CHAPTER 1
INTRODUCTION

1.1 Overview

Bio-succinic acid is recognized by the U.S. Department of Energy as one of the top 12 building block chemicals that could be converted into intermediate compounds such as 1,4-butanediol and tetrahydrofuran (Werpy and Petersen, 2004; Bozell and Petersen, 2010; Takahashi et al., 2021). It is currently proposed as a potential suitable substitute for current petrochemical production, which is mainly used in a variety of applications including food ingredients, agriculture, green solvents, pharmaceutical products, and completely biodegradable plastics (Song et al., 2006; Okino et al., 2008; Cheng et al., 2012; Jiang et al., 2013, 2014). For this reason, its commercial demand is expected to increase at a compound annual growth rate of 27.4% (768 million metric tons) by the year 2025, up from succinic acid manufacturing in the year 1990 (18,000 metric ton) (Sharma et al., 2020). In general, most of the utilized succinic acid in the chemical industry is produced from petroleum-derived maleic anhydride, which is limited in natural feedstock, too expensive, and has negative environmental consequences for widespread use as a platform chemical (Chen et al., 2010; 2017). To overcome the mentioned problems, succinic acid can be produced through microbial fermentation using renewable feedstocks as a carbon source, with *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, and *Manheimia succiniciproducens* achieving the highest succinic acid titer (Carvalh et al., 2016; Guettler et al., 1996). Unfortunately, for optimal growth and high succinate production, these microorganisms require expensive nutrients as well as external supplies of carbon dioxide, hydrogen, or the mixture of gases, resulting in high costs in industrial-scale succinate production. Furthermore, these microorganisms are at the difficulty of metabolic engineering for eliminating by-products thus causing a trouble in succinate production and its purification (Kim et al., 2004).

Currently, a lot of efforts have been made to develop recombinant *Escherichia coli* strains capable of producing succinic acid efficiently. One of the most well-known *E. coli* strains developed, *E. coli* KJ122 strain was constructed by a combined metabolic engineering and metabolic evolution, resulting in high succinic acid at the titer of 80 g/L with the molar yield of 1.46 mol per mole of glucose consumed, 85% of the maximum theoretical yield (Jantama et al., 2008ab). However, this strain has a limited or an inefficient utilization for wide- range carbon sources due to its catabolic repression by glucose and natural incapability. Presently, *Klebsiella* is a good producer and has received a lot of attention for the production of commercially fermentable products such as 1,3-propanediol, 2,3-butanediol, and lactic acid due to its fast growth, undesirable any special or expensive source of nutrients during growth, availability of well-established methods for genetic tractability and an ability to utilize a broad substrate spectrum (Wojtusik et al., 2015; Cho et al., 2015a, b; 2012; Lawrence et al., 1996; Celinska, 2012). In recent years, a metabolically engineered *K. oxytoca* KMS006 (Δ*adhE* Δ*pta-ackA* Δ*ldhA*) strain, a derivative of *K. oxytoca* M5A1, has been demonstrated to be a biosafety strain as a production host for succinic acid production (Jantama et al., 2015). However, the concentration of succinic acid was not satisfactory due to its impaired growth during anaerobic growth, and it produced several by-products such as 2,3-butanediol, formic acid, and acetic acid under microaerobic conditions. Therefore, the goal of this study is to establish *K. oxytoca* KMS006 as a novel potential microbial platform for industrial-scale succinic acid production. *K. oxytoca* KMS006 was purposefully developed by combining metabolic engineering of the main gene associated with by-product generation and metabolic evolution to increase succinic acid yield.

1.2 Research objectives

The objectives of this study are to enhance succinic acid production in *K. oxytoca* KMS006 through a combination of metabolic engineering and metabolic evolution. Succinate fermentation was optimized for parameters affecting succinic acid final titer, yield, and productivity to achieve high succinic acid production. Thus, this work focused on:

1. Investigating effects of disrupting the 2,3-butanediol, acetyl-CoA, acetic acid, and lactic acid pathways on succinic acid production in mineral salts medium under anaerobic conditions in *K. oxytoca* KMS006.

2. Evolving the mutant strain under anaerobic conditions in mineral salt medium containing 5% and 10% w/w glucose to obtain an evolved strain exhibiting better growth and succinic acid production simultaneously.

3. Investigating transcription levels or mutations of any genes affecting succinic acid production after the combining genetic manipulation and evolutionary adaptation on the metabolites of engineered strain by comparing between the parental strain (*K. oxytoca* KMS006) and the evolved strain.

4. Investigating the ability of mutant strains to produce succinic acid from a wide range of carbon sources to provide global information for future strain development using alternative carbon sources.

5. Improving the evolved strain's succinic acid production by optimizing the parameters such as initial OD_{550}, CO_2 supply, aeration and agitation rates affecting succinate fermentation in a 5 L bioreactor containing mineral salts medium and 100 g/L glucose.

6. Improving the production of succinic acid by applying batch fermentation using untreated sugarcane molasses as a carbon source.

1.3 Scope and limitations

K. oxytoca KMS006 was modified by deleting the main genes involves in the four biosynthetic pathways, which include the 2,3-butanediol pathway (catabolic **α**-acetolactate synthase (*budB*) and alpha-acetolactate decarboxylase (*budA*), on acetyl-CoA pathway (pyruvate formate-lyase (*pflB*), acetic acid pathway (propionate kinase (*tdcD*)), and lactic acid pathways (L-lactate dehydrogenase (*pmd*)). As a specific selective pressure, the engineered strain was evolved in AM1 medium containing 5-10 g/L of glucose and 100 mM potassium bicarbonate to obtain a newly strain with improved growth and high succinate production. The internal metabolic flux of newly strain was investigated by examining transcription levels of key enzyme activities in the primary three pathways, which include the reductive branch of the TCA cycle, the oxidative branch of the TCA cycle, and acetate synthesis. The new strain was tested

for their capacity to produce succinic acid from a variety of carbon sources by performing under anaerobic conditions in AM1 medium containing 50 g/L of glucose, sucrose, maltose, fructose, lactose, xylose, and glycerol. Batch fermentation was carried out in a 5 L bioreactor with AM1 medium containing 100 g/L glucose, and parameters affecting succinate fermentation, such as initial OD_{550}, CO_2 supply, and agitation rates, were optimized. In addition, untreated sugarcane molasses was used as an alternative carbon source to reduce the cost of the succinic acid process.