Inc., USA) and refractive index detector. The temperature of column and RI detector was set to 55 °C and 35 °C. Samples were injected automatically with a volume of 10 μL. A 0.05 M H_2SO_4 solution was employed as the mobile phase, with a flow rate of 0.6 mL/min.

The 1 mL samples were centrifuged at 14000 rpm for 5 minutes by a laboratory type centrifuge (Mikro 220 R, Hettich Lab Technology, Germany) before HPLC analyses. In order to avoid column deformation and contamination, the supernatant of the sample was diluted and passed through nylon 0.22 μm filters. As an internal

reference, an arabinose solution with a concentration of 0.5 g L^{-1} was employed while dilution of samples to confirm that the HPLC analysis findings were accurate and reliable.

3.4 Data Analysis

The findings of the tests were presented as the mean of the replicates performed. The one-way analysis of variance (ANOVA) were used to the data in order to provide statistical interpretation. Minitab 17 (Minitab Inc., UK) was used to do the analysis of data. In order to compare the data, the Tukey test with a 95% confidence level was applied. At a p-value less than 0.05 ($p<0.05$), statistical differences between experimental results and tests were judged significant.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Succinic Acid Production with Free *A. succinogenes* Cells

Free cell experiments were carried out in two stages as batch form in shake flasks and in bioreactor. Those containing lactose, glucose and whey in the media used were compared in both stages.

4.1.1 Shake Flask Fermentation

The results of shake flask experiments using different carbon sources are shown in the Figures 4.1, 4.2, 4.3 and 4.4. In all experiments, corresponding starting sugar concentrations were 35 g L^{-1} and working volumes were 200 mL in 250 mL flasks. In all fermentations $MgCO_3$ were added in the broth at the start of experiment with a concentration of 40 g L^{-1}. Because of the alkaline characteristic of $MgCO_3$, starting pH values were measured as approximately 8.0 in all mediums.

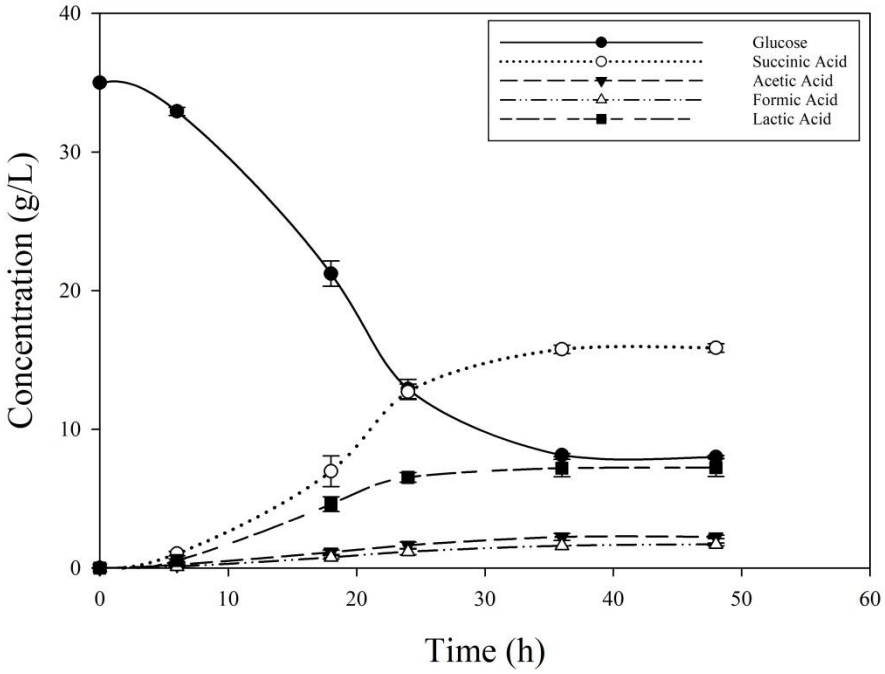


Figure 4.1 Substrate consumption and organic acid production results for shake flask fermentations using glucose as carbon source

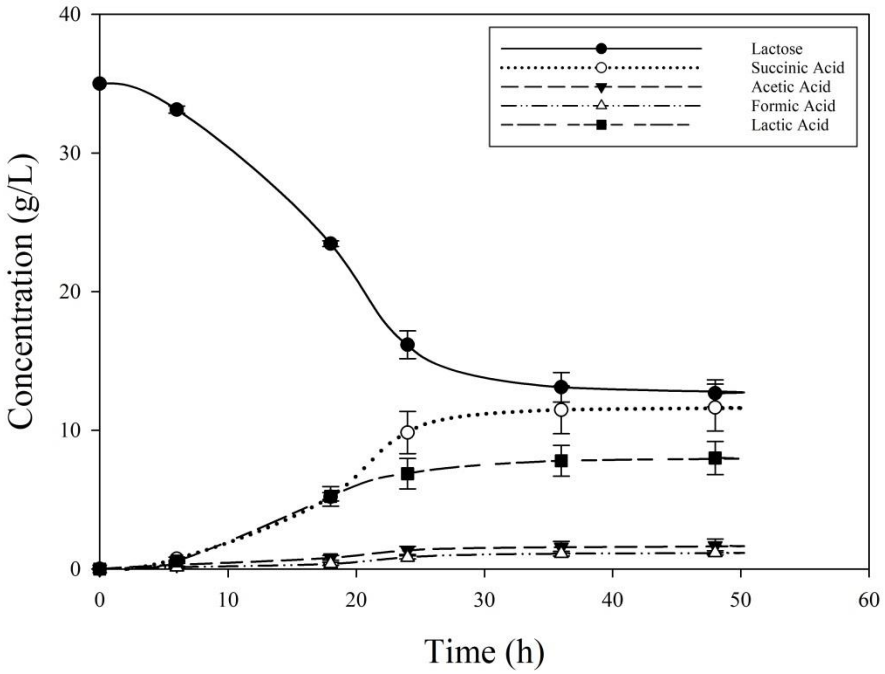


Figure 4.2 Substrate consumption and organic acid production results for shake flask fermentations using lactose as carbon source

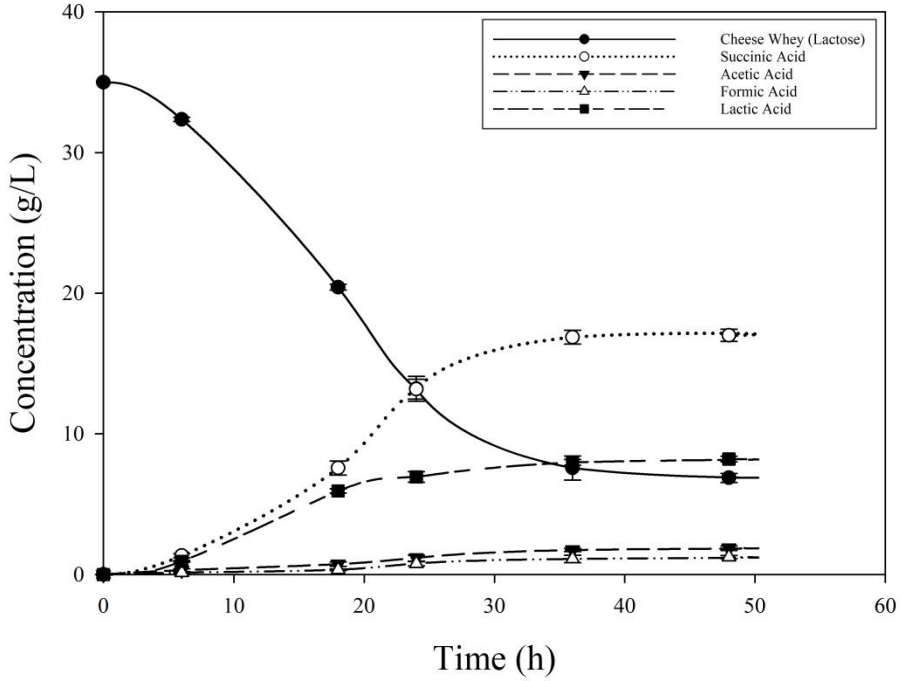


Figure 4.3 Substrate consumption and organic acid production results for shake flask fermentations using cheese whey (lactose) as carbon source

As seen from the results, succinic acid was generated as the main end product in all experiments. Succinic acid was accompanied by acetic acid, formic acid and lactic acid as by-products. Lactic acid was observed as the highest accumulated by-product in all experiments with all carbon sources. Highest succinic acid production with a concentration of 17.0 g L^{-1} was observed in cheese whey containing medium along with 8.20 g L^{-1} lactic acid, 1.87 g L^{-1} acetic acid and 1.23 g L^{-1} formic acid in 48 hours of fermentation. Yield of succinate per gram of sugar consumption for glucose, lactose and cheese whey containing media were; 0.45, 0.33 and 0.49 g g^{-1}, respectively. After 24 hours of fermentation organic acid production and sugar consumption rates were significantly dropped ($p<0.05$) and almost completely stopped after 36 hours. This was due to the fact that in shake flasks pH control was not applicable and after 24 hours, because of the organic acid accumulation, pH of media was dropped to 5.0-5.3 range. It is known that below 6.0 pH, *A. succinogenes* cells are losing their optimum viability (Van Der Werf et

al., 1997). This was likewise the reason for low yields obtained. Since cells cannot work properly, in all experiments carbon sources were remained unconsumed after the termination of fermentations (8.0 g L^{-1} glucose, 12.7 g L^{-1} lactose, 6.9 g L^{-1} cheese whey lactose concentrations). Interestingly, utilization of lactose was relatively low compared to cheese whey and glucose containing medium. Even though cheese whey was also containing same amount lactose, whey containing medium gave the highest production results and exceeded the results obtained from lactose significantly ($p<0.05$). It was concluded from this result that the complex mineral and nitrogen sources in whey have a beneficial effect on cells. This conclusion was also supported by the comparison of results obtained from fermentations with glucose. Glucose has widely considered as the preferred carbon source for optimal growth and production (Jansen and van Gulik, 2014). However, according to the results, the values obtained from whey exceeded that of glucose, but this difference was not considered significant ($p>0.05$).

Apart from pH control one of the main problems faced while conducting fermentations in shake flasks was the lactic acid accumulation in high concentrations. Lactic acid production is not desired in succinic acid fermentations with *A. succinogenes*. Possible reason for high lactic acid accumulation was that the anaerobic conditions could not be maintained. In shake flasks keeping the conditions anaerobic was not applicable. In the literature it was stated that *A. succinogenes* has the ability to synthesize lactic acid when oxygen is present in the system (Van Der Werf et al., 1997). This phenomenon was also supported by the results obtained from the bioreactor fermentation experiments which will be discussed in the next section.

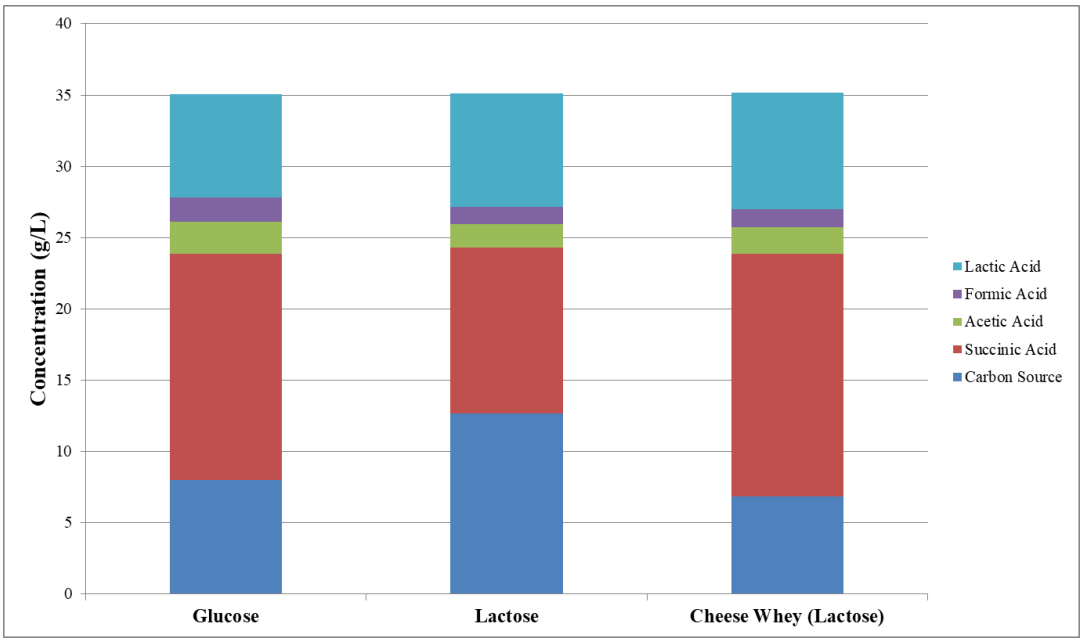


Figure 4.4 Final organic acid and sugar concentrations after 48 hours of fermentation in shake flasks with different carbon sources

4.1.2 Batch Fermentation Experiments in Bioreactor

Bioreactor batch fermentations were performed in 3 stages. In these stages, media containing glucose, lactose and whey, respectively, were used. In the first step, pH control agent (NaOH, KOH, $Ca(OH)_2$) and indirect CO_2 ($NaHCO_3$ and $MgCO_3$) source were selected by using the medium containing glucose as a reference. Selected neutralizing agent and indirect CO_2 sources were used in subsequent batch fermentation experiments. In the second stage, in order to measure the effect of initial sugar concentration on production, fermentations were conducted with 60 and 35 g L^{-1} glucose and lactose-containing media. In the third and final step, the experiments were repeated with whey media containing 35 g L^{-1} lactose and effect of different carbon sources was evaluated.

4.1.2.1 Effect of Indirect CO_2 Source

Presence of dissolved CO_2 in the system is very crucial for fermentative succinic acid production since *A. succinogenes* succinic acid metabolism is a CO_2 fixating metabolism (hypothetically 1 mole of consumed CO_2 results in 1 mole of succinic acid generation) (Longanesi et al., 2018; Pateraki et al., 2016). Because of the high operation costs of constant CO_2 gas sparging, it has considered as more feasible to use carbonate salts as indirect CO_2 source for succinic fermentation. In order to evaluate the effects of different indirect CO_2 sources on fermentation efficiency batch fermentation experiments were conducted with two different carbonate buffers. Fermentation condition parameters were chosen as; 35 g L^{-1} glucose containing medium, 2 L working volume in a 2.5 L bioreactor with an agitation speed of 150 rpm, 37.2 °C temperature, 5 M NaOH for pH adjustment, 40 g L^{-1} of $MgCO_3$ or $NaHCO_3$ as carbonate buffers. The results of the experiments performed with both CO_2 sources are shown in the Figure 4.5.

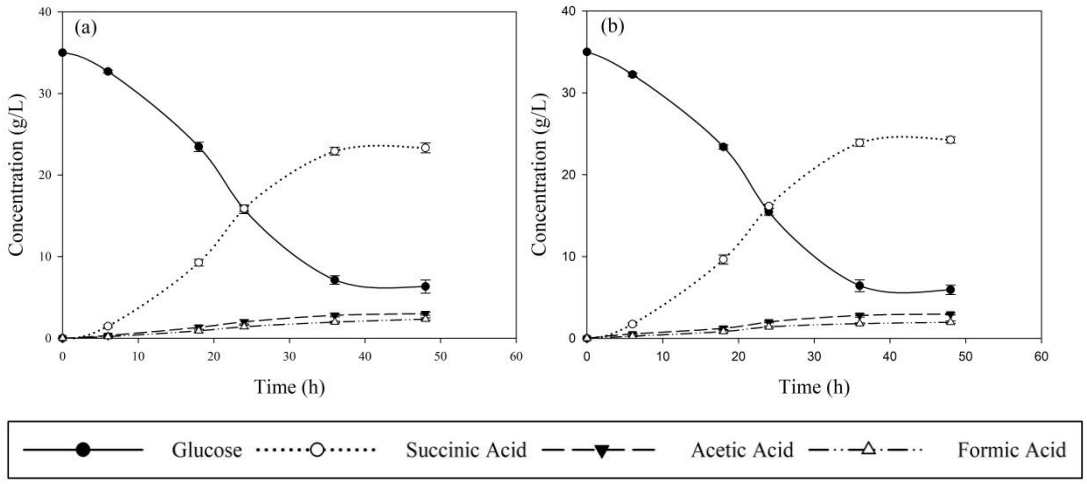


Figure 4.5 Glucose utilization and organic acid synthesis profiles of fermentations using varied indirect CO_2 sources; a) $NaHCO_3$, b) $MgCO_3$

In both cases succinic acid were found as the main end product followed by acetic and formic acid as by-products. At the initial experiments lactic acid accumulation was also observed like in the case of shake flask experiments. There have been

instances that contaminations of lactic acid bacteria were encountered; however, in these instances, there was little or no succinic acid generation was seen; instead, fermentations resulted in solely lactic acid accumulation. Every sample was subjected to a light microscope examination to ensure that there was no contamination, and any contaminated batches were immediately halted. The presence of high levels of lactic acid generation throughout the process when the contamination was not observed demonstrated that *A. succinogenes* is capable of producing lactic acid. According to some sources, lactic acid generation happened in instances when a totally anaerobic atmosphere could not be maintained for an extended period. According to, Van der Werf et al. lactate dehydrogenase activity is present in *A. succinogenes*, however, this activity can be as low as 9 nmol min^{-1} when the anaerobic process conditions were maintained (Van Der Werf et al., 1997). As reported in the literature, when a dual-phase fermentation system was constructed – initiation the fermentation as aerobic and then switching the system conditions to anaerobic – significant quantities of lactic acid production were achieved by using *A. succinogenes* as fermentation agent, demonstrating high level lactic acid production ability of *A. succinogenes* (Q. Li et al., 2010a; Wang et al., 2014). In batch fermentation experiments, when there was sufficient oxygen available, *A. succinogenes* was more likely to synthesize lactic acid (data not shown). When oxygen was removed from the system during batch trials, the synthesis of lactic acid was switched over to succinic acid. This was confirmed by subsequent experiments, in which the process was initiated and operated under anaerobic conditions, which were maintained to the greatest extent possible by flushing the reactor with gas CO_2 following every sample collection for 30 minutes. As an outcome, production of lactic acid was eliminated, providing further support for mentioned studies. In later experiments, fermentation conditions were kept anaerobic as much as possible and lactic acid production was prevented successfully.

As can be seen from Figure 4.5 organic acid production and glucose consumption trends were similar in both fermentations with $NaHCO_3$ and $MgCO_3$. When

$MgCO_3$ was used, slightly higher succinic acid concentration and yield was obtained (24.3 g L^{-1}, 0.69 g g^{-1}) compared to $NaHCO_3$ (23.3 g L^{-1}, 0.67 g g^{-1}), but this difference was not significant ($p>0.05$). Acetic acid and formic acid concentrations obtained were almost similar for both $NaHCO_3$ and $MgCO_3$ experiments; 3.0 g L^{-1} AA, 2.3 g L^{-1} FA and 3.0 g L^{-1} AA, 2.0 g L^{-1} FA respectively. These results were consistent with similar studies in the literature (Q. Li et al., 2010b; Liu et al., 2008b; Omwene et al., 2021; Samuelov et al., 1991).

In all fermentations lag phase was observed approximately 6 hours. During the lag phase organic acid production rate was very low. Production rate was continuously increased until 24 hours and later slowed down. Unlike shake flask experiments fermentation rates were not decrease drastically till 36 hours of fermentation. Since the pH of the systems were controlled automatically cells were able to produce organic acids with a relatively high rate. However fermentation rates were slowed down significantly between 36-40 hours in both tests with $NaHCO_3$ and $MgCO_3$ resulted in unutilized glucose concentrations of 6.3 g L^{-1} and 5.9 g L^{-1} in the medium respectively, at the end of fermentations. Despite the fact that it was not statistically significant, the concentration and yield of succinic acid generated by using $MgCO_3$ outperformed those produced with $NaHCO_3$. The positive effect of $MgCO_3$ on the production of succinic acid may be due to the release of Mg^{2+} ions along with CO_2, which are both crucial cofactors for phosphoenolpyruvate carboxykinase (PEPC) that is the first enzyme in the reductive path of the TCA cycle which is the path for succinic acid synthesis (Pateraki et al., 2016).

4.1.2.2 Effect of Neutrilizing Agent

In order to evaluate the pH control agent effect on succinic acid synthesis three different neutralizing agents (KOH, $Ca(OH)_2$, and NaOH) were experimented in batch fermentations. All fermentation conditions were kept similar as explained previously with the use of 40 g L^{-1} $MgCO_3$ as an indirect CO_2 source. Final organic

acid and sugar concentrations obtained from the systems at the end of 48 hours of fermentations are presented in Figure 4.6.

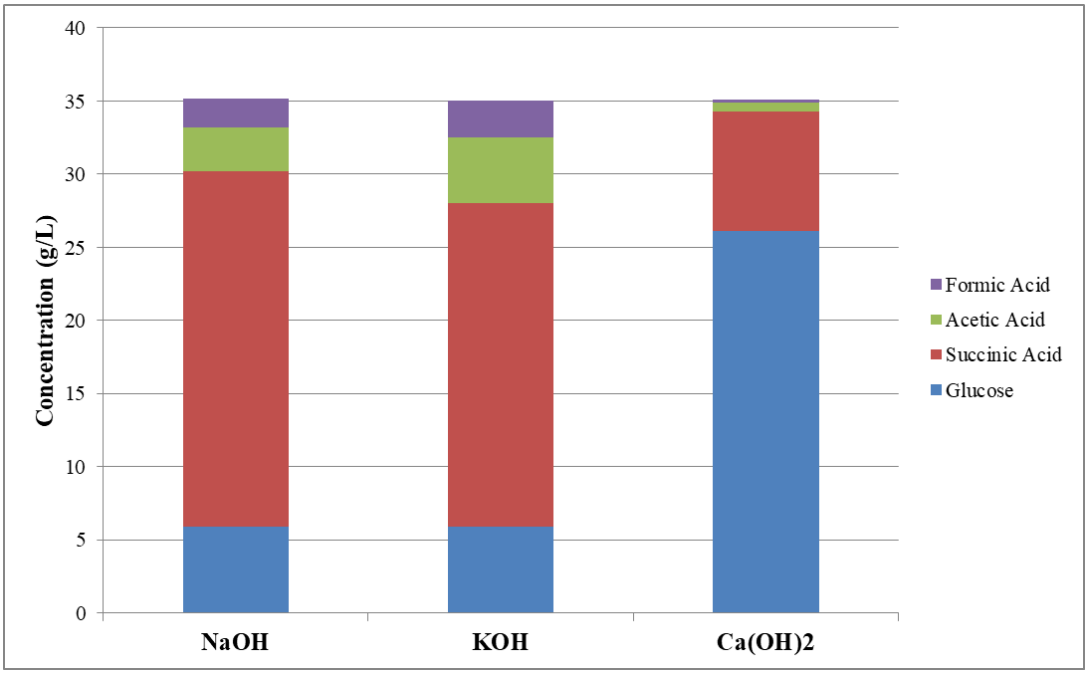


Figure 4.6 Organic acid and glucose distribution in the system after 48 h of fermentation with different neutralizing agent usage

The first and the foremost observation from the results were that almost no cell viability were observed during the fermentations where $Ca(OH)_2$ was used as the pH controller. Very low succinic acid production with a concentration of 8.2 g L^{-1} was obtained. Also negligible amounts of by-product formation (formic acid and acetic acid) was observed (<1 g L^{-1}) and 26.1 g L^{-1} of glucose remained unconsumed after the termination of fermentation. Between the 6-12 hours succinic acid production rate was increased, however it was decreased significantly when the pH of the system dropped under the control levels and when $Ca(OH)_2$ was introduced to the bioreactor. This observation shows the inhibitory effect of $Ca(OH)_2$ on *A. succinogenes* cell viability.

On the other hand, high amounts of succinic acid were obtained from the fermentations with NaOH and KOH. Usage of NaOH as neutralizing agent yielded

in higher succinic acid and lower by-product formation (24.3 g L^{-1} SA, 3.0 g L^{-1} AA and 2.0 g L^{-1} FA) with a final yield of 0.50 g g^{-1}. When KOH was used as pH adjuster 22.1 g L^{-1} succinic acid was synthesized with a yield of 0.46 g g^{-1} along with 4.5 g L^{-1} acetic acid and 2.5 g L^{-1} formic acid as by-products. In the literature the positive effect of sodium ions on succinic acid synthesis was demonstrated (Podkovyrovi and Zeikus, 1993) and also it was stated that presence of sodium ions might have an effect on cell osmolarity (Andersson et al., 2009; Liu et al., 2008a). Thus, the significant influence of NaOH on production of succinic acid may be due to high amount of sodium ions released when NaOH introduced to the system. Although this difference between final succinic acid yield of fermentations with NaOH and KOH was considered as statistically significant ($p=0.037$) KOH was chosen as the neutralizing agent in the following experiments. The reason for this choice was related to further experiments with immobilized cells and will be explained in the later sections. Briefly, it was later found that NaOH had a negative effect on Ca-alginate bead stability and for this reason KOH was used as main pH controller. For accurate comparison between free cell and immobilized cell fermentations, experiments that investigate the effect of initial sugar concentration and different carbon sources were repeated with the use of KOH.

4.1.2.3 Effect of Initial Sugar Concentration

Initial sugar concentration effect on succinic acid fermentation was investigated by initiating the system with different starting concentrations of glucose and lactose containing media. For this, two different starting concentrations (35 g L^{-1} and 60 g L^{-1}) were tested. Rest of the fermentation conditions were kept similar as explained in previous sections and controlled automatically. As neutralizing agent 5 M KOH solution and as an indirect CO_2 source 40 g L^{-1} $MgCO_3$ was used. Succinic acid accumulation and substrate consumption results of fermentations are shown in Figure 4.7, 4.8 and Table 4.1.

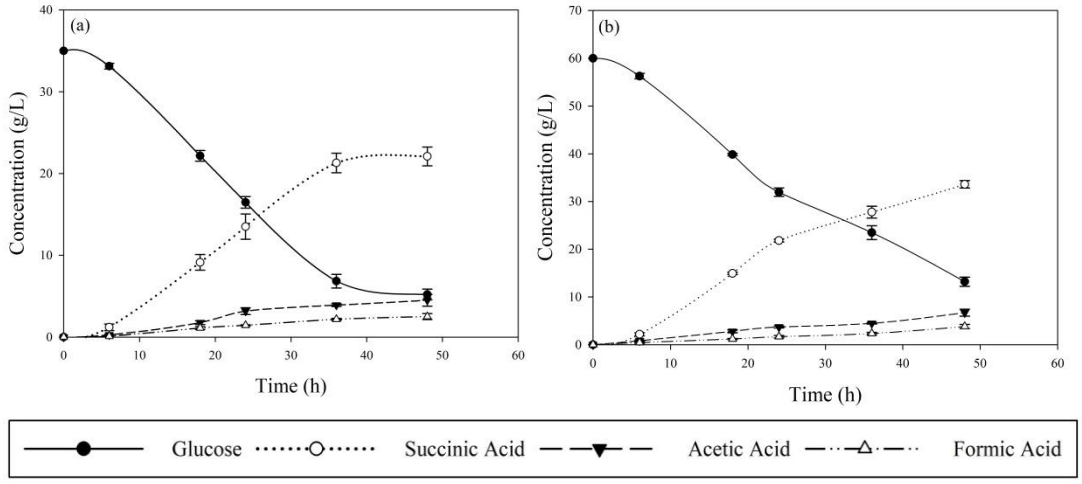


Figure 4.7 Glucose consumption and organic acid accumulation profiles of batch experiments with varied initial glucose concentrations; a) 35 g L^{-1}, b) 60 g L^{-1}

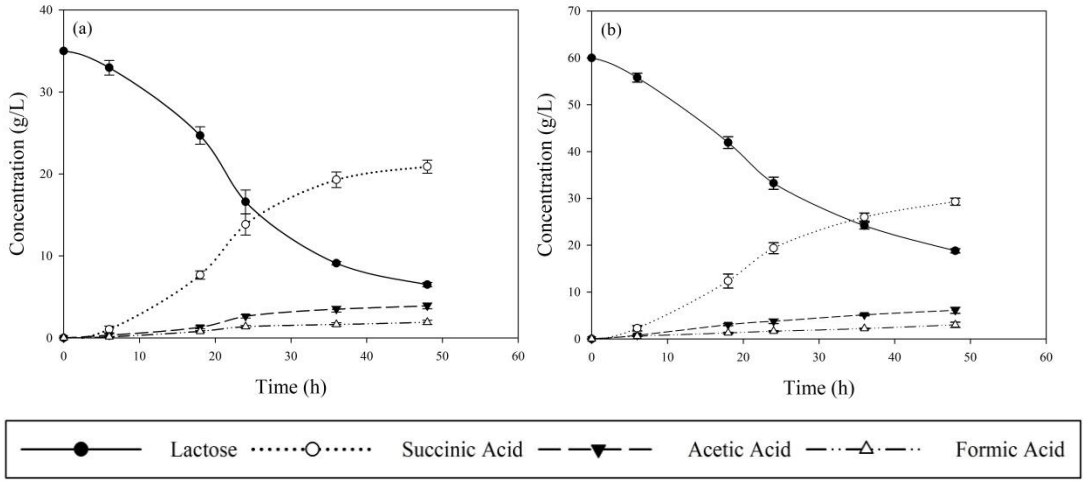


Figure 4.8 Lactose consumption and organic acid accumulation profiles of batch experiments with varied initial lactose concentrations; a) 35 g L^{-1}, b) 60 g L^{-1}

Table 4.1 Batch fermentation results for different initial glucose and lactose concentrations

Carbon Source	Concentration (g L^{-1})	SA (g L^{-1})	Productivity (g L^{-1} h^{-1})	Yield (%)	SA/AA	SA/FA
Glucose	35	22.1±0.66*	0.46±0.013	63.1±1.9	4.91±0.57	9.87±0.73
Glucose	60	33.6±0.45	0.70±0.009	56.0±0.7	5.01±0.24	8.84±0.44
Lactose	35	20.9±0.45	0.44±0.009	59.7±1.3	5.36±0.17	11.0±0.70
Lactose	60	29.3±0.43	0.61±0.009	48.8±0.7	4.80±0.38	9.77±1.2

* *Standard error was calculated by conducting each experiment three times.*

According to the findings, final succinic acid titers and productivities were significantly ($p<0.05$) in experiments with 60 g L^{-1} of initial sugar concentrations in both cases. Productivities of 0.70 g L^{-1} h^{-1} and 0.61 g L^{-1} h^{-1} were achieved from 60 g L^{-1} initial glucose and lactose containing media respectively. On the other hand, sugar to succinic acid yields obtained from 35 g L^{-1} initial concentrations (0.63 g g^{-1} for glucose, 0.60 g g^{-1} for lactose) were significantly ($p<0.05$) higher than the experiments with 60 g L^{-1}.

At the end of 48 hours in both fermentations with 60 g L^{-1} starting sugar content, high concentrations of glucose (13.2 g L^{-1}) and lactose (18.8 g L^{-1}) remained unconsumed in the system meaning that 22.0% of total glucose and 31.3% of total lactose could not be utilized. These results were significantly ($p<0.05$) higher than the ones obtained from 35 g L^{-1} starting concentrations. At the end of the 35 g L^{-1} fermentations, 5.2 g L^{-1} glucose (14.8%) and 6.5 g L^{-1} (18.6%) were remained unutilized in the fermentation broth. Fermentations were prolonged till 56 hours in order to investigate cell viability, however, organic acid production or sugar consumption was not observed after around 50 hours. This means that cells had lost viability and the results were evaluated as the experiments were stopped at the end

of 48 hours. Possible reason for the loss of cell viability might be the end-product inhibition effect on cells. Lin et al., reported a critical concentration for total organic acids for *A. succinogenes* cells' optimal viability which was 45.6 g L^{-1} (Lin et al., 2008). Also it was reported that formic acid concentrations above 2.7 g L^{-1} would significantly decrease cell density of *Saccharomyces cerevisiea* by 80% (Maiorella et al., 1983). In the baker's yeast *Saccharomyces cerevisiae*, it was discovered that the acetate and formate ions flowed readily through the cell membrane, but pyruvate, succinate and glucose were unable to reach the outer layer of the cell membrane. Also found was that the formic acid concentrations in *A. succinogenes* cultures were the same both intracellular and extracellular formate levels (Van Der Werf et al., 1997). As a result, it is likely that the same phenomenon is at function in this situation. In the experiments where initial glucose concentration was 60 g L^{-1}, total organic acid concentration was obtained as high as 46.3 g L^{-1} at the end of fermentation with a formic acid concentration of 4.3 g L^{-1} which supports these studies. It was decided that, relatively lower by-product formation and higher succinic acid yields obtained from 35 g L^{-1} starting concentrations makes it more feasible and advantageous even though higher succinic acid productions were obtained from 60 g L^{-1} fermentation media.

4.1.2.4 Effect of Carbon Source

Three different fermentation media were tested for the evaluation of the different carbon source effect on succinic acid production. Cheese whey, glucose and lactose were used as carbon sources in a previously explained fermentation media with starting sugar concentrations of 35 g L^{-1}. Figures 4.9, 4.10 and 4.11 illustrate the profiles of substrate utilization and organic acid generation obtained from experiments with glucose, lactose and cheese whey, respectively. Succinic acid was shown to be the most prevalent end product in all of the experiments. As the anaerobic conditions were kept, the only significant by-products were acetic acid and formic acid.

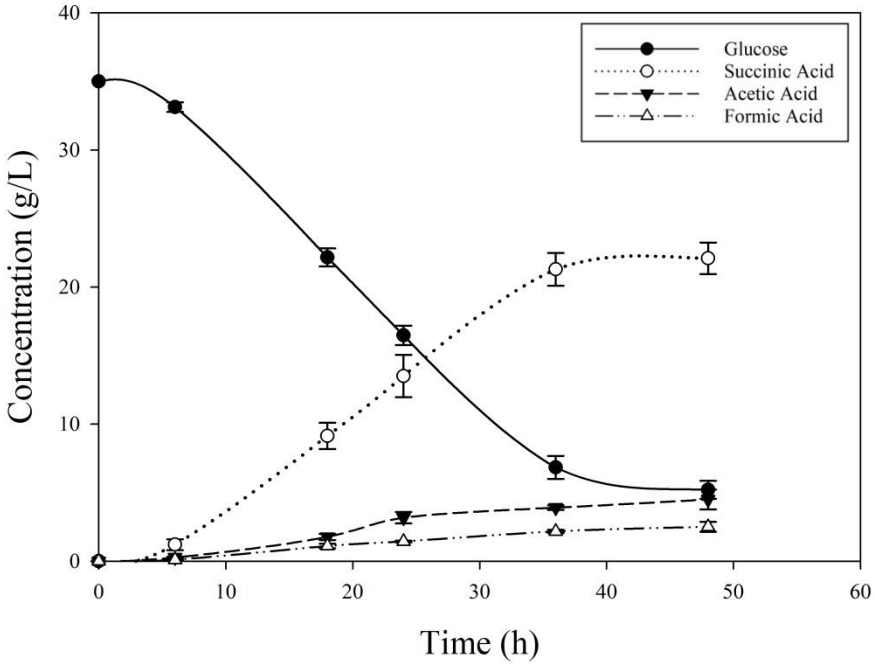


Figure 4.9 Glucose utilization and organic acid synthesis profiles of batch bioreactor fermentations with free cells

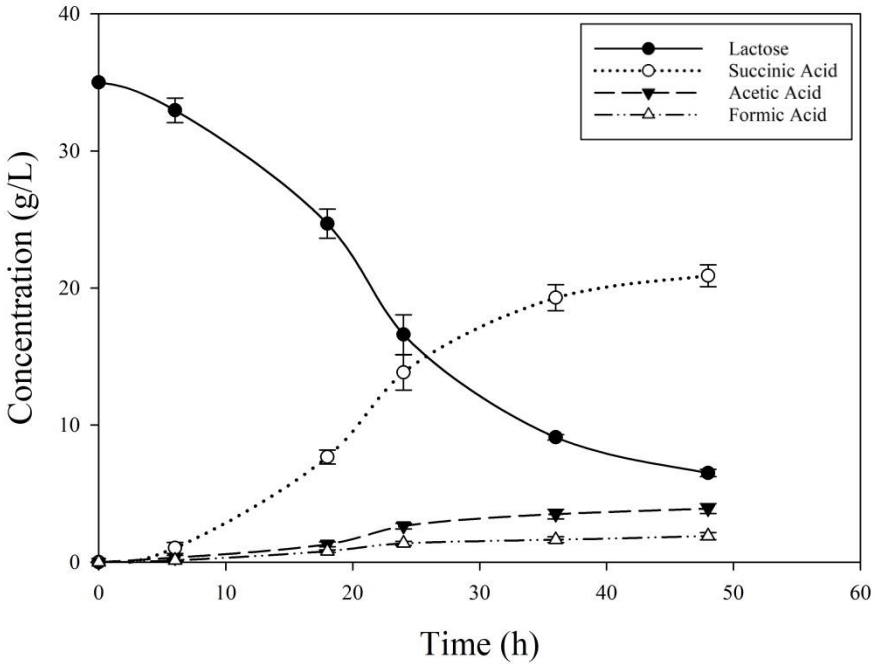


Figure 4.10 Lactose utilization and organic acid synthesis profiles of batch bioreactor fermentations with free cells

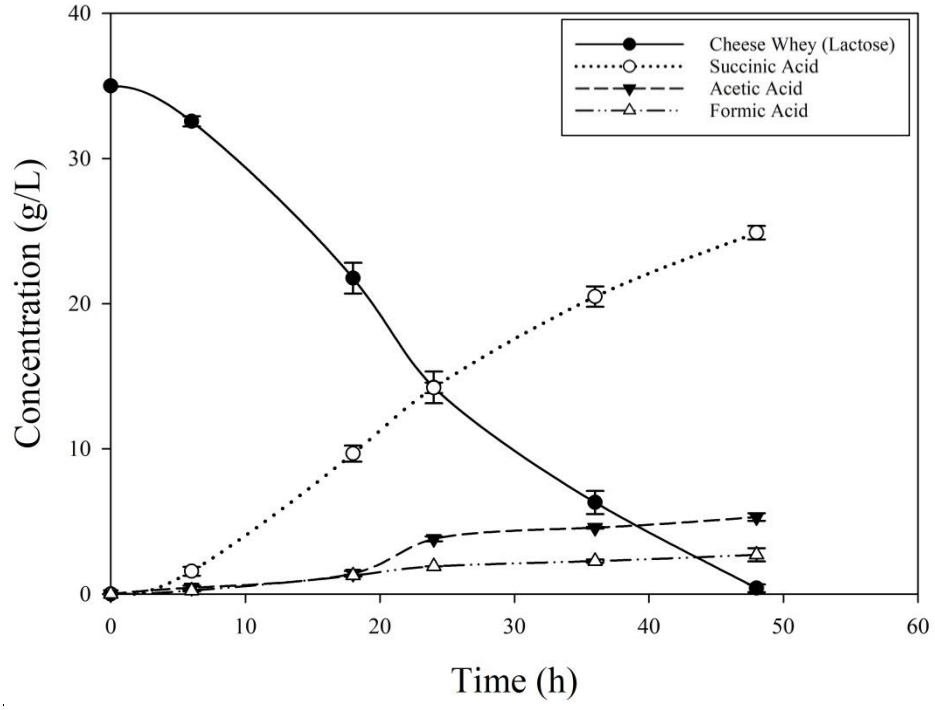


Figure 4.11 Cheese whey utilization and organic acid synthesis profiles of batch bioreactor fermentations with free cells

According to the results, *A. succinogenes* exhibited a lag phase of 4-6 hours in all fermentations for all carbon sources, with negligible formation of end products during this time. The results of the experiments have demonstrated that the fermentations using cheese whey, lactose and glucose were all followed the similar pattern. However, cheese whey fermentation (24.9 g L^{-1}, 0.52 g L^{-1} h^{-1}) had the highest production and productivity, with a yield of 0.71 g g^{-1},that was significantly ($p<0.05$) outpaced the results obtained from fermentations with glucose and lactose. Defined media with glucose was demonstrated the second highest outputs with a final succinic acid titer of 22.1 g L^{-1}, 0.46 g L^{-1} h^{-1} productivity and 0.63 g g^{-1} of glucose to succinic acid yield. Fermentations with lactose gave the lowest succinic acid titer, productivity and yield results with 20.9 g L^{-1}, 0.43 g L^{-1} h^{-1}, and 0.60 g g^{-1}, respectively. Acetic acid, along with formic acid, was the most common by-product in all of the trials. The ratios of succinic acid to formic acid and succinic acid to acetic acid were similar for every carbon source (Table 4.2).

Typically, fermentations began to slow down after 36-40 hours and after that organic acid production rate decreased to none till 48-50 hours. Interestingly, practically all of the lactose present in the cheese whey containing medium was utilized approximately after 40 hours in all of the trials. In contrast, when experiments were conducted using pure glucose and lactose-containing medium, 6.5 g L^{-1} lactose and 5.2 g L^{-1} glucose was found unconsumed after the termination of fermentations.

Table 4.2 Batch fermentation results using different carbon sources in fermentation media

Carbon Source (35 g L^{-1})	Succinic Acid (g L^{-1})	Productivity (g L^{-1} h^{-1})	Yield (%)	SA/AA	SA/FA
Cheese Whey	24.9±0.26*	0.52±0.005	71.1±0.7	4.70±0.09	9.22±0.94
Lactose	20.9±0.45	0.43±0.009	59.7±1.3	5.36±0.17	11.0±0.70
Glucose	22.1±0.66	0.46±0.013	63.1±1.9	4.91±0.57	9.87±0.73

* *Standard error was calculated by conducting each experiment three times.*

The yield achieved from fermentations using cheese whey as the sole carbon source was the most significant outcome from the batch fermentation experiments performed in this study. Final succinic acid yield, productivity and concentration obtained from cheese whey exceeded the results of glucose and lactose significantly ($p<0.05$). This finding supports the hypothesis that cheese whey contains sufficient amounts of complex nitrogen sources and nutrients which have the ability to boost the activity of *A. succinogenes* cells (Lee et al., 2003).

Succinic acid production with *A. succinogenes* by using whey as the substrate was investigated recently by Louaste and Eloutassi, Longanesi et al. and Omwene et al. (Longanesi et al., 2018; Louasté and Eloutassi, 2020; Omwene et al., 2021). Louaste and Eloutassi were managed to achieve 0.62 g g^{-1} yield with cheese whey, while Longanesi et al. and Omwene et al. achieved 0.61 g g^{-1} and 0.54 g g^{-1}, respectively. In this study obtained yield (0.71 g g^{-1}) of succinic acid from cheese

whey exceeded these findings significantly. It is concluded from the results of batch fermentations experiments that cheese whey is a more favorable substrate, exceeding the yield and titers obtained from both glucose and lactose. Also from an economical point of view, it is possible to decrease costly nitrogen source usage (such as yeast extract) with the use of whey in fermentations thanks to its beneficial nitrogen and nutrient capacity. Table 4.3 represents studies conducted to produce succinic from whey including the results obtained from this study for comparison.

Table 4.3 Compilation of the studies on fermentative succinic acid production by using cheese whey as substrate

Microorganism	Fermentation Mode	Substrate	Succinic Acid ($g\ L^{-1}$)	Yield ($g\ g^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	References
A. succinogenes 130Z	Batch	Whey	24.9	0.71	0.52	This study
A. succinogenes 130Z	Batch	Whey	15.7	0.54	0.33	(Omwene et al., 2021)
A. succinogenes 130Z	CO_2 sparging, batch	Whey	21.3	0.44	0.43	(Wan et al., 2008)
A. succinogenes ATCC 29,305	CO_2 sparging, fed-batch	Whey	34.7	0.91	1.02	(Samuelov et al., 1999)
A. succinogenes 130Z	Batch	Whey	7.48	0.68	0.46	(Longanesi et al., 2018)
A. succinogenes 130Z	CO_2 sparging, batch	Whey	13.5	0.62	0.81	(Louasté and Eloutassi, 2020)
M. succiniciproducens MBEL55E	CO_2 sparging, fed-batch	Whey	13.4	0.71	1.18	(Lee et al., 2003)
Anaerobiospirillum succiniciproducens	CO_2 sparging, batch	Whey	18.6	0.93	0.24	(Lee et al., 2000)

4.2 Succinic Acid Production with Immobilized *A. succinogenes*

Immobilized fermentation experiments were conducted in three different modes in bioreactor. In the first stage batch fermentations were conducted using cheese whey, lactose, and glucose as carbon sources. In the second and third stages, repeated-batch and continuous fermentation experiments were performed only using cheese whey containing medium.

4.2.1 Batch Fermentations with Immobilized *A. succinogenes*

Fermentations immobilized cells were carried out in a batch mode with different carbon sources similar to the fermentations with free cells. In order to allow for comparison, the starting sugar content of all media (glucose, lactose and cheese whey lactose concentrations) was fixed to 35 g L^{-1}, and rest of the fermentation parameters were kept constant.

Ca-alginate beads were obtained in the same manner as previously reported prior to the start of the studies. Following the commencement of the fermentation process, the beads began to disintegrate and were eventually totally broken down over a period of time. The unfortunate reality is that this was true for all experiments with all different substrates. It was necessary to experiment with alternative agitation speeds, pH-controlling agents, and working volumes (Table 3.3) in order to pinpoint the root of the issue. However, none of these trials were succeeded in preventing the beads getting lost. On the other hand, some interesting results were observed during these experiments.

At the start of the experiments with fermentation conditions as 150 rpm agitation, 2 L working volume and NaOH as neutralizing agent, it was observed that in the medium that contains pure lactose, Ca-alginate beads have lost their stability much faster compared to the ones with glucose and cheese whey. All beads were broken down as fast as 4 hours after the initiation of fermentation while beads were hold

their stability longer, around 8-10 hours in glucose and cheese whey containing broth. Despite all the experiments and attempts, the reason behind this phenomenon could not be found. The answer to the question of why the beads disintegrate faster in the presence of pure lactose is left for further research.

Another observation from immobilized batch trials was the negative effect of NaOH on Ca-alginate bead strength. In order to observe neutralizing agent's effect on bead stability from start, pH of the fermentation media were decreased at the start of the experiments to 6.70 by using 1 M H_2SO_4 solution. Beads were broken down faster when NaOH was used as neutralizing agent compared to the trials with KOH and $Ca(OH)_2$. For glucose and cheese whey containing media it took around 4-5 hours for disintegration of beads, while with KOH, beads lasted for 8-10 hours and with $Ca(OH)_2$ beads significantly lasted longer around 20-22 hours. It was clearly seen that NaOH affects the stability of beads. Although, bead strength was significantly increased when $Ca(OH)_2$ was used, almost no production of organic acids or sugar consumption was observed in the presence of $Ca(OH)_2$. This was also the case in the free cell experiments. Both of these results indicates that $Ca(OH)_2$ has a significant inhibitory effect on *A. succinogenes* cell viability and it cannot be used as neutralizing agent in succinic acid fermentations. Despite the fact that beads were still lost in the experiments with KOH, when compared to other two agents it gave the best results and it was decided to use KOH as sole neutralizing agent in further experiments.

With the usage of KOH as pH adjuster and glucose as reference carbon source, different working volumes and stirring speeds were tested in order to see if the loss of beads were related to mechanical parameters. However, with all working volume-stirring speed combinations (Table 3.3) fragmentation of the beads still could not be prevented.

These results were in opposition to comparable research done by Ercole et al. and Alexandri et al. (Alexandri et al., 2017; Ercole et al., 2019). When alginate was employed as an immobilization material for the production of succinic acid with *A.*

succinogenes utilizing glucose as carbon source, 70 hours of fermentation were successfully completed without any bead disintegration in 1 L flasks with a total working capacity of 200 mL, according to the research conducted by Ercole and colleagues (Ercole et al., 2019). Additionally, Alexandri et al. also employed alginate for the immobilization of *A. succinogenes* in order to ferment spent sulphite liquor, and they managed to use the immobilized cells in five repeated-batch fermentation experiments (Alexandri et al., 2017). On the contrary, other studies supported the findings of the present study, Corona-Gonzalez et al. investigated the immobilization of *A. succinogenes* in a variety of matrices and found that Ca-alginate had poor mechanical qualities and was easily disturbed (Corona-González et al., 2014). In the opinion of Cha et al., the poor tolerance of alginate beads to disruption, along with the dispersion of divalent cations from the gel matrix, results in a progressive stability loss of Ca-alginate beads (Cha et al., 2012). Whichcukit et al., reported that alginate beads containing riboflavin were lost their stability and cracked by the time of 13 hours in a release solution containing a mixture of 0.5% xanthan in 3% sucrose solution and visually observed their further disintegration after 20 hours with use of magnetic resonance imaging (Wichchukit et al., 2013).

In the work of Wichchukit et al., whey proteins were utilized to increase bead durability by mixing a solution of whey protein isolate with sodium alginate solution (50-50 %) and a higher endurance was observed in beads obtained from whey-alginate combination, which suggests that whey proteins increase the stability of alginate beads (Wichchukit et al., 2013). In the present study, in order to prevent the loss immobilization matrix same idea was applied. Since the main carbon source experimented in this research was cheese whey, already present whey proteins in whey was exploited to support alginate bead strength. To do this, following the autoclaving of the medium to both sterilization and denaturation of whey proteins, incubation of fermentation medium with cheese whey in 100 mL flasks was carried out till the lactose in the broth was fully depleted. Immediately following incubation, this media was combined with a 2% alginate solution at a

rate of 40% by volume (40% alginate solution, 60% whey fermentation medium), creating the final mixture. A magnetic stirrer was used to keep the mixture homogenous and prevent the precipitation of denatured proteins. Using this combination, the bead preparation operation is carried out as described previously. Obtained beads from mixture were approximately 4 mm in diameter. Transparencies of beads were less compared to the beads obtained from pure alginate and the colors of beads were closer to white. Fermentations were initiated with these beads obtained with alginate-whey mixture.

Fermentations were effectively performed with the use of beads obtained with whey-alginate combination. In all experiments with all carbon sources –including the fermentations with pure lactose in which beads obtained with pure alginate were disrupted much faster-, there was no disintegration of beads were observed. Like in the case of the free cell fermentations, the maximum production of succinic acid was obtained from cheese whey containing medium with a final yield and concentration of 0.75 g g^{-1} lactose and 26.2 g L^{-1}, respectively. Final succinic acid concentrations and yields obtained from synthetic media of glucose and lactose were; 24.5 g L^{-1}, 0.70 g g^{-1} glucose and 22.3 g L^{-1}, 0.64 g g^{-1} lactose. Organic acid accumulation and sugar consumption profile of immobilized system fermentations with glucose, lactose and cheese whey containing media were demonstrated in Figure 4.12, 4.13 and 4.14, respectively. The productivity and yield results obtained with all carbon sources were shown in Table 4.4.

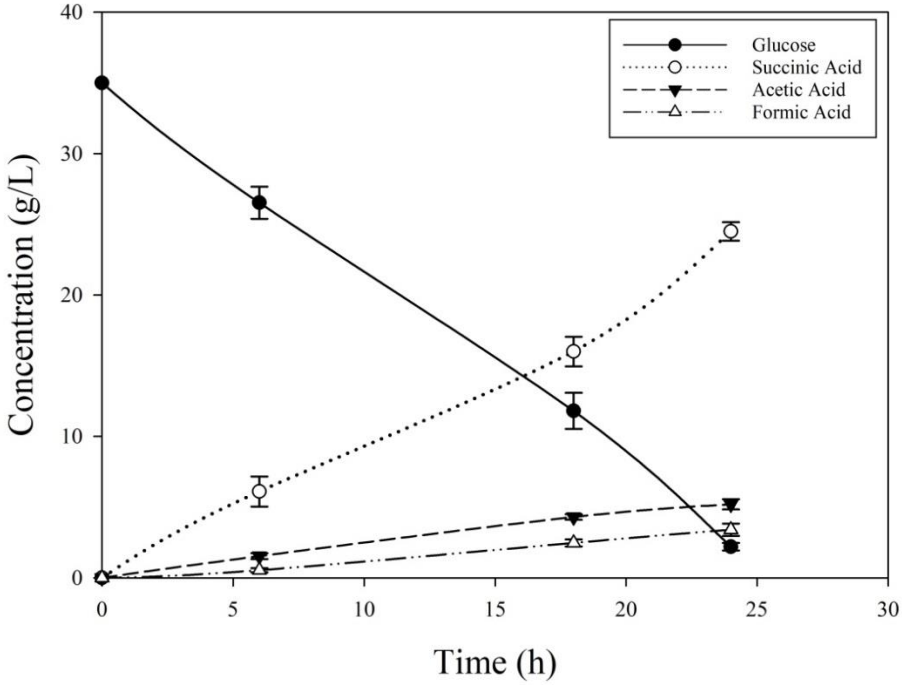


Figure 4.12 Substrate consumption and organic acid accumulation profile of batch immobilized cell fermentation with glucose as carbon source

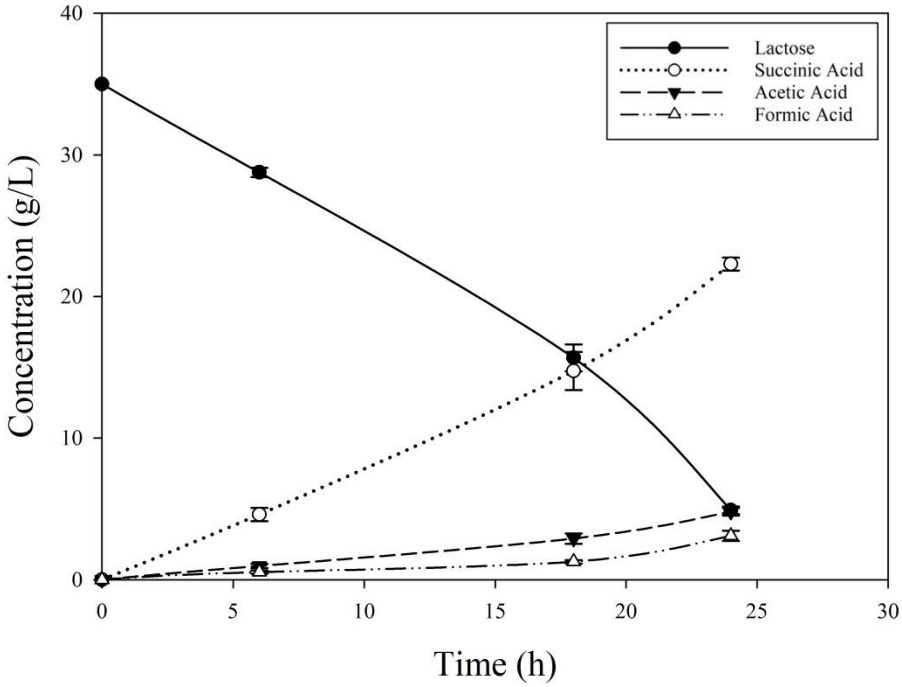


Figure 4.13 Substrate consumption and organic acid accumulation profile of batch immobilized cell fermentation with lactose as carbon source

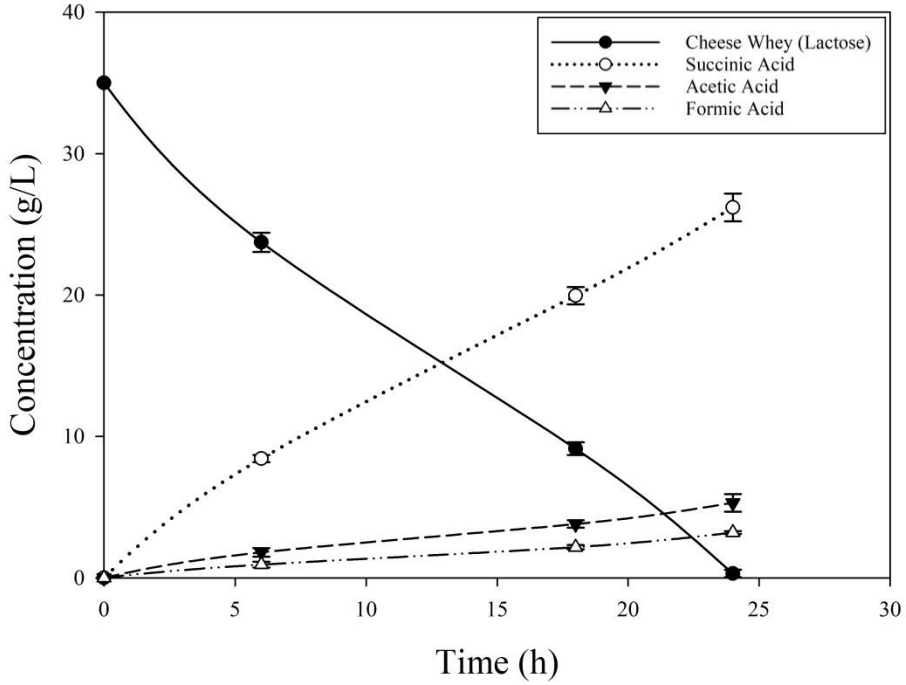


Figure 4.14 Substrate consumption and organic acid accumulation profile of batch immobilized cell fermentation with cheese whey as carbon source

Experiments with immobilized cells were gave a slightly higher yields than those achieved in free cell experiments, although these increases were not significant ($p>0.05$). Productivity, on the other hand, increased significantly with all carbon sources ($p<0.05$). As can be seen from Figures 4.12, 4.13 and 4.14, immobilized systems did not experience the 6-hour lag phase that occurred in free cell fermentations and all fermentations were completed in 24-28 hours. After the initiation of fermentation, in the first 6 hours 8.7 g L^{-1} of succinic acid was produced in cheese whey containing medium and all lactose in the system were completely consumed by the end of 24 hours demonstrating the efficiency of the process. In all experiments with different substrates, an increase in productivities of nearly twofold was found. With cheese whey, lactose and glucose as carbon sources, productivity values of 1.09 g L^{-1} h^{-1}, 0.93 g L^{-1} h^{-1} and 1.02 g L^{-1} h^{-1} were achieved, respectively. The productivities obtained from immobilized cell experiments significantly outperformed those obtained in previous investigations with alginate immobilized *A. succinogenes*. Alexandri et al., was achieved 0.39 g

L^{-1} h^{-1} productivity from fermentation of spent sulphite liquor and Ercole et al., was achieved 0.77 g L^{-1} h^{-1} with the use of glucose as substrate (Alexandri et al., 2017; Ercole et al., 2019). It can be concluded from the results that compared to free cell submerged fermentations, using cell immobilization and alginate-whey mixture as immobilization support is an efficient way to produce succinic acid.

Table 4.4 Batch fermentation results with immobilized cells using different carbon sources in fermentation media

Carbon Source (35 g L^{-1})	SA (g L^{-1})	Productivity (g L^{-1} h^{-1})	Yield (%)	SA/AA	SA/FA
Cheese Whey	26.2±0.57*	1.09±0.023	74.9±1.6	4.94±0.43	8.19±0.32
Lactose	22.3±0.26	0.93±0.011	63.7±0.8	4.65±0.21	7.19±0.60
Glucose	24.5±0.38	1.02±0.015	70.0±1.0	4.71±0.14	7.21±0.58

**Standard error was calculated by conducting each experiment three times.*

4.2.2 Repeated-batch Fermentations with Immobilized *A. succinogenes*

Batch fermentations with immobilized cells in an alginate-whey mixture were carried out repeatedly in order to assess the stability and reusability of the beads used in the experiments. For the repeated-batch studies, a cheese whey-containing fermentation medium was employed. Lactose content in the media was 35 g L^{-1} initially. In each fermentation cycle, the beads were rinsed and washed with a 2% $CaCl_2$ solution and kept in the solution for 1 hour before the addition of the fresh medium to the bioreactor to initiate the process again. The organic acid and lactose content profiles of five repeated batches are depicted in Figure 4.15.

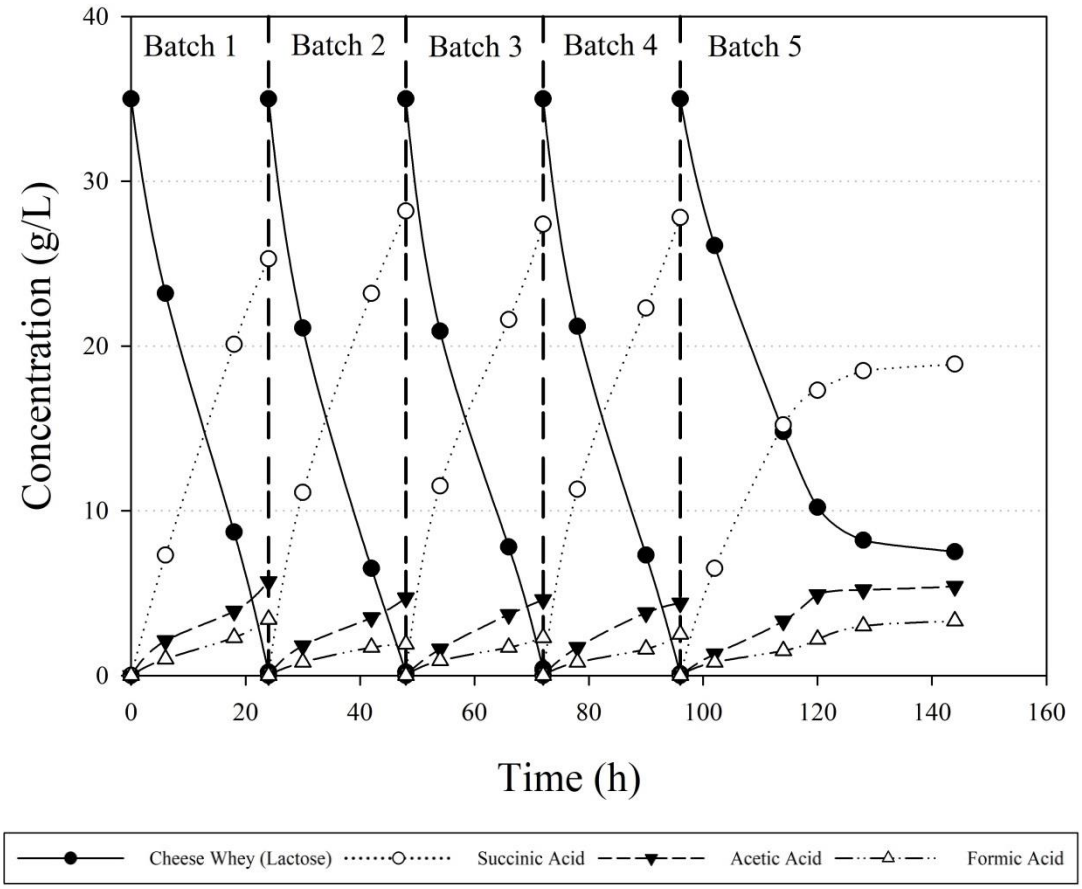


Figure 4.15 Lactose consumption and organic acid accumulation profiles of 5 repeated batch fermentations using immobilized *A. succinogenes*

As can be observed in Figure 4.15., the fermentation rate reduced dramatically after the fifth batch, and the fermentation process was ended after 48 hours. Following the same pattern as batch trials, the lactose in the broth was totally consumed in the first four batches within 24 hours. Despite the fact that the final succinic acid concentrations in the first four batches were almost similar and no significant difference observed ($p>0.05$), the second batch (28.2 g L^{-1}) produced the most succinic acid, whereas the first batch produced the least succinic acid (25.3 g L^{-1}) (Table 4.5). The first four batches provided yields of roughly 0.80 g g^{-1}; however, the significant drop ($p<0.05$) in production was observed in the fifth batch resulted in a yield of 0.54 g g^{-1}. Productivity dropped as low as 0.06 g L^{-1} h^{-1} in the final 24 hours of the 5th batch trial. The fact that 7.5 g L^{-1} lactose remained unutilized in the

system after the fermentation was terminated suggested that the cells were no longer viable at the end of 5th batch. According to the findings of Alexandri et al., after the fourth batch, both productivity and yield was declined as a result of the high number of beads that broken throughout the fermentation process (Alexandri et al., 2017). On other hand, no drop in the number of beads was seen in the present study, indicating that the use of whey in conjunction with alginate was effective in strengthening the beads. On the other hand, when the number of batch cycles is increased, it is possible that the specific growth rate and succinic acid production will decrease over time due to: a) an increase in succinic acid and by-products concentration in the beads, which will inhibit the growth of cells and further synthesis of succinic acid; and in each batch cycle the maximum concentration of cells within a bead is gradually increase. This latter tendency, in conjunction with cell aging, has the potential to result in a decrease in growth rate. Indeed, at the start of each new cycle the cells that were present in the beads were considerably older compared to the ones in the previous batches (Ercole et al., 2021).

Table 4.5 Organic acid, yield and productivity results of repeated-batch fermentation experiments

Batch	Succinic Acid ($g\ L^{-1}$)	Acetic Acid ($g\ L^{-1}$)	Formic Acid ($g\ L^{-1}$)	Yield ($g\ g^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)
1	25,3±0.153*	5,7±0.208	3,4±0.100	0,72±0.004	1,05±0.006
2	28,2±0.361	4,7±0.306	1,9±0.094	0,81±0.010	1,18±0.015
3	27,4±0.265	4,6±0.153	2,3±0.153	0,78±0.008	1,14±0.011
4	27,8±0.458	4,4±0.203	2,5±0.208	0,79±0.013	1,16±0.019
5	18,9±0.202	5,4±0.058	3,3±0.150	0,54±0.006	0,39±0.004

**Standard error was calculated by conducting each experiment three times.*

In terms of by-product creation, there was no evidence of lactic acid generation, with acetic acid serving as the primary byproduct. In Figure 4.15., it can be seen that the final concentrations of both by-products were consistent, with the exception of succinic acid titer, which rose from the first to the fourth batch run. Every batch produced formic acid with a final concentration of roughly 2.5-3 $g\ L^{-1}$, whereas acetic acid was produced within a range of 5 $g\ L^{-1}$. In the 2nd to 4th batch

runs both yields and productivities were exceeded the obtained results from immobilized batch and free cell batch experiments. The reason behind this increase may be the adaptation of cells to the environment. The cells that have been immobilized have already acclimated to the fermentation environment after the incubation-preparation of beads and also after the 1st batch run. Also pH in the immobilization matrix might decrease faster when reused in dispersed systems as a result of the well-known phenomenon of mass transfer, eventually leads to an increase in succinic acid synthesis (Ercole et al., 2021). Indeed, Galazzo and Bailey discovered that the internal pH of cells contained in alginate is somewhat lower than that of cells suspended in a fermentation broth freely, and that this condition has an effect on the permeability of the cell membrane (Galazzo and Bailey, 1990). The permeabilization of the cell membrane has been proposed by several researchers: penetration of protons through the membrane would have an impact on ATP usage rates since the cell spends ATP at a high rate in order to maintain constant intracellular pH which promoted the increase in cellular metabolism. Thus, it has been shown that the metabolic activity of immobilized cells increase as compared to free cells (Angelova et al., 2000; Dishisha et al., 2012). These beneficial occurrences, however, may be thwarted by the occurrence of unfavorable phenomena. The transport phenomena of carbon source into the entrapped cells and the transport phenomena of succinic acid out of the beads should reduce the effectiveness of the immobilized fermentation. The use of proper bead size and porosity, and also the mechanical stability, can, on the other hand, limit the negative effects (Galaction et al., 2011).

In this study, it can be concluded that usage of whey along with alginate to immobilize cells effectively increase mechanical stability of immobilization matrix. Even at the end of 5th fermentation batch no breaking of beads were observed. A total of 144 hours fermentation process was performed and 0.89 g L^{-1} h^{-1} of average productivity was obtained. A maximum of 1.18 g L^{-1} h^{-1} succinic acid productivity was resulted in 2nd batch cycle (Table 4.5). An average total yield of 0.76 g g^{-1}

resulted from five repeated batch cycles with a maximum yield of 0.81 g g^{-1} in 2nd batch were outperformed results of both immobilized and free batch fermentations.

4.2.3 Continuous Fermentations with Immobilized *A. succinogenes*

Continuous fermentation experiments were performed by using whey-alginate immobilized *A. succinogenes* cells and cheese whey containing media as substrate. Initially, experiments were started with 2 L working volume. In this working volume 250 g of immobilized beads were used and the lactose concentration in media was 35 g L^{-1}. Inlet stream also contains cheese whey fermentation media with 35 g L^{-1} lactose and this media was fed into the reactor with a dilution rate (D) of 0.05 h^{-1}.

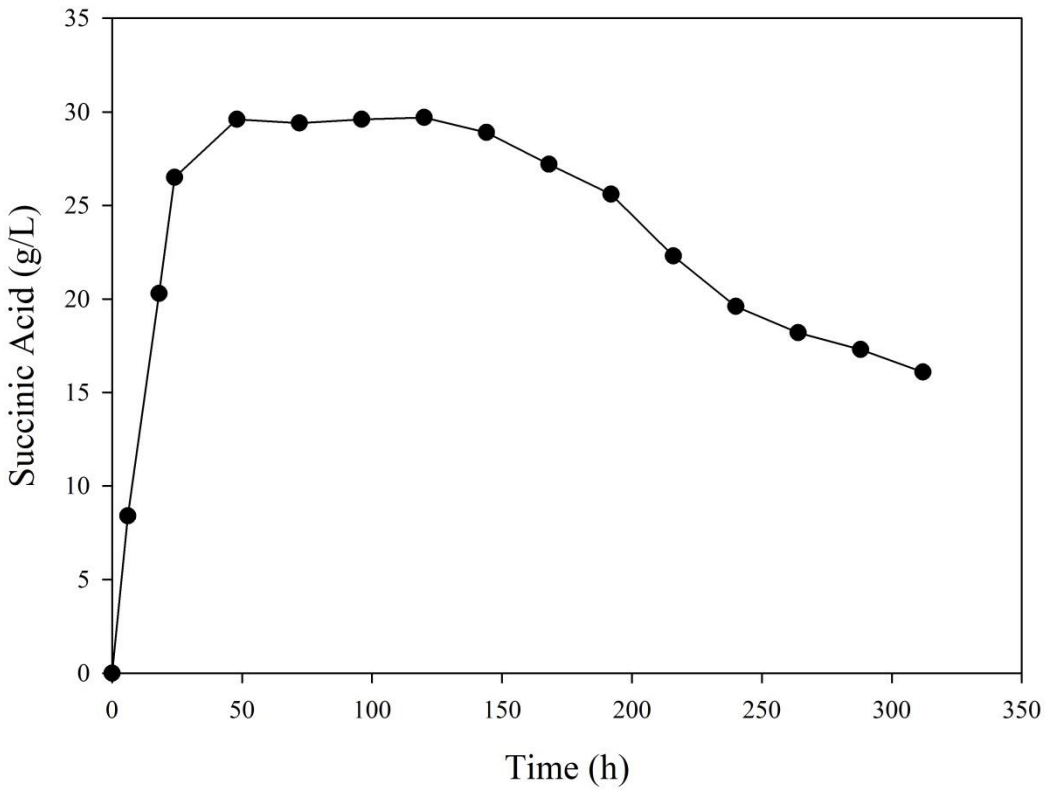


Figure 4.16 Succinic acid concentration profiles in continuous fermentation for 13 days (312 hours)

Inlet stream feeding was initiated after 18 hours of batch fermentation when there was 5.3 g L^{-1} of lactose remained present in the system and 20.3 g L^{-1} of succinic acid accumulated. After the initiation of continuous system, in the first 24 hours of fermentation, quasi steady state conditions were successfully achieved, and 29.6 g L^{-1} of succinic acid was produced in the first 24 hours, with a productivity of 1.23 g L^{-1} h^{-1}. When compared to the repeated-batch and batch studies, these findings were slightly better but not significant. Acetate and formate was produced as by-products, with titers of 4.2 g L^{-1} and 2.0 g L^{-1} in the fermentation broth, respectively. There was no evidence of lactic acid generation. Continuous fermentation experiments were conducted with a minimum of 9 days and maximum of 13 days. During all of the experiments, the beads began to break down after a week or so (7^{th}-9^{th} day). This might be related to the swelling of the beads as a result of the increased biomass in the immobilization matrix (Alexandri et al., 2017). Also washing of beads with $CaCl_2$ solution in a continuous system was not applicable. Not enough $CaCl_2$ in the system may also led the diffusion of calcium out of the gel structure increased alginate-water interaction, resulting in increased swelling (Wichchukit et al., 2013) and discharge of calcium also caused erosions on the beads (Mishra et al., 2016).

Fermentation studies were carried out for an extended period of time even after the beads had been broken down in order to determine the viability of the cells following the disruption. It was possible to execute operations for a total of 13 days. Following the loss of beads, the pace of production began to decline gradually, although the generation of organic acids remained unabated. On the tenth day of fermentation, 19.6 g L^{-1} of succinic acid was obtained, which was a drop from the previous day's value of 25.6 g L^{-1} obtained on the 8^{th} day of fermentation. Following the loss of the beads in the system, the growth of a biofilm was detected. Ferone et al. was stated that biofilm formation could not be avoided when the bioreactor was operated for more than 10 days, which was also supported by other similar studies (Bradfield and Nicol, 2014; Ferone et al., 2019b; van Heerden and Nicol, 2013). Urbance et al. was stated that *A. succinogenes* has the

natural ability to form biofilms (Urbance et al., 2004). The creation of biofilms following the loss of immobilization matrices demonstrated conclusively that the immobilization of cells was a good method for preventing biofilm formation in the first place.

Succinic acid yield was ranged between 0.73-0.84 g g^{-1} before the breaking down of beads and fell all the way to 0.46 g g^{-1} at the last day of fermentation when experiment was prolonged for 13 days. After this point biofilm formation was considerably high. Even though, it has been known that biofilms of *A. succinogenes* have beneficial effects on fermentation rate (Bradfield et al., 2015; Bradfield and Nicol, 2014; Maharaj et al., 2014), the bioreactor used in the experiments were not suitable for biofilm fermentation. The decrease in yields and productivity may be explained by this reason, since after high amounts of biofilm formation sufficient agitation and cell-substrate interaction could not be maintained in the used CSTR. After the maximum of 13^{th} day of continuous fermentation experiments were terminated. The average yield of 0.76 g g^{-1} was obtained with immobilized cells was slightly higher than the yield achieved in batch studies. These results were largely consistent with those obtained by previous continuous system trials of a similar kind (Table 4.6).

By raising both the rate of dilution and the concentration of cheese whey, the titer of succinic acid may be boosted (data not shown). But these were not chosen since at greater concentrations, bead swelling happened more quickly and beads began to break down sooner; the same event was observed at higher dilution rates as well. The preservation of immobilized cells was given top priority in this work, and as a result, the system was operated at a low dilution rate (0.05 h^{-1}).

Findings of the studies in the literature regarding the succinic acid production by using immobilized *Actinobacillus succinogenes* have been listed in Table 4.6 along with the results obtained from this study.

Table 4.6 Compilation of studies on succinic acid production by using immobilized *A. succinogenes*

Substrate	Immobilization support	Process mode	Yield ($g\ g^{-1}$)	Productivity ($g\ L^{-1}\ h^{-1}$)	Succinic acid titer ($g\ L^{-1}$)	References
Cheese Whey	Whey-alginate beads	Batch	0.75	1.09	26.2	This study
Cheese Whey	Whey-alginate beads	Repeated-batch	0.81	1.18	28.2	This study
Cheese Whey	Whey-alginate beads	Continuous	0.84	1.23	29.6	This study
Glucose	Alginate beads	Continuous	0.86	35.6	31.0	(Ercole et al., 2021)
Glucose	Tygon rings	Continuous	0.98	22.0	43.0	(Ferone et al., 2018)
Spent sulphite liquor	Alginate beads	Batch, Repeated-batch, Fed-batch	0.81	0.39	36.8	(Alexandri et al., 2017)
Glucose	Fibrous-bed	Batch, Fed-batch	0.89	2.80	98.0	(Q. Yan et al., 2014a)
Glucose	Stainless-steel wool	Continuous	0.91	-	48.5	(Bradfield and Nicol, 2014)
Glucose	Poraver ® beads	Continuous	0.90	10.8	32.5	(Maharaj et al., 2014)
Corn stover (xylose, glucose, arabinose, galactose)	Polypropylene protruding arms	Continuous	0.78	1.80	39.6	(Bradfield et al., 2015)
Glucose	Silicone tube	Continuous	0.74	9.00	9.70	(Brink and Nicol, 2014)
Glucose	Genulite TM Groperl	Continuous	0.69	6.40	12.0	(van Heerden and Nicol, 2013)
Glucose	PCS disks	Repeated-batch	0.86	0.90	35.1	(Urbance et al., 2004)
Glucose	Agar beads	Batch, Repeated-batch	0.61	2.65	21.5	(Corona-González et al., 2014)
Xylose	Stainless-steel wool	Continuous	0.55	3.40	10.9	(Salvachúa et al., 2016)
Lactose	Glaxstone®	Batch, Repeated-batch	0.58	0.72	8.00	(Longanesi et al., 2018)

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

In this study, fermentative production of succinic acid by using cheese whey as main carbon source was investigated. *Actinobacillus succinogenes* was used as succinic acid producing microorganism. In order to compare the efficiency of production, batch fermentation experiments with pure glucose and lactose containing media as substrate were performed along with cheese whey. *A. succinogenes* cells were immobilized using alginate and immobilization support (alginate beads) durability was improved by using whey in combination with alginate. Batch immobilized fermentation experiments in bioreactor with 2.5 L working volume were done by using synthetic media of glucose and lactose and also with cheese whey. Repeated-batch and continuous fermentations were performed with improved immobilized cells with the use of cheese whey containing media as fermentation broth.

Batch fermentations in shake flasks (200 mL working volume) and bioreactor (2.5 L working volume) using free *A. succinogenes* cells has showed that cheese whey is a favorable substrate for succinic acid fermentation. Achieved production yields (0.71 g g^{-1}) of succinic acid with cheese whey was exceeded the results obtained from glucose (0.63 g g^{-1}) and lactose (0.60 g g^{-1}) in both shake flasks and the bioreactor. In these experiments it was observed that presence of oxygen in the fermentation system leads to the production of lactic acid and it was determined that anaerobic environment is necessary for efficient production of succinic acid with *A. succinogenes*. As a result of optimization experiments it was observed that the usage of KOH as a neutralizing agent, $MgCO_3$ as an indirect CO_2 source and 35 g L^{-1} initial sugar concentration (compared to 60 g L^{-1}) gave the best results.

Immobilized cell experiments with the use of pure alginate showed that Ca-alginate beads had a poor stability in fermentation broth and broken down during fermentation process. Different fermentation conditions (neutralizing agent, working volume, stirring speed) were tested to analyze the response in durability of alginate beads. The solution to the problem of low strength of the beads was found with by preparing beads with a mixture of fermented whey and alginate. Batch fermentations were successfully performed with strengthened beads and 0.75 g g^{-1} succinic acid yield with 1.09 g L^{-1} h^{-1} of productivity achieved by using cheese whey as carbon source indicating the efficiency of immobilization in fermentation.

Further experiments were performed using immobilized cells in repeated-batch and continuous fermentation modes with cheese whey. Increase in the strength of beads was once more approved via using the same beads in 5 consecutive cycles in repeated-batch fermentations and 13 days of continuous fermentation. In repeated-batch experiments maximum 0.81 g g^{-1} succinic acid yield was achieved with a productivity of 1.18 g L^{-1} h^{-1} exceeding the batch fermentation results. In continuous fermentations, system was operated maximum of 312 hours and 0.76 g g^{-1} of average succinic yield was obtained.

This study gives new insights on the fermentation-based manufacture of succinic acid. The efficacy of succinic acid generation from cheese whey, lactose, and glucose has been demonstrated in batch experiments with immobilized and free cells, repeated-batch experiments with immobilized cells, and continuous fermentations with immobilized cells of *A. succinogenes*. Among these it was proven that cheese whey is a favorable and a valuable renewable resource for fermentative succinic acid production. The immobilization in calcium-alginate beads using cheese whey combined with alginate has been shown to be a good method of increasing the resilience of the beads. *A. succinogenes* immobilized on a solid support may be an attractive option for efficient biosuccinic acid production because to the high yields and productivities attained, as well as the cell reusability and reduced fermentation time. However, further studies are required to apply large

scale production using these methods. Some challenges and future perspectives are; reducing the by-product formation to increase fermentation efficiency, reducing the neutralizing agent addition to reduce downstream processing costs, metabolic engineering applications to increase efficiency and optimization of the supplementation of nitrogen/vitamin sources. These parameters are important prospective topics to investigate in order to develop more economical succinic acid production bioprocess.

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APPENDICES

A. CHEMICALS AND SUPPLIER INFORMATION

Table A.1 The list of chemicals and materials with their suppliers

Chemicals	**Producers**
D-(+)-Glucose monohydrate	Sigma-Aldrich (St. Lois, MO, USA)
D-(+)-Lactose monohydrate	Sigma-Aldrich (St. Lois, MO, USA)
D-(+)-Galactose	Fluka Chemie GmbH (Germany)
L-(+)-Arabinose	Fluka Chemie GmbH (Germany)
Cheese Whey	Açkar Dairy Products Co. (Turkey)
TSB Broth	Merck (Darmstadt, Germany)
Yeast Extract	Merck (Darmstadt, Germany)
Magnesium carbonate ($MgCO_3$)	Emir Kimya (Turkey)
Sodium bicarbonate ($NaHCO_3$)	Emir Kimya (Turkey)
Sodium hydroxide (NaOH)	Merck (Darmstadt, Germany)
Potassium hydroxide (KOH)	Emir Kimya (Turkey)
Calcium hydroxide ($Ca(OH)_2$)	Emir Kimya (Turkey)
Dipotassium hydrogen phosphate (K_2HPO_4)	Merck (Darmstadt, Germany)
Potassium dihydrogen phosphate (KH_2PO_4)	Merck (Darmstadt, Germany)
Sodium chloride (NaCl)	Merck (Darmstadt, Germany)
Calcium chloride ($CaCl_2$)	Merck (Darmstadt, Germany)
Magnesium chloride hexahydrate ($MgCl_2$*6 H_2O)	Merck (Darmstadt, Germany)
Succinic acid disodium salt	Sigma-Aldrich (St. Lois, MO, USA)
D-(-)-Lactic acid	Sigma-Aldrich (St. Lois, MO, USA)
Formic acid	Sigma-Aldrich (St. Lois, MO, USA)
Acetic acid	Merck (Darmstadt, Germany)
Sulfuric acid (H_2SO_4)	Merck (Darmstadt, Germany)
Nutrient agar	Merck (Darmstadt, Germany)
Glycerol	Merck (Darmstadt, Germany)

B. STANDARD CURVES OF HPLC ANALYSIS

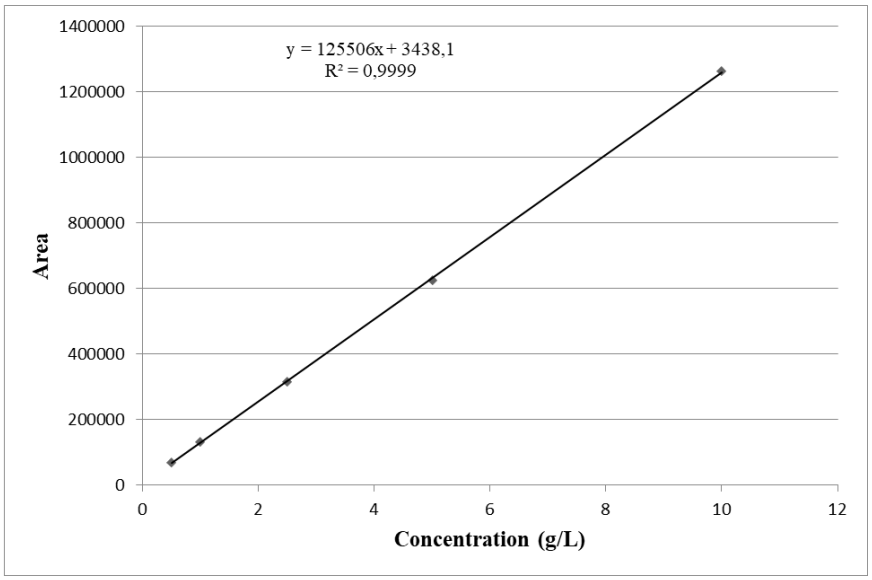

Figure B.1 Standard curve of glucose

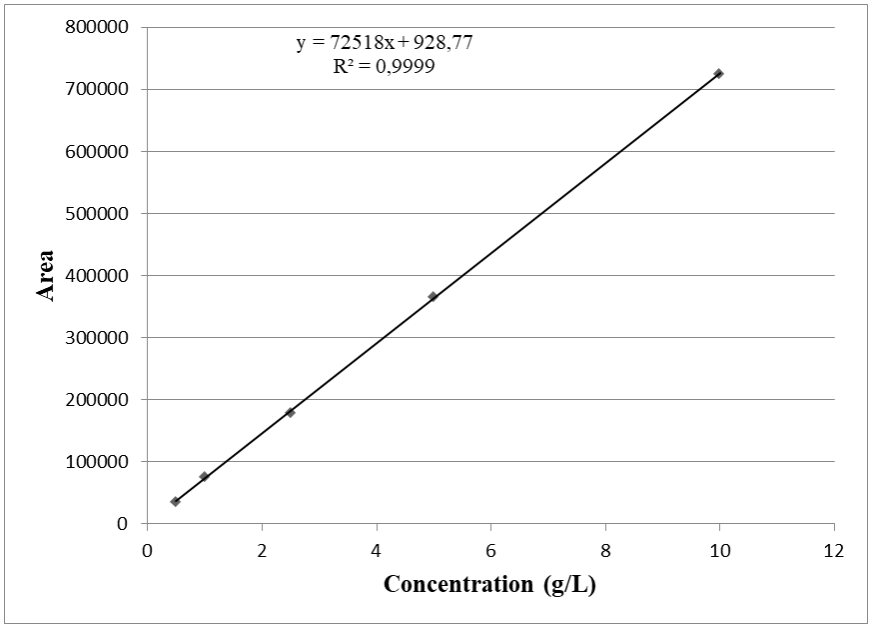

Figure B.2 Standard curve of lactose

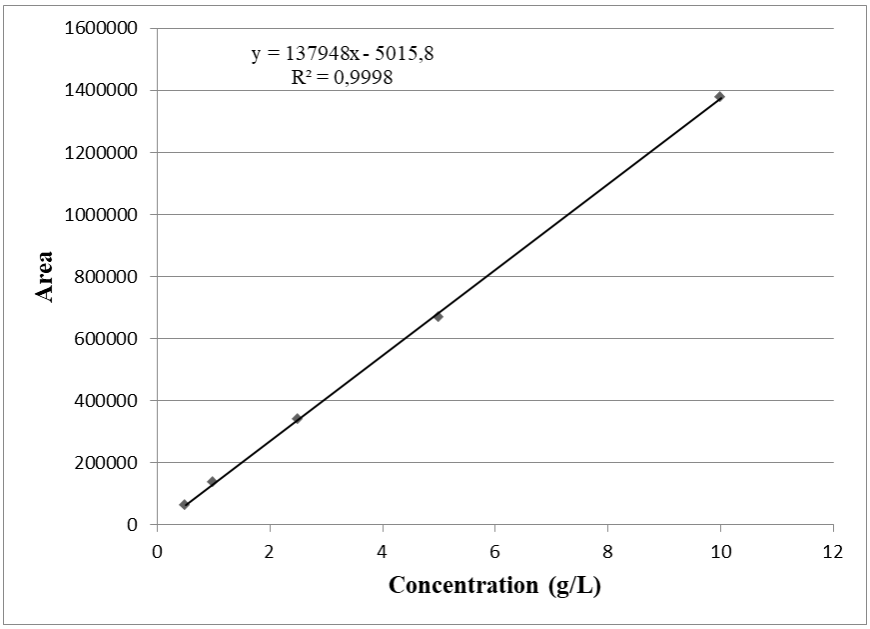

Figure B.3 Standard curve of galactose

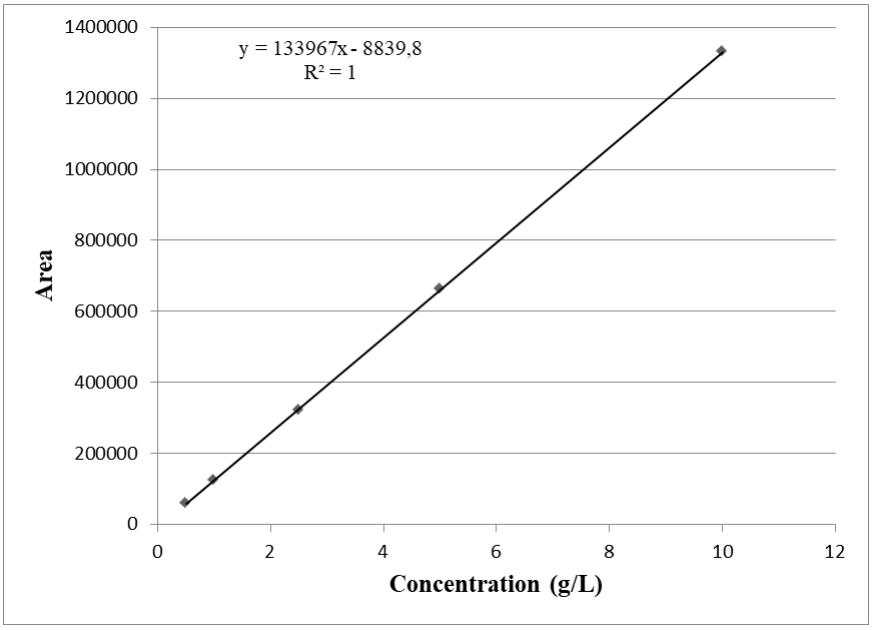

Figure B.4 Standard curve of arabinose

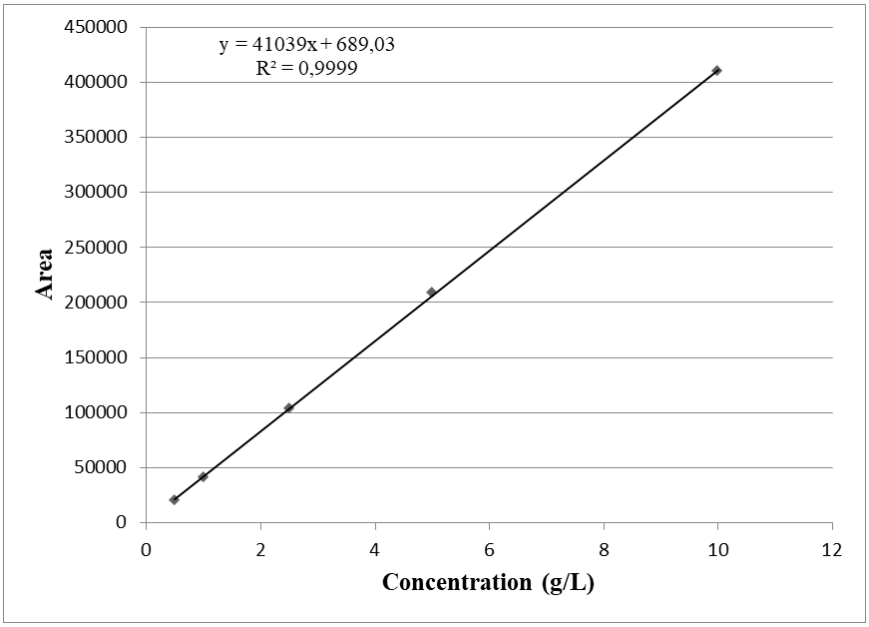

Figure B.5 Standard curve of succinic acid

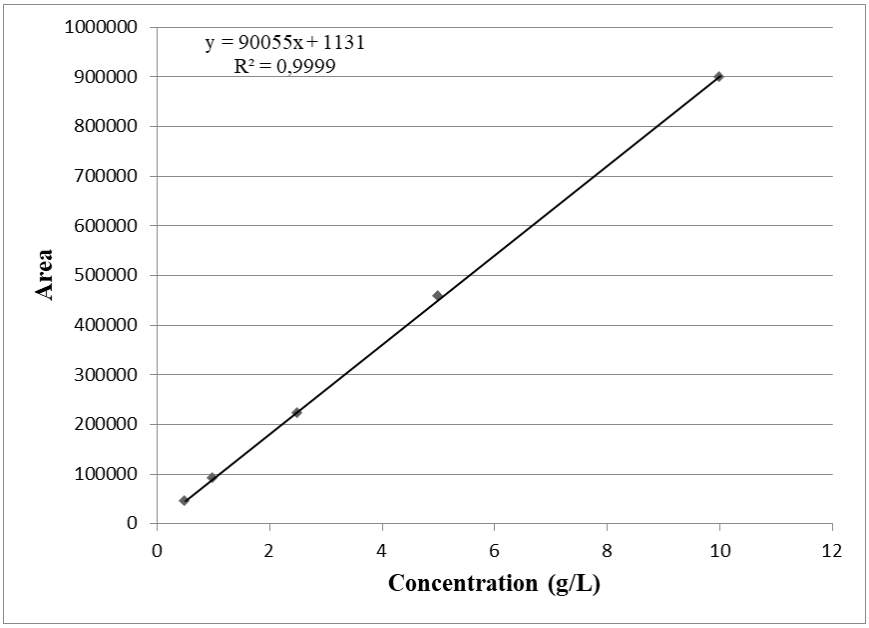

Figure B.6 Standard curve of lactic acid

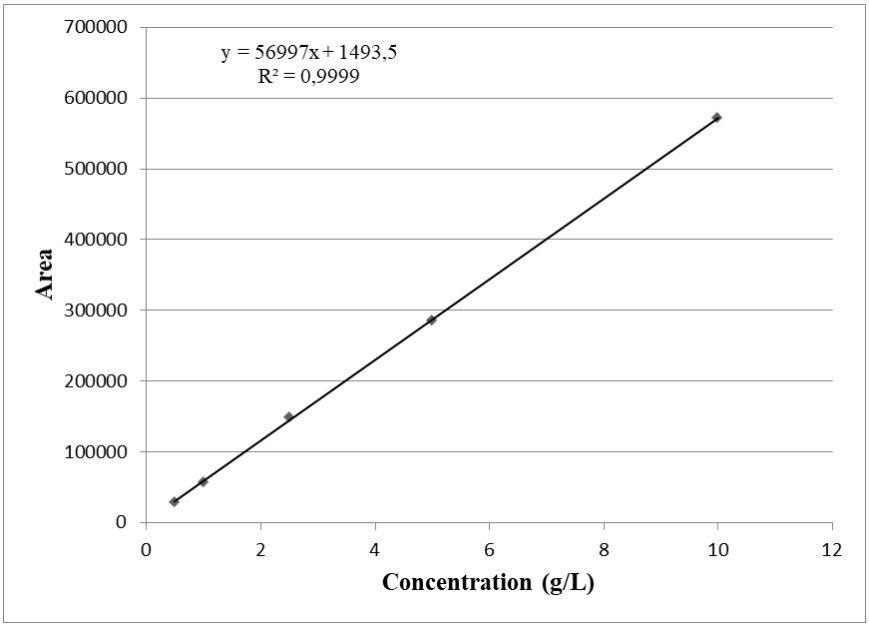

Figure B.7 Standard curve of acetic acid

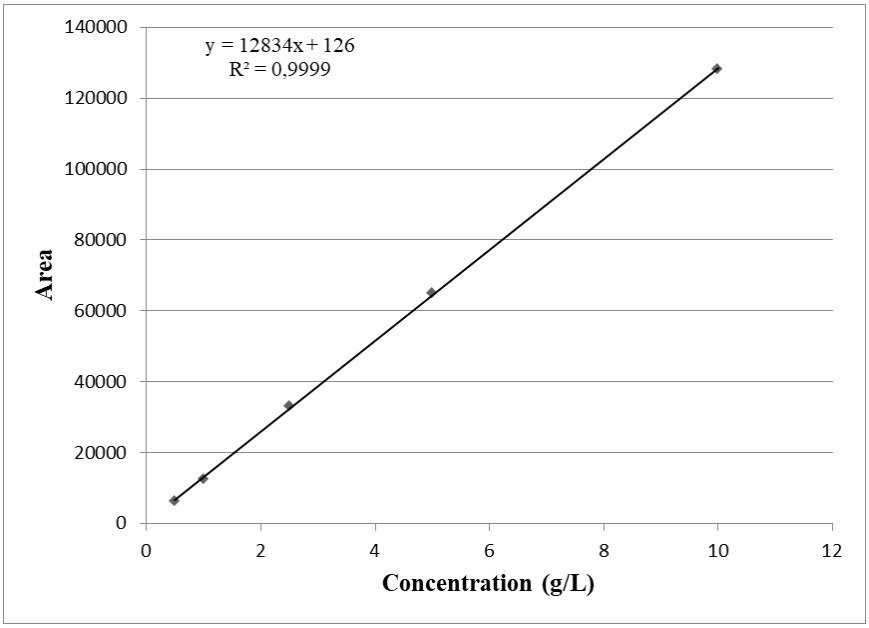

Figure B.8 Standard curve of formic acid

C. OPTICAL DENSITY ANALYSIS

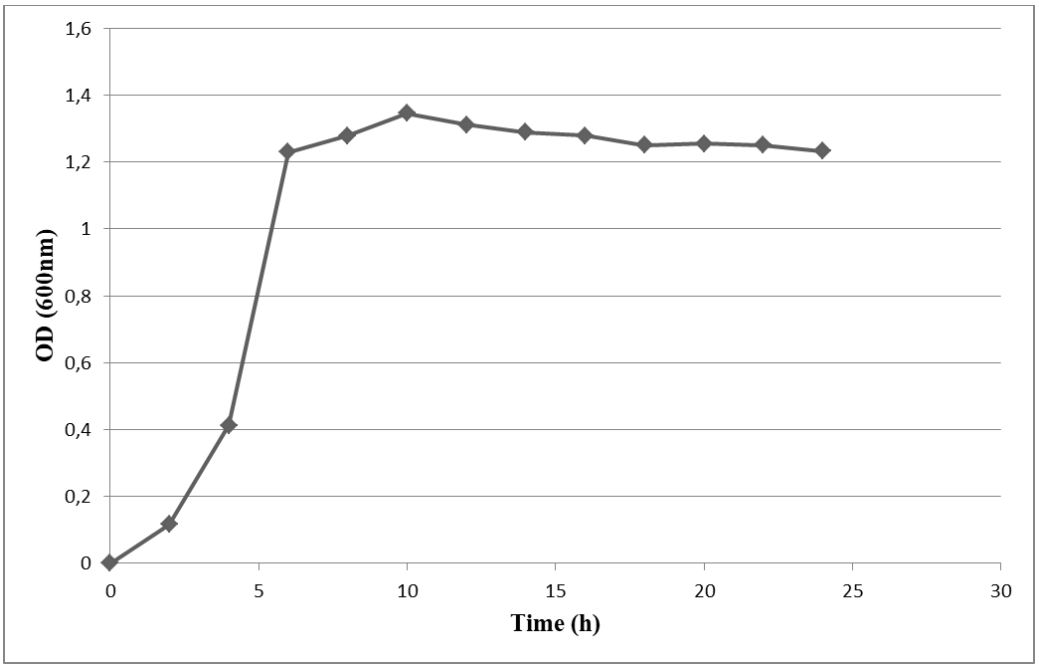

Figure C.1 Growth curve for *Actinobacillus succinogenes* 130Z (OD values were determined by diluting the sample until it reaches an OD_{600} = 0.6 and calculated accordingly)

D. STATISTICAL ANALYSIS OF DATA

Table D.1 One way ANOVA for carbon source effect on succinic acid production in shake flasks, batch bioreactor with free cells and batch bioreactor with immobilized cells

Source	DF	SS	MS	F	P
Carbon Source	8	558.17	69.7708	90.66	0.000
Error	18	13.85	0.7696		
Total	26	572.02			
S = 0.877285		R-Sq = 97.58%		R-Sq(adj) = 96.50%	

Table D.2 Tukey pairwise comparisons with 95% confidence of succinic acid production in shake flask, free cell batch bioreactor and immobilized cell batch bioreactor fermentations with different carbon sources (35 g L^{-1} initial concentration)

Carbon Source	Fermentation Mode	N (number of trials)	Mean	Grouping
Whey	Immobilized Cell - Batch	3	26.2	A
Whey	Free Cell - Batch	3	24.9	A
Glucose	Immobilized Cell - Batch	3	24.5	A, B
Lactose	Immobilized Cell - Batch	3	22.3	B,C
Glucose	Free Cell - Batch	3	22.1	B,C
Lactose	Free Cell - Batch	3	20.9	C
Whey	Shake Flask	3	17.0	D
Glucose	Shake Flask	3	15.9	D
Lactose	Shake Flask	3	11.6	E

Means that do not share a group letter are significantly different.

Table D.3 One way ANOVA for indirect CO_2 source effect on succinic acid production in batch experiments in bioreactor with free cells

Source	DF	SS	MS	F	P
CO_2 Source	1	1.307	1.3067	5.16	0.086
Error	4	1.013	0.2533		
Total	5	2.320			
S = 0.503322		R-Sq = 56.32%		R-Sq(adj) = 45.40%	

Table D.4 Tukey pairwise comparisons with 95% confidence of succinic acid production with different indirect CO_2 sources in batch bioreactor fermentations with free cells

CO_2 Source	N (number of trials)	Mean	Grouping
$MgCO_3$	3	24.3	A
$NaHCO_3$	3	23.3	A

Means that do not share a group letter are significantly different.

Table D.5 One way ANOVA for neutralizing agent effect on succinic acid production in batch experiments in bioreactor with free cells

Source	DF	SS	MS	F	P
Neutralizing Agent	1	7.042	7.0417	9.56	0.037
Error	4	2.947	0.7367		
Total	5	9.988			
S = 0.858293		R-Sq = 70.50%		R-Sq(adj) = 63.12%	

Table D.6 Tukey pairwise comparisons with 95% confidence of succinic acid production with different neutralizing agents in batch bioreactor fermentations with free cells

Neutralizing Agent	N (number of trials)	Mean	Grouping
NaOH	3	24.3	A
KOH	3	22.1	B

Means that do not share a group letter are significantly different.

Table D.7 One way ANOVA for initial sugar concentration effect on succinic acid yield in batch experiments in bioreactor with free cells

Source	DF	SS	MS	F	P
Neutralizing Agent	3	0.033832	0.011277	23.40	0.000
Error	8	0.003856	0.000482		
Total	11	0.037687			
S = 0.0219532		R-Sq = 89.77%		R-Sq(adj) = 85.93%	

Table D.8 Tukey pairwise comparisons with 95% confidence of succinic acid yield with different initial sugar concentrations (35-60 g L^{-1}) in batch bioreactor fermentations with free cells

Carbon Source	N (number of trials)	Mean	Grouping
35 g L^{-1} Glucose	3	0.63	A
35 g L^{-1} Lactose	3	0.60	A,B
60 g L^{-1} Glucose	3	0.56	B
60 g L^{-1} Lactose	3	0.49	C

Means that do not share a group letter are significantly different.

Table D.9 One way ANOVA for initial sugar concentration effect on succinic acid productivity in batch experiments in bioreactor with free cells

Source	DF	SS	MS	F	P
Neutralizing Agent	3	0.141885	0.047295	138.81	0.000
Error	8	0.002726	0.000341		
Total	11	0.144610			
S = 0.0184584		R-Sq = 98.12%		R-Sq(adj) = 97.41%	

Table D.10 Tukey pairwise comparisons with 95% confidence of succinic acid productivity with different initial sugar concentrations (35-60 g L^{-1}) in batch bioreactor fermentations with free cells

Carbon Source	N (number of trials)	Mean	Grouping
60 g L^{-1} Glucose	3	0.70	A
60 g L^{-1} Lactose	3	0.61	B
35 g L^{-1} Glucose	3	0.46	C
35 g L^{-1} Lactose	3	0.44	C

Means that do not share a group letter are significantly different.

Table D.11 One way ANOVA for carbon source effect on succinic acid yield in batch experiments in bioreactor with free and immobilized cells

Source	DF	SS	MS	F	P
Carbon source	5	0.049980	0.009996	19.44	0.000
Error	12	0.006171	0.000514		
Total	17	0.056151			
S = 0.0226779		R-Sq = 89.01%		R-Sq(adj) = 84.43%	

Table D.12 Tukey pairwise comparisons with 95% confidence of succinic acid yield in free cell batch bioreactor and immobilized cell batch bioreactor fermentations with different carbon sources (35 g L^{-1} initial concentration)

Carbon Source	Fermentation Mode	N (number of trials)	Mean	Grouping
Whey	Immobilized Cell - Batch	3	0.75	A
Whey	Free Cell - Batch	3	0.71	A
Glucose	Immobilized Cell - Batch	3	0.70	A
Lactose	Immobilized Cell - Batch	3	0.64	B
Glucose	Free Cell - Batch	3	0.63	B
Lactose	Free Cell - Batch	3	0.60	B

Means that do not share a group letter are significantly different.

Table D.13 One way ANOVA for carbon source effect on succinic acid productivity in batch experiments in bioreactor with free and immobilized cells

Source	DF	SS	MS	F	P
Carbon source	5	1.37450	0.274900	441.37	0.000
Error	12	0.00747	0.000623		
Total	17	1.38197			
S = 0.0249566		R-Sq = 99.46%		R-Sq(adj) = 98.78%	

Table D.14 Tukey pairwise comparisons with 95% confidence of succinic acid productivity in free cell batch bioreactor and immobilized cell batch bioreactor fermentations with different carbon sources (35 g L^{-1} initial concentration)

Carbon Source	Fermentation Mode	N (number of trials)	Mean	Grouping
Whey	Immobilized Cell - Batch	3	1.09	A
Glucose	Immobilized Cell - Batch	3	1.02	B
Lactose	Immobilized Cell - Batch	3	0.93	C
Whey	Free Cell - Batch	3	0.52	D
Glucose	Free Cell - Batch	3	0.46	D,E
Lactose	Free Cell - Batch	3	0.43	E

Means that do not share a group letter are significantly different.

Table D.15 One way ANOVA for succinic acid production in 5 consecutive repeated-batch fermentation cycle

Source	DF	SS	MS	F	P
Batch	4	179.364	44.8410	156.79	0.000
Error	10	2.860	0.2860		
Total	14	182.224			
S = 0.534790		R-Sq = 98.43%		R-Sq(adj) = 97.80%	

Table D.16 Tukey pairwise comparisons with 95% confidence of succinic acid production in 5 consecutive repeated-batch fermentations

Batch	N (number of trials)	Mean	Grouping
2nd	3	28.2	A
4th	3	27.8	A
3rd	3	27.4	A
1st	3	25.3	B
5th	3	18.9	C

Means that do not share a group letter are significantly different.