REENGINEERING OF *ESCHERICHIA COLI* TO PRODUCE SUCCINATE FROM XYLOSE-CONTAINING MEDIUM AND ITS PURIFICATION BY NANOFILTRATION

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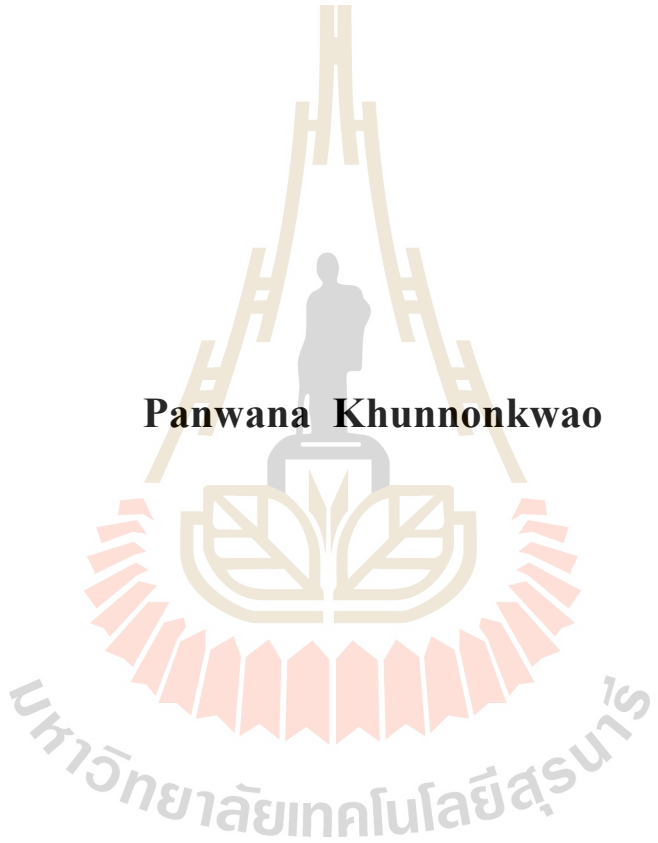

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วิศวกรรมของเอสเชอริเชีย โคไล เพื่อผลิตซักซิเนตจากอาหารเลี้ยงเชื้อที่มีน้ำตาลไซโลสและการทำบริสุทธิ์ด้วยกระบวนการกรองชนิดนาโน

นางสาวพรรวณา ขุนโนนเขวา

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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พรรวณา ขุนโนนเขวา : วิศวกรรมของ *Escherichia coli* เพื่อผลิตซักซิเนตจากอาหารเลี้ยงเชื้อที่มีน้ำตาลไซโลสและการทำบริสุทธิ์ด้วยกระบวนการกรองชนิดนาโน (REENGINEERING OF *ESCHERICHIA COLI* TO PRODUCE SUCCINATE FROM XYLOSE-CONTAINING MEDIUM AND ITS PURIFICATION BY NANOFILTRATION) อาจารย์ที่ปรึกษาที่มหาวิทยาลัยเทคโนโลยีสุรนารี : รองศาสตราจารย์ ดร.เขมวิทย์ จันต๊ะมา, อาจารย์ที่ปรึกษาที่ UNIVERSITÉ PAUL SABATIER : DR. HÉLÈNE ROUX-DE BALMANN, 236 หน้า.

Escherichia coli สายพันธุ์ KJ122 ได้ถูกดัดแปลงพันธุกรรมเพื่อให้สามารถผลิตซักซิเนตในระดับความเข้มข้น และผลผลิตที่สูงในอาหารเลี้ยงเชื้ออย่างง่ายที่มีกลูโคส ด้วยการหมักอย่างง่ายภายใต้สภาวะไร้ออกซิเจน อย่างไรก็ตามสายพันธุ์นี้ไม่สามารถใช้น้ำตาลไซโลสอย่างมีประสิทธิภาพเนื่องจากการยับยั้งกระบวนการสลาย ดังนั้นเพื่อที่จะเพิ่มประสิทธิภาพการนำเข้าและใช้น้ำตาลไซโลสของ *E. coli* สายพันธุ์ KJ122 ยีนที่ควบคุมการขนส่งน้ำตาลไซโลสเข้าสู่เซลล์ (*xylFGH*) ถูกยับยั้งด้วยเทคนิคการตัดสายพันธุกรรมทิ้ง ได้สายพันธุ์กลายพันธุ์ที่มีชื่อว่า KJ12201 (*E. coli* สายพันธุ์ KJ122 ที่ถูกตัดยีน *xylFGH*) แสดงความสามารถในการเจริญ การใช้น้ำตาลไซโลสและการผลิตซักซิเนตที่สูงมากเมื่อเทียบกับสายพันธุ์พ่อแม่ หลังจากการวิวัฒนาการของกระบวนการสร้างและสลาย พบว่าอีโคไลสายพันธุ์ KJ12201-14T สามารถใช้น้ำตาลไซโลสความเข้มข้น 10 เปอร์เซ็นต์อย่างมีประสิทธิภาพในการผลิตซักซิเนตที่มีความเข้มข้นสูงถึง 84 กรัมต่อลิตร โดยมีการสะสมของอะซิเตทที่ความเข้มข้น 11 กรัมต่อลิตร ในอาหารเลี้ยงเชื้ออย่างง่าย (AM1) ภายใต้สภาวะการหมักแบบไร้ออกซิเจนแบบกะ ในระหว่างกระบวนการหมักแบบกึ่งกะ พบว่า *E. coli* สายพันธุ์ KJ12201-14T ผลิตซักซิเนตที่ความเข้มข้น 84 กรัมต่อลิตร โดยมีผลผลิตอยู่ที่ 0.85 กรัมของซักซิเนตต่อกรัมของน้ำตาลทั้งหมดที่ใช้ไป และอัตราการผลิตที่ 1.0 กรัมต่อลิตรต่อชั่วโมง จากผลการทดลองดังกล่าวแสดงให้เห็นว่า *E.coli* สายพันธุ์ KJ12201 น่าจะเป็นสายพันธุ์ที่มีศักยภาพในการผลิตซักซิเนตจากน้ำตาลไซโลสและไฮโดรไลเสตที่มีน้ำตไซโลสซึ่งได้จากวัสดุลิกโนเซลลูโลสที่คุ้มค่าทางเศรษฐกิจ

ในการศึกษาครั้งนี้ได้ทำการศึกษาเกี่ยวกับการทำบริสุทธิ์ของซักซิเนตจากน้ำหมักด้วยกระบวนการกรองชนิดนาโน การทดลองครั้งนี้ได้ทำการทดลองกับแผ่นเยื่อ NF45 และน้ำหมักสังเคราะห์ที่มีซักซิเนตและสิ่งเจือปนต่างๆ เช่น เกลืออนินทรีย์ กลูโคส และเกลือของกรดอินทรีย์รวมไปถึงอะซิเตท ทั้งนี้ได้ทำการศึกษาผลกระทบของสภาวะการดำเนินการ เช่น ค่าความเป็นกรดด่าง ความดัน ตลอดจนองค์ประกอบของน้ำหมักต่อประสิทธิภาพของกระบวนการกรองชนิดนาโน

ทั้งนี้กลไกการถ่ายเทมวลสารของตัวถูกละลายผ่านแผ่นเยื่อถูกศึกษาเพื่ออธิบายค่ารีเทนชั่นของตัวถูกละลายต่างชนิดกันซึ่งเป็นองค์ประกอบของน้ำหมัก จากการทดลองพบว่าในสารละลายที่มีตัวถูกละลายหนึ่งชนิด ค่ารีเทนชั่นเพิ่มขึ้นเมื่อเพิ่มความดันและค่าความเป็นกรดด่างของสารป้อน และลดลงเมื่อความเข้มข้นของสารป้อนสูงขึ้น ตัวอย่างเช่น ค่ารีเทนชั่นของซักซิเนต และอะซิเตทในสารละลายผสมไม่แตกต่างจากค่าที่วัดได้ในสารละลายที่มีตัวถูกละลายหนึ่งชนิดที่ความเข้มข้นของเกลือต่ำประมาณ 0.1 โมลาร์ ดังนั้นสามารถทำบริสุทธิ์ของซักซิเนตได้ดี ในทางตรงกันข้ามค่ารีเทนชั่นลดลงเนื่องจากผลกระทบจากการคัดเลือกเมื่อความเข้มข้นของซักซิเนตสูงขึ้น ดังนั้นค่ารีเทนชั่นของซักซิเนต และอะซิเตทมีค่าใกล้เคียงกันเกินไปที่จะทำให้เกิดการแยก จากผลการศึกษากลไกการเทมวลสารข้างต้นได้ถูกนำมาใช้เพื่อหาวิธีการในการทำบริสุทธิ์ของซักซิเนตจากน้ำหมัก การแยกซักซิเนตออกจากอะซิเตทถูกดำเนินการในสองขั้นตอน โดยการทำไดอะฟิลเตรชันของน้ำหมักที่ทำการเจือจางจะดำเนินการในขั้นตอนแรก และการทำให้เข้มข้นจะถูกดำเนินการในขั้นตอนต่อมา ซึ่งการแยกด้วยกระบวนการนี้ทำให้ค่าบริสุทธิ์ของซักซิเนตเพิ่มขึ้นจากร้อยละ 85 เป็นมากกว่าร้อยละ 99.5 ในขณะที่สามารถรักษาผลผลิตมากกว่าร้อยละ 92 จากงานวิจัยนี้แสดงให้เห็นว่ากระบวนการกรองชนิดนาโนสามารถนำมาใช้ในการทำบริสุทธิ์ซักซิเนตจากน้ำหมักได้อย่างมีประสิทธิภาพ

สาขาวิชาเทคโนโลยีชีวภาพ
ปีการศึกษา 2559

ลายมือชื่อนักศึกษา พรรวณา ชนโนนงาว
ลายมือชื่ออาจารย์ที่ปรึกษา (at SUT)
ลายมือชื่ออาจารย์ที่ปรึกษา (at UPS)
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PANWANA KHUNNONKWAO : REENGINEERING OF *ESCHERICHIA COLI* TO PRODUCE SUCCINATE FROM XYLOSE-CONTAINING MEDIUM AND ITS PURIFICATION BY NANOFILTRATION. THESIS ADVISOR AT SURANAREE UNIVERSITY OF TECHNOLOGY : ASSOC. PROF. KAEMWICH JANTAMA, Ph.D., THESIS ADVISOR AT UNIVERSITÉ PAUL SABATIER : HÉLÈNE ROUX-DE BALMANN, Ph.D., 236 PP.

SUCCINATE/FERMENTATION/*ESCHERICHIA COLI*/METABOLIC ENGINEERING/XYLOSE/NANOFILTRATION

Escherichia coli KJ122 strain was previously engineered to produce high titers and yields of succinate in mineral salts medium containing glucose under simple-batch anaerobic conditions. However, this strain does not efficiently utilize xylose due to catabolic repression. To improve the xylose uptake and its utilization of *E. coli* KJ122, *xylFGH* genes were inactivated by the gene deletion technique. The mutant strain named KJ12201 (*E. coli* KJ122 Δ*xylFGH*) exhibited high abilities in fast growth, xylose consumption and succinate production compared to those of the parental strains. After performing metabolic evolution, *E. coli* KJ12201-14T efficiently consumed 10% xylose to produce a high succinate concentration at 84 g/L with an accumulated acetate concentration at 11 g/L in mineral salts medium (AM1) under batch fermentation. During fed-batch fermentation, *E. coli* KJ12201-14T produced succinate at a concentration, yield, and overall productivity of 84 g/L, 0.85 g/g, and 1.0 g/L/h, respectively. These results demonstrated that *E. coli* KJ12201 would be a potential strain for the economic bio-based succinate production from xylose and other xylose-rich hydrolysates derived from lignocellulosic materials.

The succinate purification from fermentation broth by nanofiltration (NF) was also investigated. The experiment was carried out with a NF45 membrane and various synthetic fermentation broths containing succinate salt and different impurities such as inorganic salts, glucose, and other organic acid salts including acetate. The influence of the operating conditions (pH, pressure) as well as the broth composition on the NF performances was evaluated. The mechanisms governing the transfer of the solutes through the membrane were studied in order to explain the different solute retentions observed according to the fermentation broth composition. In single-solute solutions, the succinate retention increases with increasing pressure and feed pH and decreases with increasing feed concentration. For instance, at a low salts concentration at 0.1 M, it was observed that the retentions of succinate and acetate in the mixture are identical to those in single solutions. Thus, a good purification of succinate can be obtained. On the contrary, with higher succinate concentrations, the retention was decreased due to the screening effect. Retentions of those solutes were then too close to achieve a separation. Based on abovementioned mechanisms observed, a methodology was proposed to perform the succinate purification from fermentation broth. The succinate/acetate separation was carried out in two steps. A diafiltration of the diluted fermentation broth was initially performed, and the concentration step followed. With this process, it was possible to increase the succinate purity from 85% to more than 99.5% while maintaining a total yield higher than 92%. From this work, it was shown that NF could be effectively used for the succinate purification from fermentation broth.

School of Biotechnology

Academic Year 2016

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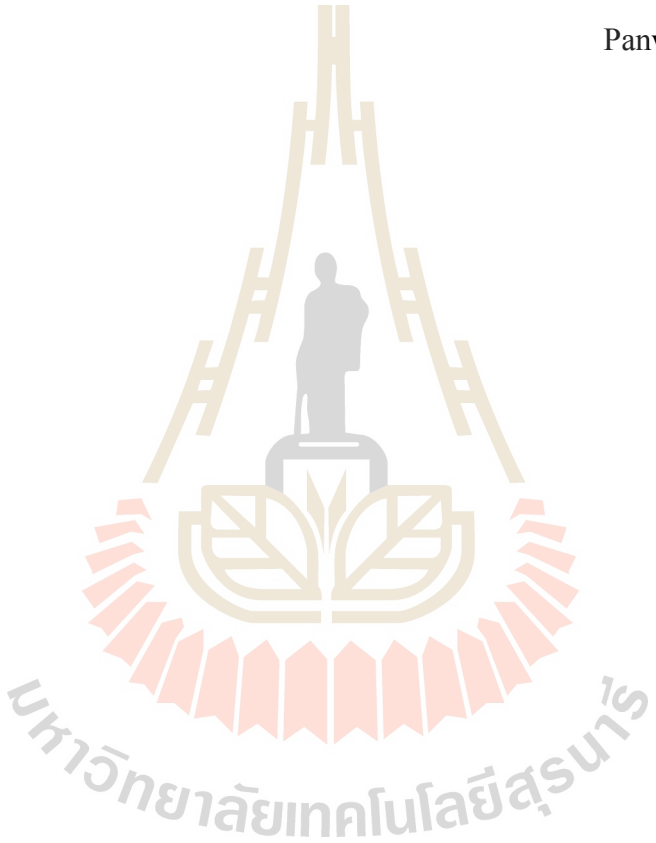
Last but not least, I wish to express my infinite thanks and appreciation to my lovely parents, my brother, my closed relatives, my friends as well as my closed friend for their loves and supports, without them I would not be able to be anything. To them I say “we meet to part, but more importantly we part to meet again”.

Panwana Khunnonkwao



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LIST OF ABBREVIATIONS

ATP	=	Adenosine triphosphate
ADP	=	Adenosine diphosphate
°C	=	Degree Celsius
cAMP	=	Cyclic adenosine monophosphate
CRP	=	Catabolic repression protein
CDW	=	Cell dry weight
CO_2	=	Carbon dioxide
C_{Ace}	=	Concentration of acetate (M)
C_{Suc}	=	Concentration of succinate (M)
DNA	=	Deoxyribonucleic acid
E. coli	=	*Escherichia coli*
EDTA	=	Ethylenediaminetetraacetic acid
g/g	=	Gram per gram
g/L	=	Gram per liter
GalP	=	Galactose permease
GatC	=	Galactitol permease
h	=	Hour
HPLC	=	High performance liquid chromatography
HAce	=	Acetic acid
HPyr	=	Pyruvic acid

LIST OF ABBREVIATIONS (continued)

H_2Suc	=	Succinic acid
LB	=	Luria-Bertani medium
M	=	Molar
mM	=	Milli-molar
ml	=	Millilitre
NADH	=	Nicotinamide adenine dinucleotide (reduced form)
NAD^+	=	Nicotinamide adenine dinucleotide (oxidative form)
OD	=	Optical cell density
OAA	=	Oxaloacetic acid
PCR	=	Polymerase Chain Reaction
PCK	=	Phosphoenolpyruvate carboxykinase
PDH	=	Pyruvate dehydrogenase
PEP	=	Phosphoenolpyruvate
PFLB	=	Pyruvate formate lyase
POXB	=	Pyruvate oxidase
PTA	=	Phosphate acetyltransferase
PPC	=	Phosphoenolpyruvate carboxylase
PPP	=	Pentose phosphate pathway
PYK	=	Pyruvate kinase
rpm	=	Revolution per minutes
TAE	=	Tris-acetate-EDTA
TBE	=	Tris-borate-EDTA

LIST OF ABBREVIATIONS (continued)

UV	=	Ultraviolet
V	=	Volt
v/v	=	Volume per volume
w/v	=	Weight per volume
L_{po}	=	Water permeability ($L.m^{-2}.h^{-1}.bar^{-1}$)
J_w	=	Water permeate flux ($m^3.s^{-1}.m^{-2}$)
ΔP	=	Pressure difference (Bar)
R	=	Retention
R_{obs}	=	Observed retention
$R_{obs,succinate}$	=	Observed retention of succinate
$R_{obs,acetate}$	=	Observed retention of acetate
C_p	=	Concentration of solute in permeate (M)
C_f	=	Concentration of solute in feed (M)
C_r	=	Concentration of solute in retentate (M)
A	=	Area (m^2)
V	=	Volume (m^3)
V_0	=	Volume of feed (L)
V_P	=	Volume of permeate (L)
V_R	=	Volume of retentate (L)
KAce	=	Potassium acetate
KPyr	=	Potassium pyruvate
K_2Suc	=	Potassium succinate
Na_2Suc	=	Sodium succinate

CHAPTER I

INTRODUCTION

1.1 Background and signification of research problem

Succinic acid has been identified by the U.S. Department of Energy as one of the top 12 building block chemicals that could be produced from renewable feedstocks (Werpy and Petersen, 2004). Current succinic acid production by the hydrogenation of petroleum-derived maleic anhydride is too expensive for widespread use as a platform chemical. Inexpensive microbial processes could provide succinic acid as a renewable building block molecule for conversion into chemical intermediates, specialty chemicals, food ingredients, green solvents, pharmaceutical products, and biodegradable plastics (Zeikus *et al*., 1999). Potentially high volume products that can be made from succinic acid include tetrahydrofuran, 1,4-butanediol, succindiamide, succinonitrile, dimethylsuccinate, N-methyl-pyrrolidone, 2-pyrrolidone, 1,4-diaminobutane, and γ-butyrolactone (Sauer *et al*., 2008). The microbial production of succinic acid from carbohydrates offers the opportunity to be both greener and more cost effective than petroleum-based alternative products. For an economical production, lignocellulosic biomass is a promising feedstock for succinate production considering its great availability, sustainability, and low cost compared with refined sugars (Li *et al*., 2011; Zheng *et al*., 2009; Zheng *et al*., 2010).

Xylose is the second most abundant sugar in nature and a major constituent of hemicellulose in lignocellulosic biomass. In *E. coli*, xylose transport mechanisms

require ATP as an energy source for uptake and phosphorylation of xylose. Previous work has shown that ATP was insufficient to accomplish the conversion of xylose to succinate, so that there was an ATP deficit for succinate production (Liu *et al.*, 2012). Hasona *et al*. (2004) found that an *E. coli* mutant lacking pyruvate formate lyase (PFL) was unable to grow anaerobically with xylose as the sole carbon source. Due to the *pflB* deletion strain can not convert pyruvate to acetyl co-A, the required precursor for acetate production, and could not produce the additional ATP (Gonzale *et al*., 2000). In other words, *E. coli* AFP184 was a metabolically engineered strain for succinate production by deleting the *pflB*, *ldhA*, and *ptsG* genes. AFP184 can ferment xylose with a high growth rate (Anderson *et al*., 2007). Liu *et al.* (2012) also showed that the engineered *E. coli* BA204, a *pflB*, *ldhA*, and *ppc* deletion strain overexpressing the ATP-forming phosphoenolpyruvate carboxykinase (PEPCK) from *Bacillus subtilis* 168, can produce succinate from xylose with enhancement of the ATP supply. However, both strains produce low succinate yield and productivity.

Jantama *et al.* (2008) previously reported that a metabolically engineered *E. coli* KJ122 produced impressive titers and yields of succinate from glucose under simple anaerobic conditions. However, Wang *et al.* (2013) reported this strain performs poorly with xylose due to an insufficient ATP supply. Therefore, the improving xylose consumption in this strain is desirable for succinate production.

Succinate production by fermentation has distinct advantages. However, the fermentative broth does not contain only succinate but also other substances and by-products such as sugar, inorganic salts or other organic acid salts like acetate. Thus product recovery is an important step and needs more attention because it needs high costs to achieve the high quality requirement for succinate. In order to reduce the cost,

numerous studies on succinate separation have been conducted using different techniques including reactive distillation, simultaneous-moving bed adsorption, membrane technology, ion exchange, electrodialysis (ED) and direct distillation. For the solvent extraction, it is limited by undesirable distribution coefficients and by using hazardous solvents. Absorption process also has the disadvantages due to a short lifetime of adsorbents, low capacity, and additional filtration. ED is used to recover succinate from non-ionized compounds with proper ion exchange membrane. Direct distillation is an energy-intensive process, and it causes product formation. Membrane technologies such as Nanofiltration (NF) have been proving their advances in the fields of separation and purification. NF is a recent membrane process that is expected to be more competitive and overall more sustainable when compared with the chemical separation processes (Li and Shahbazi, 2006; Abels *et al*., 2013). However, NF is a recent membrane process, the mass transfer mechanisms are still to be understood especially when complex solutions, like fermentation broths, are concerned. For example, results obtained with single-solute solutions can not be directly applied to predict those in mixed-solute solutions (Bargeman *et al.,* 2005). Indeed, the fermentation broth is a mixture containing a lot of components such as neutral and charged solutes. Previous work was investigated that NF could be applied for separation of lactate from fermentation broth (Umphuch *et al*., 2010; Bouchoux *et al*., 2005). Experimental investigation is thus required, at the laboratory scale first to screen for operating conditions and membrane choice and then at the pilot scale to evaluate the performances of the NF purification at larger scale.

Since the demand of succinate in many applications is high and increasing every year, it is important to note that production of succinate by fermentation should be

developed especially as how it relates to the utilization of agricultural renewable to produce higher-value fermentation derived succinate. Also, the product recovery should be further investigated for more cost effectiveness of succinate purification thus achieving low cost-high purity succinic acid supplying to chemical industry. Therefore, this research was focused on the succinate production from xylose and its separation by using NF process.

1.2 Research objectives

Succinic acid is an industrially important product with a large and rapidly expanding market due to its attractive and valuable multi-function properties. The economics of succinic acid production by fermentation is dependent on many factors, of which the cost of the raw materials is very significant. Lignocellulosic biomass is a promising feedstock for succinic acid production considering its great availability, sustainability, and low cost compared with refined sugars. Despite these advantages, the commercial use of xylose for succinic acid production is still problematic. Therefore, this study investigated the improving xylose fermentation in *E. coli* KJ122 by metabolic engineering coupled with metabolic evolution. However, the fermentation broth does not only contain succinate but also other impurities. Therefore, this work was to investigate the separation and purification of succinate from fermentation broth by NF. The better understanding in mass transfer mechanisms in NF for succinate purification was expected. In addition, the influence of operating conditions (pH, pressure) as well as of the fermentation broth composition were investigated. The innovative technology should be further developed and transferred for scaling up the operation unit close to an industrial scale. The activity implemented in this work was summarized in Fig. 1.1.

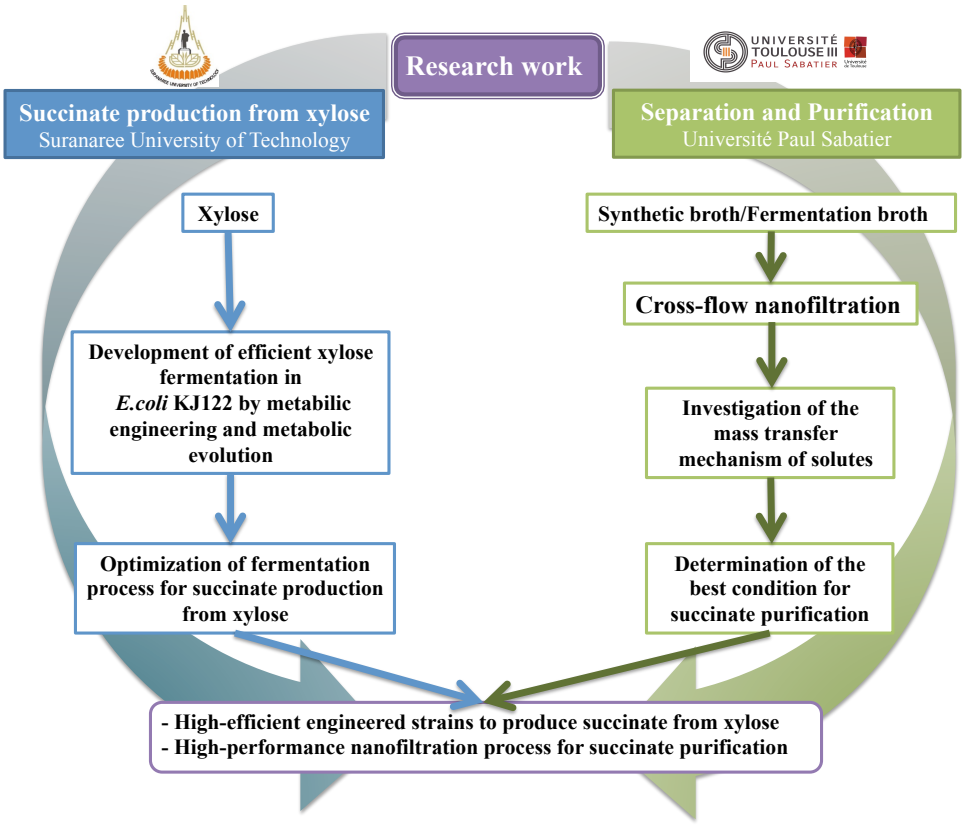

Figure 1.1 Flow schematic diagram for an overview activity implemented in this research.

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CHAPTER II

LITERATURE REVIEW

2.1 Succinic acid and its applications

Succinic acid is a dicarboxylic acid having the molecular formula of $C_4H_6O_4$ and otherwise known as butanedioic acid. It is a common metabolite formed by plants, animals and microorganisms. It is an intermediate compound in the tricarboxylic acid cycle (TCA). It is also one of the fermentation products of energy metabolism (Zeikus *et al*., 1999). It has been synthesized from petrochemical based maleic acid, but its fermentation production is drawing much attention in response to the current need to develop sustainable process using renewable resources (Willke and Vorlop, 2004). This is an important point, as succinic acid can be produced from renewable, environmentally sound carbohydrates rather than relying on limited petrochemical hydrocarbons. It is synthesized by carbondioxide fixation based carboxylation of C_3 metabolism. This unique carbondioxide fixation makes fermentative succinic acid production even more attractive. As the importance of succinic acid for use as a biodegradable polymer has increased, the biological production by fermentation has been focused on the alternative to the petrochemical based process (Zeikus *et al*., 1999).

Succinic acid is among the new bio-derived building-block chemicals that could replace the current maleic anhydride C_4 platform. The main interest in succinic acid lies in its derivatives, since it can be transformed into a lot of interesting products:

1,4-butanediol (BDO), γ-butyrolactone (GBL), tetrahydrofuran (THF), *N*-methyl-2-pyrrolidone (NMP), 2-pyrrolidone (2-Pyrr), succinimide, succinic esters, maleic acid (M.A.)/maleic anhydride (M.Anh.) and several others (Fig. 2.1).

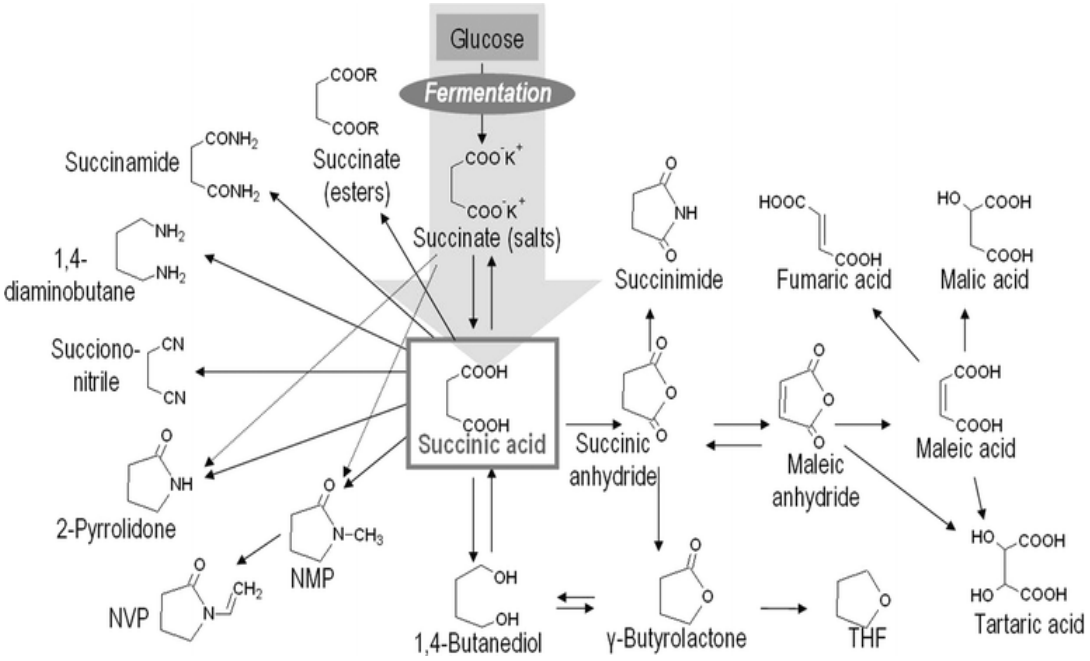

Figure 2.1 Succinic acid and its derivatives (Kamm and Kamm, 2007).

2.2 Succinate production

Currently, the large use of succinic acid and its derivatives is around 20,000-30,000 tones per years with the potential price of $400,000,000 per year (Kidwell *et al*., 2008; Zeikus *et al*., 1999). This rate increases by 10% per year and the market size is estimated to be more than 270,000 tons per year (Willke and Vorlop, 2004). A commercialized succinic acid is mainly produced by chemical process from butane or oxidation of benzene through maleic anhydride. The price of succinic acid is reported to be in the range of $5.9-8.8 /kg depending on its purity. Confronted with the rising price of petroleum and pollution, there are many researchers tending to make succinic acid from renewable resources using microorganism and green biotechnology. The

large scale of fermentative succinate was produced in early 1980 (Zeikus, 1980). Fermentative succinate production is about 5,000 ton per year and is sold at $2.20/kg to the food market. As expected, natural succinate price would be decreased by $0.55/kg if production size would be above 75,000 tones due to utilizing cheap carbon substrates such as corn, starch, molasses, and sugars (Zeikus *et al*., 1999; Kidwell *et al*., 2008). Bio-based succinate needs consumption of CO_2 during fermentation so this process would contribute to reduce green house gas. Moreover, it declines pollution from the manufacturer by constituting many commodities based on benzene and intermediate petrochemical of over 250 benzene-derived chemicals (Ahmed and Morris *et al*., 1994). In this decade, fermentative succinate has much been developed in commercial scale using engineering *E. coli* strains as a biocatalyst. In 2011-2012, hundred tones of succinic acid is expected to be produced using *E. coli* as a catalyst and glucose as a feedstock in an administration plant which was developed by DSM and France's Roquette Frères. Meanwhile, Myriant from the USA tested the scale-up production of succinic acid in the 20,000 L bioreactor, also used *E. coli* as a biocatalyst and renewable resources as a feedstock (http://www.myriant.com/product/ Replacement-products.cfm). In Japan, Mitsubishi has also attempted to industrialize fermentation production of succinic acid, which will be used as monomer units of the company’s biodegradable plastics (Xu and Guo, 2010). For industrial scale of succinate production, the costs of substrates and downstream processing are crucial constraints to become economically viable for succinate production. Purification has cost around 60%-70% of total bio-production process (Sauer *et al*., 2008).

2.3 Succinate producers

Succinic acid is an intermediate in the tricaboxylic acid cycle and one of the fermentation end products of anaerobic of metabolism by several anaerobic and facultative microorganisms (Song and Lee, 2006). Even though succinate can be produced by fungi and bacteria, only bacteria are used in fermentation for food and beverages. Various bacteria have been reported to produce succinic acid including typical gastrointestinal bacteria, rumen bacteria, some lactobacillus strains and *E. coli* strain (Kaneuchi *et al*., 1988; Agarwal *et al*., 2006). There are many bacteria which have been found to produce high succinate as a major product in fermentation (Guettler *et al*., 1998). Efficient strains such as *Actinobacillus succinogenes* (Guettle *et al*., 1996; Guettler *et al*., 1998). *Anaerobiospirillum succiniciproducens* (Glassner and Datta *et al.*, 1992; Urbance *et al*., 2003), and *Mannheimia succiniciproducens* (Lee *et al*., 2003; Lee *et al*., 2006) naturally produce as high productivity as 4 g/L.h with impressive titers at 300-900 mM and high yields more than 1.1 mol succinate/mol glucose.

2.4 Succinate producing pathways in microorganisms

Succinic acid producing bacteria produce not only succinate but also by-products such as lactic acid, acetic acid, formic acid and ethanol during fermentation. The rumen bacterium, *A. succiniproducen* produced a succinate yield up to 1.2 mol/mol glucose, accompanied with by-products of acetate, pyruvate, formate and ethanol (Guettler *et al*., 1996). Unlike rumen bacteria, *E. coli* produces succinate as a minor product as low as 0.12 mol/mol glucose (Zeikus *et al*., 1999). In theory, proximally 1.71 mol succinate is produced per mol glucose consumed plus CO_2 (Eq. 2.1). Based on redox balance, 24 electrons in glucose are converted to 14 electrons in succinate (Mckinlay *et al*., 2007).

$$7\ C_6H_{12}O_6 + 6\ CO_2 \rightarrow 12\ C_4H_6O_4 + 6\ H_2O \quad (2.1)$$

Succinate from glucose fermentation is primarily determined by carbon partitioning at the phosphoenolpyruvate (PEP) node (Fig. 2.2). In rumen bacteria, more than half of the phosphoenolpyruvate formed from glucose is carboxylated to oxaloacetate and converted to succinate, the primary fermentation product. However, requirements for complex nutrients by these bacteria increase both the cost and process complexity. Native strains of *E. coli* ferment glucose effectively in simple mineral salts medium but produce succinate only as a minor product (Neidhardf, 1996). In *E. coli*, half of the PEP from glucose is metabolized directly to pyruvate by the PEP-dependent phosphotransferase system for glucose uptake. Most of the remaining PEP is used for ATP production by pyruvate kinases for ATP. To preserve redox balance, the resulting pyruvate is converted to formate, acetate, lactate, ethanol, and small amounts of succinate.

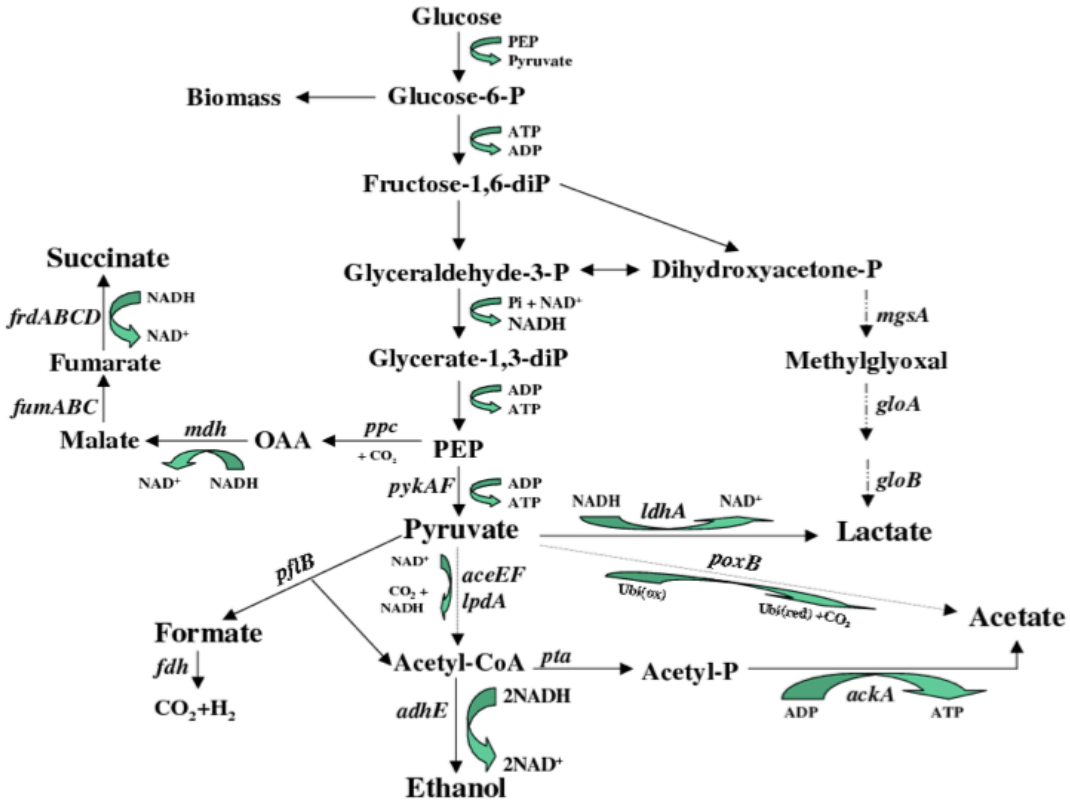

Figure 2.2 Central metabolic pathway of *E. coli*. Solid arrows represent central fermentative pathways. Dotted arrow represents microanaerobic pathway (*poxB*) and dash arrow represents minor lactate producing pathway (*mgsA, gloAB*).

Genes: pyruvate kinase (*pykAF*), lactate dehydrogenase (*ldhA*), pyruvate formate-lyase (*pflB*), phosphate acetyltransferase (*pta*), acetate kinase (*ackA*), alcohol dehydrogenase (*adhE*), PEP carboxylase (*ppc*), acetyltransferase/dihydrolipoamide acetyltransferase component of the pyruvate dehydrogenase complex (*aceEF/lpdA*), malate dehydrogenase (*mdh*), fumarase (*fumABC*), fumarate reductase (*frdABCD*), formate dehydrogenase (*fdh*), methyglyoxal synthase (*mgsA*), glyoxylase (*gloAB*), and pyruvate oxidase (*poxB*) (Jantama *et al*., 2008a).

2.5 Development of succinic acid producing strains from *E. coli*

E. coli has been considered as the best studied bacterium because the microorganism has an ability to grow fast without a requirement of complex nutrients and it is easy to manipulate its metabolic pathways by genetic engineering. However, the feasibility of increasing succinate production yield in this microorganism through metabolic engineering has not yet been fully developed.

Under anaerobic conditions, glucose was fermented to PEP and the carboxylation of PEP to oxaloacetate by *ppc* is the primary fermentative route for succinate. Meanwhile NADH is generated in glycolysis pathway for cell growth and being as intermediate (co-enzyme) in succinic acid production. Unfortunately, no more than 0.2 mol of succinate is produced per mol of glucose consumed by *E. coli* during fermentation (Lee *et al.*, 2004).

Many investigators have described genetic engineering approaches to improve succinate production in *E. coli* by adding foreign genes (Vemuri *et al*., 2002; Kim *et al*., 2004; Sanchez *et al*., 2005; Wang *et al*., 2006). The key to these improvements is increasing the carboxylation of PEP and pyruvate to a four-carbon dicarboxylic acid precursor of succinate. *E. coli* has four native carboxylation pathways that could potentially serve this function (Fig. 2.3). Overexpression of pyruvate carboxylase in strain AFP11 extremely increased succinate titer (99.2 g/L) and increased succinate titer (99.2 g/L) and productivity (1.3 g/L/h). Pyruvate could be reconverted to PEP by ATP with the formation of PPi and AMP, but the energy is wasted by this process (Zhang *et al*., 2009). Since *E. coli* excretes succinate in small amount, gene knockouts in succinate competition pathway were done to improve its yield. For example, AFP111, a mutant in *ptsG* system (glucose phosphotransferase), pyruvate-formate

lyase, and lactate dehydrogenase, could produce succinate at concentration of 36 g/L (Chartterjee *et al*., 2001).

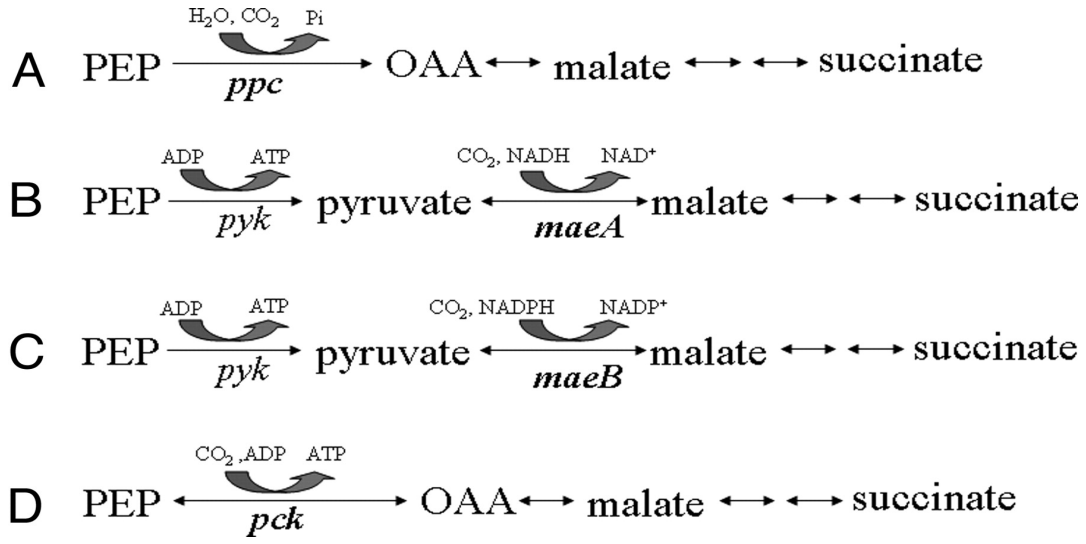

Figure 2.3 Carboxylation pathways potentially available for succinate production in *E. coli*. A. PEP carboxylase (primary fermentation route). B. NADH linked malic enzyme (gluconeogenic). C. NADPH-linked malic enzyme (gluconeogenic). D. PEP carboxykinase (gluconeogenic). Genes encoding carboxylation activities are shown in bold (Zheng *et al.*, 2009).

Furthermore, gene inhibition was intensively studied in central pathway of *E. coli*. Sanchez *et al.*, 2005 reported that SBS550MG was efficient in succinate production at 1.6 mol/mol glucose consumed with productivity of 10 mM/h. This strain was deactivated the fermentation by-products genes such as *ldhA* encoding lactae dehydrogenase, *adhE* encoding alcohol dehydrogenase, *ack-pta* encoding acetate kinase and phosphate acetyl transferase and the targeting glyoxylate shunt flux by deleting the transcriptional repressor of the glyoxylate shunt (*iclR*) and over expressing *Bacillus subtilis* citrate synthase and *B. retli* pyc from a multi-copy plasmids. The overexpressions of some key enzymes for succinate development were

observed. Overexpressed fumurate reductase (*frdABCD*) activated fumarate to succinate in recombinant *E. coli* with the conversion rate at 93% (Goldberge *et al*., 1983; Wang *et al*., 1998). Carbonic anhydrase from *Anabaena* sp.7120 was overexpressed in *E. coli* to provide bicarbonate for improved succinate production (Wang *et al.*, 2009).

For an alternative carboxylation pathway, malate dehydrogenase (*mdh*) was expressed in JCL1208 strain to dissipate accumulated pyruvate to succinate resulting in 108 mM succinate with a molar yield of 0.98 mol succinate per mol glucose consumed (Millard *et al*., 1996). In 2009, Wang observed that the specific glucose consumption rate and specific productivity were increased with overexpressing *mdh* in the *ldhA* double mutant NZN111 resulting in 1.14 mol succinate produced per mol glucose consumed. Also, *E. coli* was genetically engineered to produce succinic acid under aerobic condition. In 2005, Lin *et al.* constructed strain HL27659K that was eliminated in succinate dehydrogenase (*sdhAB*), phosphate acetyltransferase-acetate kinase (*pta-ackA*), pyruvate oxidase (*poxB*), glucose transporter (*ptsG*), *iclR* and overexpressed phosphorenolpyruvate carboxylase (*ppc*) to promote the glyoxylate shunt and to make succinate as a main product. This strain produced less than 100 mM succinate with yield (0.91 mol succinate/ mol glucose consumed) and required oxygen in fermentation (Lin *et al*., 2005). Considering the cost of materials (medium), purification, and waste disposal, novel strain of *E. coli* C was genetically engineered and developed by growth-based selection (metabolic evolution) for increased succinate production in a mineral salts medium supplemented to 1 mM betaine as an osmoprotectant (Jantama *et al*., 2008b). KJ122 was reported to be efficient in metabolizing glucose to succinate in high titer, yield, and productivity but excreting

less by-products. Hence, this strain can be comparable to the best succinate producing rumen bacteria.

2.6 *E. coli* KJ122

E. coli KJs' strains were developed to be not only efficient strains, but they also acted as an ideal biocatalyst for industrial succinate production based on fast growth, no requirement of expensive nutrients, but less mixed organic acids under simple batch conditions. There are some dominant KJs' strains which are noticeable in high succinate production. Strain KJ073, derived from wide type *E. coli* ATCC 8739, was constructed by combination of metabolic engineering and metabolic evolution. Strain KJ073 was knockout in genes encoding alternative NADH oxidizing pathway such as Δ*ldhA::FRT* Δ*adhE::FRT* Δ*ackA::FRT* Δ*(focA-pflB)::FRT* Δ*mgsA and* Δ*poxB.* The strain produced succinate with molar yield of 1.2 per mole of glucose consumed; however, after genes deletion, there are all FRT fragments remain (Jantama *et al.*, 2008a). Strain KJ091 was genetically improved by removing all FRT fragments in which this strain was similar to KJ073 for producing succinate (Jantama *et al.*, 2008b). Further development, KJ122 was constructed and able to be a high succinate producer (Fig. 2.4). Strain KJ122 (Δ*ldhA* Δ*adhE* Δ*ackA* Δ*(focA- pflB)* Δ*mgsA* Δ*poxB* Δ*tdcDE* Δ*citF* Δ*aspC* Δ*sfcA*) produced succinate yield (1.46 mol/mol glucose), succinate titer (80 g/L) and average volumetric productivity (0.9 g/L.h) at 96 h.

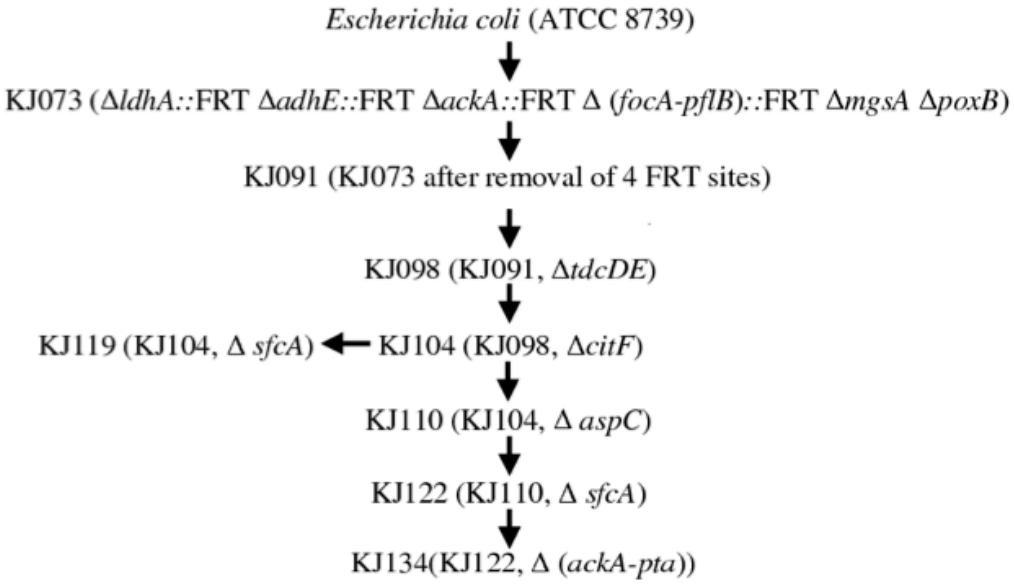

Figure 2.4 Strain construction of KJ122 (Jantama *et al*., 2008a, b).

Further, Zhang *et al.* (2009) found that increased succinate production in KJs' strains were due to increasing expression of PEP carboxykinase (*pck*) and inactivation of the glucose phosphoenolpyruvate-dependent phosphotransferase sytem (PTS) after metabolic evolution. In addition, the glucose uptakes system was replaced by an alternative pathway, galactose permease (*GalP*) in KJ strains. *GalP* was noted to use ATP rather than PEP for phosphorylation. This provided an energy mechanism to increase the pool size of PEP and to facilitate redox balance. Furthermore, improved succinate yield made the succinate producing pathway in *E. coli* that was closed to succinate producing rumen bacteria (Zhang *et al.*, 2010).

2.7 Succinate production from lignocellulosic biomass

2.7.1 Composition of lignocellulosic biomass

The global production of plant biomass, of which over 90% is lignocellulose, amounts to ~200 × 10^9 tons per year, where ~8-20 × 10^9 tons of the primary biomass remains potentially accessible. Lignocellulosic biomass is organic material derived from a biological origin, and represents the most abundant global source of biomass that has been largely unutilized (Lin and Tanaka, 2006). It is mainly composed of cellulose (insoluble fibers of β-1,4-glucan), hemicellulose (noncellulosic polysaccharides including xylans, mannans, and glucans), and lignin (a complex polyphenolic structure), which form ~90% of the dry matter, plus lesser amounts of minerals, oils, and other components (Fig. 2.5) (Balat, 2011; Molina-Sabio and Rodríguez-Reinoso, 2004; Yang *et al*., 2009). This biomass includes forest and crop residues (Chen and Lee, 1997; Melzoch *et al*., 1997), municipal solid waste (John *et al*., 2007), waste paper (McCaskey *et al*., 1994), and wood (Linko *et al*., 1984). The structural and chemical composition of lignocellulosic material has varying amounts of these components because of genetic and environmental influences and their interactions (Demirbas, 2005). The proportion of biomass constituents varies between species, and there are distinct differences between hardwoods and softwoods. The total content of cellulose and hemicellulose is higher in hardwoods (78.8%) than in softwoods (70.3%), but the total content of lignin is higher in softwoods (29.2%) than in hardwoods (21.7%) (Balat, 2009). The cellulose, hemicellulose, and lignin content depends on the type of lignocellulosic biomass, which indicates that an appropriate material should be selected for the corresponding fermentation.

Cellulose, the major component of plant biomass (30-60% of total feedstock dry matter), is a homopolysaccharide composed of *β*-D-glucopyranose units, linked by *β*-(1,4)-glycosidic bonds. The orientation of the linkages and additional hydrogen bonding make the polymer rigid and difficult to break. Hemicellulose (20-40% of total feedstock dry matter) is a short, highly branched heterogeneous polymer consisting of pentose (xylose and arabinose), hexose (galactose, glucose, and mannose), and acid sugars (Saha, 2000). Mannose is the dominant hemicellulose sugar in soft-woods, while xylose is dominant in hardwoods and agricultural residues (Taherzadeh and Karimi, 2008). Hemicellulose is more readily hydrolyzed compared to cellulose because of its branched and amorphous nature. Lignin (15-25% of total feedstock dry matter) is an aromatic polymer synthesized from phenylpropanoid precursors. The phenylpropane units of lignin (primarily syringyl, guaiacyl, and phydroxy phenol) are bonded together by a set of linkages to form a very complex matrix (Demirbas, 2008). This complex matrix consists of a variety of functional groups, e.g., hydroxyl, methoxyl, and carbonyl groups, which impart a high polarity to the lignin macromolecule (Feldman *et al*., 1991). Lignin is considered to be difficult to use as a fermentation substrate because it makes the biomass resistant to chemical and biological degradation (Taherzadeh and Karimi, 2008).

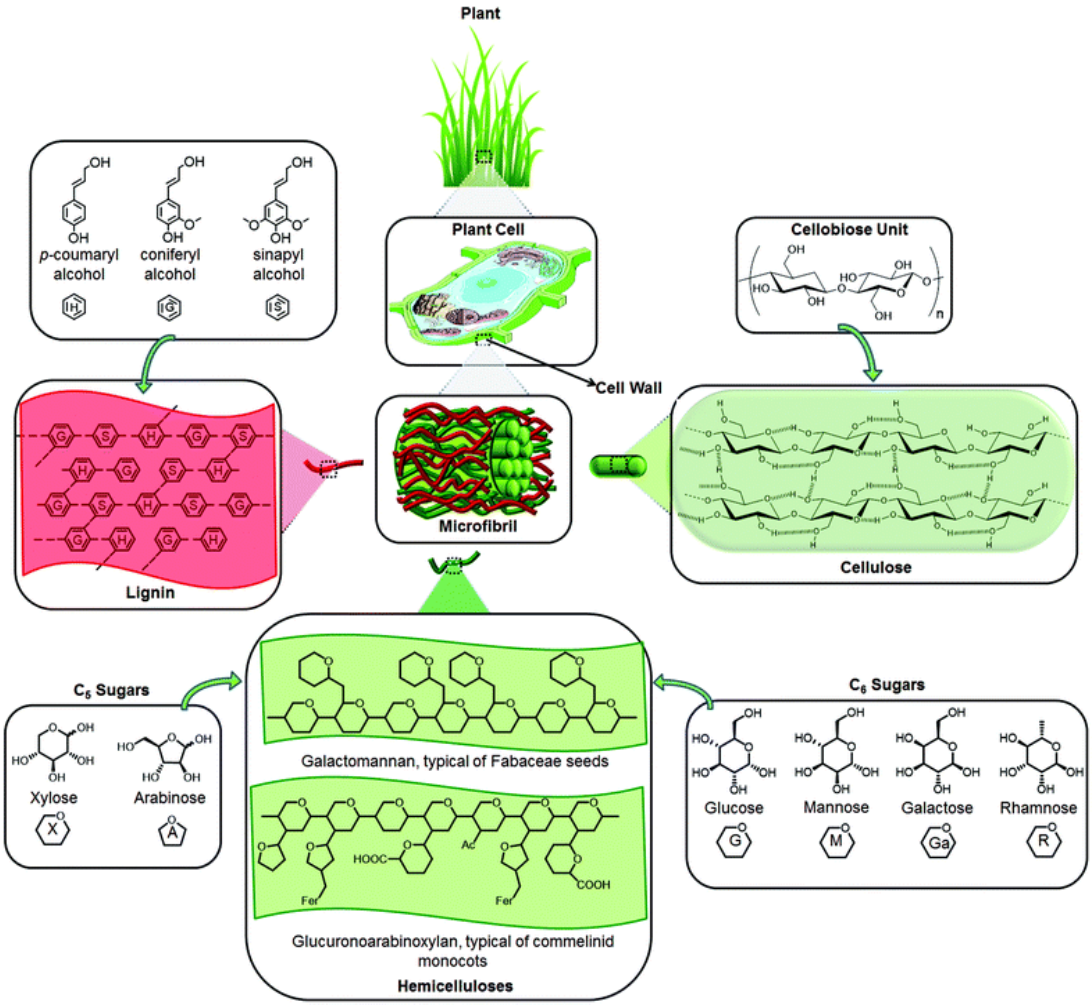

Figure 2.5 The main components and structure of lignocellulose (Furkan and Remzi, 2015).

2.7.2 Conventional processes for succinate production by engineered *E. coli* strain from lignocellulosic biomass

Despite the advantages in its sustainability and availability, the commercial use of lignocellulose in succinate production is still problematic due to its complexity. The biochemical conversion of lignocellulosic biomass requires several processing steps designed to convert structural carbohydrates to monomeric sugars, i.e., glucose, xylose, arabinose, and mannose. Further, these sugars can be fermented to succinate by engineered *E. coli* strains, with varying degrees of effectiveness. Once

the technologies are established and commercialized, a wide range of valuable products could be produced from lignocellulosic biomass. The conventional processes for succinate production from lignocellulosic biomass include the following 5 main steps (Fig. 2.6):

(1) Pretreatment-breaking down the structure of the lignocellulosic matrix.

(2) Enzymatic hydrolysis-depolymerizing lignocellulose to fermentative sugars, such as glucose and xylose, by means of hydrolytic enzymes.

(3) Improvement of co-consumption of xylose and glucose by metabolic engineering and metabolic evolution in engineered *E. coli* for succinate production.

(4) Fermentation-metabolizing the sugars to succinate, generally by engineered *E. coli.*

(5) Separation and purification of succinate to meet the standards of commercial applications.

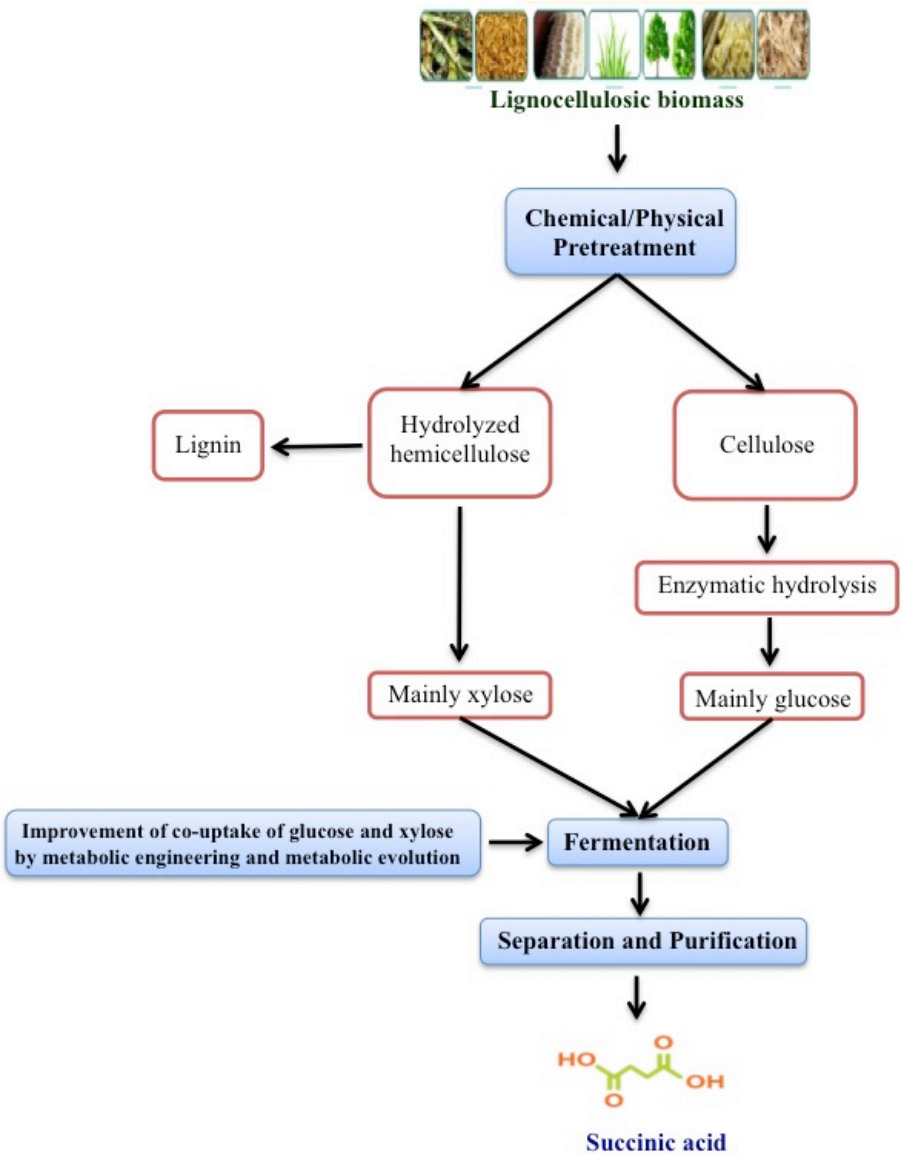

Figure 2.6 A general flow chart of the conventional process for succinate production from lignocellulosic biomass materials (modified from Abdel-Rahman *et al*., 2011).

2.7.3 Xylose uptake in *E. coli*

A xylose transport system for *E. coli* was first reported by David and Wiesmeyer in 1970 (David and Wiesmeyer, 1970). They showed that the xylose permease system is inducible and specific for D-xylose. The system was able to transport xylose against a concentration gradient by expending energy. It took almost one decade before Shamanna and Sanderson (1979) proved that there were two transport systems for xylose in *E. coli*. Based on kinetic studies, they measured apparent Km values of 110 and 24 μM for the low- and high- affinity xylose transport system, respectively (Shamanna and Sanderson, 1979). In 1980, Lam and coworkers discovered the proton linked D-xylose transport system which showed an alkaline pH change while taking up xylose. They tested different mechanisms to find if the transporter was energized by a proton-motive force, directly by ATP or acetyl phosphate, or phosphorylated by phosphoenolpyruvate. In addition to alkaline pH behavior, the energized xylose transport system was found to be inhibited by uncoupling agents and insensitive to arsenate or fluoride. Based on the observations, they concluded transport system is energized by a proton-motive force (Lam *et al.*, 1980). They reported an apparent Km value of 23.9±2.4 μM for the transport system which was consistent with previous reported values (Shamanna and Sanderson, 1979). It was postulated that the lack of other transport activity might be due to repression effects of other sugars used in the experiments, especially glucose and glycerol.

Further, Ahlem *et al.* (1982) purified periplasmic D-xylose-binding protein from *E. coli* K-12. This osmotic shock-releasable binding protein was thought to be part of the high affinity xylose transport system that was reported earlier (Shamanna and Sanderson, 1979). They also found that the binding protein was produced while

growing in media containing both xylose and glycerol. This result was consistent with L-arabinose and D-ribose transport systems which include both low affinity and high affinity mechanisms with a similar binding protein serving as part of high affinity one (Parsons and Hogg, 1974; Willis and Furlong, 1974). In 1984, Davis and coworkers did a series of experiments that confirmed the presence of at least two xylose transport systems in *E. coli*, one proton-linked and the other associated with a binding protein. Finally, Davis and Henderson, 1987 cloned and sequenced the *xylE* gene. The *xylE* contains 1473 base pairs encoding for a 491 amino acid protein which is likely the only protein responsible for xylose-H^+ symport. Like other sugar-proton symport proteins, it is highly hydrophobic and there is a high degree of homology with *AraE* in its structure. While analyzing the *E. coli* genome in 1994, Sofia and coworkers identified three genes downstream of *xylF*. These genes encoded proteins which were similar to the components of ABC-type transport systems and the *araC* regulator. As with *xylF* they proposed the ATP-binding protein gene be called *xylG* and membrane compound of transporter be called *xylH* due to homology to the arabinose transport system. They also named the regulatory protein gene *xylR* (Sofia *et al*., 1994). In 1997, Song and Park characterized the functions and regulation of *xyl* operon. They concluded that the transcription regulation of the *XylA*, *XylB* and *XylFGH* transporter is mediated by *XylR*, and the cylic-AMP receptor protein CRP.

Xylose uptake in *E. coli* occurs through two transport systems (Fig. 2.6). *XylFGH*, the other D-xylose transport system, belongs to the ATP binding cassette ("ABC") family of transporters and requires one ATP per xylose transported. *XylFGH* exhibits a high affinity with an apparent Km between 0.2 and 4 μM (Sumiya *et al*., 1995). In the transporter *xylFGH*, *xylH* is the permease of the ABC transporter (Sofia

et al., 1994), *xylF* is the periplasmic xylose-binding protein, (Ahlem *et al*., 1982) and *xylG* is the ATP-binding protein (Khankal *et al.*, 2008). *XylE* is a D-xylose proton symporter which uses the proton gradient as a source of energy. It possesses a relatively low affinity with high Km values between 63 and 169 μM (Sumiya *et al*., 1995).

After xylose permeates into the cell, xylose isomerase (*XylA*) and xylulokinase (*XylB*), which are encoded by the *xylAB* gene in the xylose-specific operon, convert xylose to xylulose 5-phosphate (Fig. 2.7) (Khankal *et al*., 2008). Then, xylulose 5-phosphate is metabolized by the enzymes of the pentose phosphate pathway (Desai and Rao, 2010) before entry into the glycolysis pathway. In *E. coli*, xylose is primarily transported through transporter *xylFGH*, which is driven by ATP (Hasona *et al*., 2004 and Desai and Rao, 2010). During this process, one molecule of xylose requires one ATP for its transportation and other ATP for phosphorylation, whereas the conversion from xylose to succinate only yields 1.67 net ATP per xylose during anaerobic fermentation (Andersson *et al*., 2007; Liu *et al*., 2012). Additionally, the imbalance of ATP consumption and generation comes from energy requirements for uptake and for phosphorylation (Tao *et al*., 2001; Underwood *et al*., 2002).

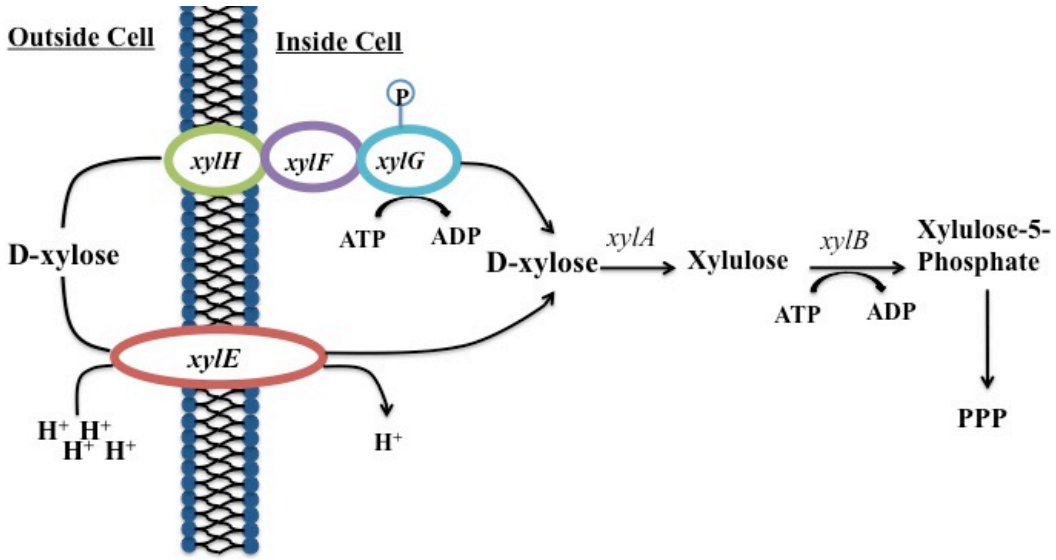

Figure 2.7 Xylose transport systems in *E. coli*. (modified from Utrilla *et al*., 2012).

2.7.4 Previously improved xylose uptake and utilization in *E. coli*

The preferential utilization of the most available nutrition may be an adaptation that allows bacteria to survive in a competitive environment. However, carbon catabolite repression (CCR) among these sugar sources hampers the efficient production of bio-products. Therefore, a reduction in the utilization efficiency of secondary preferred sugars and a long fermentation time at different culture scales (Park *et al.*, 2012). To eliminate CCR in *E. coli*, recently, substantial efforts have been devoted to the deletion of the key genes triggering CCR or to the overexpression of the genes overcoming CCR (Cirino *et al*., 2006; Görke and Stülke, 2008; Hernández-Montalvo, *et al*., 2003). The deletion of the *ptsG* gene has been used to ameliorate sugar hierarchy utilization. When this mutant is cultivated in mixed sugar (glucose/xylose), xylose can be co-utilized with glucose leads to an increase in succinate accumulation (Andersson *et al.*, 2007). However, the glucose uptake rate is lowered in the *ptsG* mutant of *E. coli*. (Nair and Zhao, 2010; Nichols *et al*., 2001; Thakker *et al*., 2013; Yao *et al*., 2011). Additionally, the replacement of the native *crp*

gene with a cAMP-independent mutant without CCR can facilitate xylose uptake and glucose-arabinose-xylose co-utilization (Cirino *et al*., 2006; Jojima *et al*., 2011; Khankal *et al*., 2009; Nair and Zhao, 2010). To co-uptake glucose and xylose, the overexpression of catabolic receptor protein (crp^+) affects the repression of *ptsG* and *ptsH* by the increased Mlc and up-regulates the glycolysis, TCA cycle, and gluconeogenesis genes, allowing the co-consumption of glucose and xylose in *E. coli* (Yao *et al*., 2011). Moreover, replacement of the native fermentation pathway in *E. coli* B with a homo-ethanol pathway from *Zymomonas mobilis* (*pdc* and *adhB* genes) resulted in a 30 to 50% increase in growth rate and glycolytic flux during the anaerobic fermentation of xylose (Tao *et al*., 2001). In addition, Wang *et al.* (2011) reported the cyanoacterial *ppc* overexpression improved the glucose/xylose utilization for succinate production. Further, the overexpression of ATP-forming phosphoenolpyruvate carboxykinase (PEPCK) from *Bacillus subtilis* 168 in the mutant *E. coli*, created to increase xylose uptake and succinate accumulation (Liu *et al*., 2012; Liu *et al*., 2013a,b; Liang *et al*., 2013; Bao *et al*., 2014). The mutation in *gatC* and *galP* also improved xylose fermentation and high organic acids accumulation in engineered *E. coli* (Utrilla *et al*., 2012; Sawisit *et al*., 2015). Moreover, the deletion of ATP-dependent xylose transporter (*xylFGH*) resulted an increase in growth rate and xylose uptake for lactate production (Utrilla *et al*., 2012).

Jantama *et al.* (2008) previously reported that a metabolically engineered *E. coli* KJ122 produced impressive titers and yields of succinate from glucose under simple anaerobic conditions. Unfortunately, this strain grew poorly on xylose due to low ATP supply in *E. coli* KJ122. Therefore, in this work investigated the improving xylose utilization and succinate production by deletion of *xylFGH* genes to enhance ATP supply in *E. coli* KJ122.

2.8 Separation and purification of succinate from the fermentation broth

Purification of succinate from the fermentation broth is the last step for biological succinate production. The separation and purification of succinate are estimated to make up more than 50% of the total costs in its microbial production (Cheng *et al*., 2012). To make fermentation-based succinate production competitive with petrochemical processes, the development of optimized producing strains and fermentation processes must be combined with cost-saving and energy-effective downstream processes to minimize the production costs. The first downstream processing step of succinate purification is clarification by centrifugation or filtration which is followed by ultrafiltration to separate microbial cells, proteins, polysaccharides, and other polymers from the fermentation broth (Fig. 2.8). For the separation and purification of succinate, different strategies such as precipitation, extraction, ion exchange, membrane process such as electrodialysis and nanofiltration. and crystallization have been investigated.

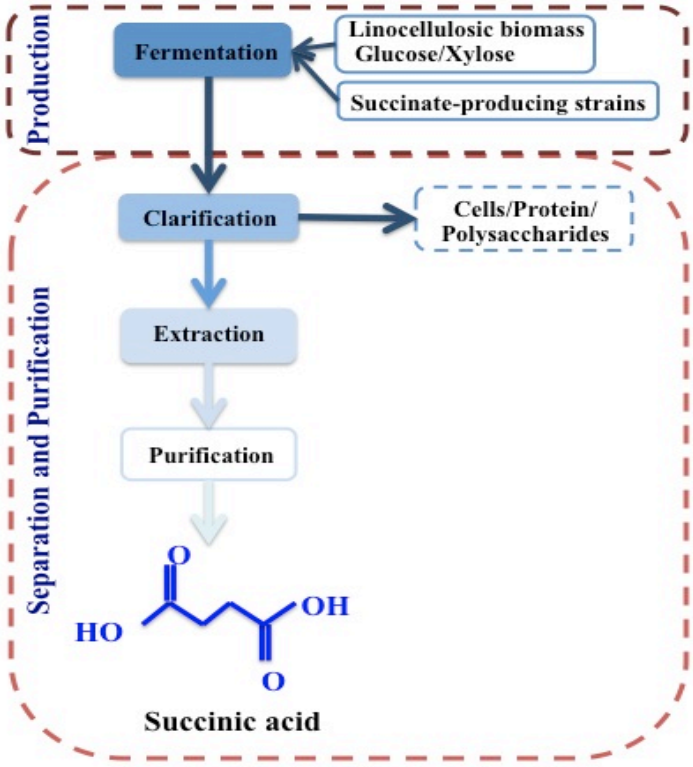

Figure 2.8 The operation units for succinic acid production.

2.8.1 Precipitation

Precipitating succinic acid is a separation process that was first proposed by Datta *et al*. (1992). In this process, after the fermentation reaches completion, solids are centrifuged and separated out of the fermentation broth. This broth is then treated with calcium hydroxide, which creates calcium succinate; a solid that precipitates out of solution (Lee *et al*., 2008). This solid is then removed from the fermentation broth and washed three times with RO water to remove residual sugar and other soluble salts from calcium succinate. A further acidification step, adding sulphuric acid to the solid, dissolves the calcium succinate and produces succinic acid. The calcium in solution reacts with sulphate to produce solid calcium sulphate, also known as gypsum (Datta *et al*., 1992). This solid can be removed from the solution and the succinic acid, dissolved in solution, can to be removed through other

separation methods, such as vacuum distillation. This precipitation method can also take place *in-situ* through the addition of a calcium buffer where it helps maintain the pH of the system (Lee *et al*., 2008).

Fermentation process with *A. succiniciproducens* yields 1.4 mol succinic acid/ mol glucose. Using precipitation with calcium hydroxide the yield of succinic acid is 1.2 mol/ mol glucose, which already means a loss of nearly 15% of the acid (Datta *et al*., 1992). Precipitation appears to be the most common and simplest method for succinic acid separation, but it is also one of the worst processes from an environmental and economic standpoint. During fermentation, the pH of the system is neutralized and buffered by the addition of chemicals such as lime, or calcium hydroxide. Then, when the slurry is treated to remove the succinic acid, large amounts of sulphuric acid are added to the solution, creating succinic acid from calcium succinate and generating calcium sulphate (Corona-Gonzalez *et al*., 2008). Gypsum is unusable from this process as it can not be sold due to discolouration and smell. Therefore, it must be disposed of in a landfill, which adds to the cost of separation. The amount of slurry and solid waste created from this process renders it unfit for commercial applications. Calcium succinate also needs to be washed after it is removed from the fermenter to reduce as much as possible contaminants to be carried into the acidification step. This washing requires a large amount of water for the process as well, another environmental issue that makes precipitation an unappealing choice for succinic acid separation (Brain *et al*., 2004).

In addition, precipitation with ammonia is possible but so far has only been described on a laboratory scale (Berglund *et al.,* 1999; Yedur *et al.,* 2001). In this case diammonium succinate is generated by controlling the pH of the

fermentation broth with an ammonia-based titration agent or by substituting the cation (counter-ion) of the succinate salt with ammonia after fermentation. The diammonium salt of succinic acid in the ultrafiltrated fermentation broth is then treated with sulphate ions or by combining it with ammonium bisulphate and/or sulfuric acid at low pH to yield the succinic acid precipitate and ammonium sulphate. The by-product ammonium sulphate can be cracked thermally into ammonia and ammonium bisulphate. The precipitated succinic acid is separated and purified after dissolution in methanol and re-crystallisation. With this approach, succinic acid can be refined with a yield of 93.3% based on the diammonium succinate available in the fermentation broth (Yedur *et al*., 2001).

The advantages of the integrated precipitation with ammonia are the lower amount of waste by-products and the possibility of recycling base and acid. The main disadvantage is the low selectivity of the precipitation with ammonia. Other organic acids present in the fermentation broth were precipitated together with succinate at the same time (Yedur *et al.,* 2001).

2.8.2 Reactive extraction

Amine-based extraction is a method of reactive extraction that separates organic acids based on their pKa values as it removes undissociated acids (Huh *et al*., 2006; Hong and Hong, 2005). It is a promising method of separation because separation is possible *in-situ* at room temperature and pressure, so no pre-treatment is required (Huh *et al*., 2004). The focus of much literature in amine-based extraction is the use of tri-n-octylamine (ToA) because previous studies have shown that it extracts succinic acid very well (Huh *et al*., 2006). Hong and Hong (2005) used reactive extraction with ToA in 1-octanol for succinic acid recovery, succinate yield of 96%

and purity of 83.3% were obtained (Table 2.1). However, ToA for reactive extraction is toxic to cells. Because of this effect on cell growth and production, other methods of succinic acid extraction need to be investigated. There are additional steps that must take place to continue the process of separating and purifying succinic acid. Bechthold *et al.* (2008) suggested that the combination of three processes (reactive extraction/ vacuum distillation/crystallization) seemed to be the most promising separation method. A yield of 73.1% and a purity of 99.8% of succinic acid crystal were obtained with this process (Table 2.1). Song *et al*. (2007) proposed a similar process in recovery of succinic acid in fermentation broth, and a yield of 67.1% with a purity of 99.5% were obtained (Table 2.1). Umpuch *et al.* (2015) also reported the complex separation process including reactive extraction using ToA, vacuum distillation and crystallization successfully purified succinic acid from the other organic acids in the simulated fermentation broth. The results showed that the yield and purity of succinic acid of 30.25% and 99.10%, respectively were obtained (Table 2.1). However, there are some problems that remain and would need to be addressed before further consideration is given to this method. The main factor is that the extraction process is sensitive to pH changes. Since the extraction rates is affected by the pH due to the dissociation of succinic acid in the aqueous phase. Then, the extraction rates decreases with increasing pH (Jun *et al*., 2007a). Because only undissociated acids can be extracted using ToA, the pH needs to be kept low to ensure that acid is removed from solution (Huh *et al*., 2006). Given that this separation method has no selectivity and removes acetic acid first, it should be considered more like a pre-treatment step rather than a process to remove succinic acid from solution. Further processing of fermentation broth is required after acetic acid removal. Finally,

this amine-based extraction to remove succinic acid from the fermentation broth is relatively new and the possibility of long-term stability needs to be studied to ensure there are no toxic or inhibitory effects on the cells, especially if extractant is used *in situ* on an industrial scale (Bechthold *et al*., 2008). Additional information is also required to determine the cost of this process on an industrial scale.

Table 2.1 Feed compositions, the operating conditions for separation, the yield and purity of succinate obtained after recovery using reactive extraction.

Feed composition	Operating conditions	Results		References
		Yield (%)	Purity (%)	
Synthetic fermentation broth (50 g/L succinic acid and 18.8 g/L acetic acid)	Reactive extraction (0.25 mol TOA/ kg 1-octanol, pH 6.5)	96	83.3	Hong and Hong, 2005
Real fermentation broth (22.3 g/L succinate, 7.0 g/L pyruvate and 1.8 g/L acetate)	Reactive extraction (0.25 mol TOA/ kg 1-octanol, pH 5.0), vacuum distillation and crystallization (pH 2.0 and 4.0°C)	73.1	99.8	Huh *et al*., 2006
Real fermentation broth (13.6 g/L succinate, 4.82 g/L pyruvate and 0.37 g/L acetate)	Reactive extraction (0.25 mol TOA/ kg 1-octanol, pH 5.0), vacuum distillation and crystallization (pH 2.0 and 4.0°C)	67.1	99.5	Song *et al*., 2007
Synthetic fermentation broth (11.67 g/L succinic acid, 2.46 g/L malic acid, 1.51 g/L pyruvic acid and 2.14 g/L acetic acid)	Reactive extraction (0.25 mol TOA/ kg 1-octanol, pH 6.0), vacuum distillation and crystallization (pH 2.0 and 4.0°C)	30.25	99.1	Umpuch *et al*., 2015

2.8.3 Ion exchange

Ion exchange resin, alumina, silica, and zeolite molecular sieve adsorption have been recently reported in some journal publications and a patent (Nam *et al*., 2011; Straathof *et al*., 2010). The key desired properties for an ideal sorbent are high capacity, complete stable regenerability, and specificity for the product.

Brian *et al*. (2004) used the resin XUS 40285 to recover succinic acid. This resin has a stable capacity of about 0.06 g of succinic acid /g of resin at moderate concentrations of 5 g/L succinic acid. It also has a good stable isotherm capacity, prefers succinate over glucose, and has good capacities at both acidic and neutral pH. Using XUS 40285 in a packed column, succinic acid was removed from simulated media containing succinic acid, acetic acid and glucose. The fermentation by-product, acetate, was completely separated from succinate. By a modified extraction procedure combining acid and hot water washes, XUS 40285 showed both good stable capacities for succinic acid over 10 cycles and >95% recovery in a batch operation (Table 2.2).

In addition, Jun *et al*. (2007b) proposed the use of SBA-15 silica as a potential adsorbent in the separation and purification processes of succinic acid. The competitive adsorption of pyruvic acid and succinic acid was studied using a synthetic fermentation broth. They found that pyruvic acid was adsorbed 3-fold better than succinic acid. So this kind of silica seems to be more suitable for the removal of contaminant acids from broth than for the separation of succinic acid.

Furthermore, after removal of cell biomass and protein impurities, cation-exchange resin Amberlite IR 120 H, a cationic resin of sulfonic (SO_3H) type based on a polystyrene-divinylbenzene copolymer, was employed to convert the fermentation

products such as succinate, formate, acetate, and pyruvate from the salt form into the free acid form. Then, succinic acid was selectively separated from the acid mixture by vacuum evaporation at 48°C to eliminate residual volatile carboxylic acids such as acetic, formic, and pyruvic acid. The crystallization of succinic acid was carried out at 4°C for 24 h. The succinic acid yield and purity were 89.5% and 99%, respectively (Lin *et al*., 2010) (Table 2.2). In addition, Li *et al*. (2010a) studied an alkalic anion exchange resin (NERCB 04) for succinic acid recovery from model solutions and real fermentation broth. In a packed column test, its adsorption capacity was 0.41 g succinic acid /g resin when the feed concentration was 50 g/L (Table 2.2). The spent resins were eluted by 0.7 M NaOH and gave 97% average regenerability. Successive column loading and regeneration process was tested for 30 times with stable adsorption capacity and regenerability. Since in the process of adsorption the hydroxyl of the resin is exchanged by the succinate, the effluent of NERCB 04 can be used to adjust the pH during fermentation, which provides a new method to couple online product removal with pH buffer process in succinic acid production (Li *et al*., 2010a). Further, Inci *et al.* (2011) studied to recover succinic acid from aqueous solutions by alumina adsorption. They found that the recovery of succinic acid increases with an increasing amount of alumina. The maximum adsorption capacity (0.02 g of succinic acid /g of resin) was obtained when using 2 g of alumina (Table 2.2). The major disadvantage of this process is the high chemicals consumption. In addition, this process is not environmentally friendly due to waste water from washing, rinsing and regeneration.

Table 2.2 Feed compositions, the operating conditions for separation, the adsorption capacity, the yield and purity of succinate obtained after recovery using ion exchange.

Feed composition	Operating conditions	Results			References
		Adsorption capacity (g succinic acid/g resin)	Yield (%)	Purity (%)	
Synthetic fermentation broth (5 g/L succinic acid, 5 g/L acetic acid and 5 g/L glucose)	Resin XUS 40285	0.06	>95	-	Brian *et al*., 2004
Real fermentation broth (70.6 g/L succinate, 2.3 g/L pyruvate, 0.3 g/L formate and 2.8 g/L acetate)	Ion exchange (resin Amberlite IR 120 H), vacuum distillation and crystallization (pH 2.0 and 4.0°C)	0.58	89.5	99	Lin *et al*., 2010
Single solution (50 g/L succinic acid)	Resin NERCB 04	0.41	97	-	Li *et al*., 2010a
Single solution (0.05 g/L succinic acid)	Resin alumina	0.02	40	-	Inci *et al.,* 2011

2.8.4 Electrodialysis

Electrodialysis (ED) is a method that has potential to separate succinate from fermentation broth after ultrafiltration. ED is a process using ion exchange membranes and electric potential difference to remove impurities e.g. sugar, protein and polysaccharide from charged solutes like succinate and lactate. It can also constitute a concentration step (Glassner and Datta, 1992).

Precipitation produces large amounts of effluents with a high salt content (Bailey and Ollis, 1986). In order to reduce this environmental impact, Bailly *et al.* (2001) investigated the concentration of organic acid salts by conventional electrodialysis (EDC). They reported that EDC can efficiently concentrate lactate from the fermentation while residal sugar (glucose) can be recycled in the feed tank. Glassner and Datta (1992) reported that the succinic acid purity increased from 51.5 to 79.6% and completely removed proteins and salts, but acetate was still remained about 19.9% (Table 2.3). In addition, Glassner *et al*. (1995) develop a succinic acid purification process using combined ED and bipolar membrane electrodialysis (BPED). ED is applied to remove impurities from succinate at a first step. It can also be used for concentration of succinate together with removal of neutral solutes. In the second step, the concentrated succinate is converted to succinic acid by using BPED. After two steps, a total succinic acid yield of 60% is obtained with high succinic acid purity (95%) (Table 2.3). Fu *et al.* (2014) also used bipolar membrane electrodialysis (BPED) was used to convert sodium succinate into succinic acid. They found that the succinic acid yield is ranged between 64 to 97% when the current density increases from 12.5 to 37.5 mA/cm^2 (Table 2.3).

As discussed above, ED has shown its competence in the production of organic acids like succinic acid. However, more improvements are needed for adapting ED to industrialization, and more research needs to be conducted for optimizing operation parameters and process integration.

Table 2.3 Feed compositions, the operating conditions for separation, the yield and purity of succinate obtained after recovery using ED.

Feed composition	Operating conditions	Results		References
		Yield (%)	Purity (%)	
Real fermentation broth (50.3 g/L succinate, 1.3 g/L formate, 13.6 g/L acetate and 1.9 g/L glucose)	ED, crystallization (pH pH 2.0 and 4.0°C)	97	79.6%	Glassner and Datta, 1992
Real fermentation broth (52.9 g/L succinate, 0.7 g/L formate, 9.5 g/L acetate and 2.3 g/L pyruvate)	ED combined with BPED, crystallization (pH pH 2.0 and 4.0°C)	60	95	Glassner *et al*., 1995
Single solution (0.05 M succinate)	BPED (using current density 37.5 mA/cm^2)	97%	-	Fu *et al.*, 2014

2.8.5 Nanofiltration

Nanofiltration (NF) is known to be a separation technique lying between ultrafiltration (UF) and reverse osmosis (RO) which presents a selectivity governed both by size and charge effects. It was reported by many research groups that most NF membranes have a high retention of compounds of molecular weight up to 150-300 g/mol and charged molecules, especially multivalent ions. According to its separation properties, NF begins to be used in organic acids (succinate, lactate and acetate) separation and purification.

Bargeman *et al*. (2005) suggested that the results obtained from single-solute solutions in NF can not be directly used to predict those for mixed-solute solutions. Since the fermentation broth is a mixture containing a lot of components such as neutral and charged solutes, the study with a mixed-solute solution representative of the fermentation broth is needed. Han and Cheryan (1995) investigated to separate acetate from model solution containing acetate (10 g/L) and glucose (10 g/L) by NF membrane. Under acidic condition (pH 2.7), NTR729 membrane has the ability to permeate the acetate while glucose was retained and recycled to the feed tank. The retention of acetate (10%) is lower than that of glucose (99%) because acetate size might be small compared to the membrane pore size (Table 2.4). Further, Kang *et al*. (2004) reported that NF can efficiently remove magnesium ions (together with glucose or lactose) from a raw lactate fermentation broth. Bouchoux *et al*. (2006) also reported that NF could achieve the purification of sodium lactate by removing divalent ion and disaccharide sugar such as lactose. However, the separation between sodium lactate and glucose was found to be hardly achievable in this conditions (Bouchoux *et al*., 2005). It was also pointed out that

glucose retention was significantly lower in mixed-solute solutions, i.e. when sodium lactate was present. In order to improve the separation of glucose and sodium lactate by NF. Umpuch *et al.* (2010) suggested that the addition of Na_2SO_4 to facilitate the permeation of lactate while reducing that of glucose. The mechanism of co-ions competition was used in this approach, SO_4^{2-} ions had more charge and made lactate ions easier to pass through the membrane. Further, Sikder *et al.* (2012) reported that NF3 could retain sucrose (R=94%) while allowing lactate (R=32%) to permeate (Table 2.4).

In addition, NF could be used to change the composition of monovalent anions and divalent anions. Kang and Chang (2005) reported that the retention of divalent anions such as succinate was higher than that of monovalent anions such as formate, acetate and lactate. The negative retention of monovalent anions was also obtained in the presence of divalent anions in some conditions. Furthermore, nanofiltration of a mixture solution in a diafiltration mode was developed for removal of by-products such as formate, acetate and lactate from simulated fermentation broth.

As already reported, NF can separate the organic acids from impurities and residual sugar in fermentation broth. Especially, in the case of mixed-salts solutions with mono and divalent anions, divalent anions were totally retained by NF membrane due to larger size and stronger electrostatic repulsion while monovalent anions permeate though the membrane. Then, high retention of divalent anions was obtained. It was demonstrated that NF membrane can be used for separation and purification of succinate (divalent salts) from impurities such as acetate (monovalent salts).

Table 2.4 Feed compositions, the operating conditions for separation, the retention and the separation factor of organic acids obtained after recovery using NF.

Feed composition	Operating conditions	Results			References
		J_V (x10^{-5} m^3 m^{-2} s^{-1})	Retention (%)	SF_{max}	
Mixture solution (10 g/L acetate and 10 g/L glucose)	Constant feed concentration - NTR729 membrane - pH 2.7 - ΔP=6-18 bar	1.7	99 (glucose) 10 (acetate)	9	Han and Cheryan, 1995
Real fermentation broth (0.95 M lactate, 4.8 mM glucose, and 1.8 mM magnesium ion)	Constant feed concentration - NF45 membrane - pH 5.5 - ΔP= 27.5 bar	0.5	89.1 (glucose) 34.4 (lactate)	6.3	Kang *et al*., 2004
Mixture solution (0.1 lactate, 0.1 M glucose and 0.25 M Na_2SO_4)	Constant feed concentration - 5 DK membrane - pH 7.0 - ΔP=2-20 bar	0.2	65 (glucose) 30 (lactate)	1.9	Umpuch *et al.*, 2010
Real fermentation broth (108.3 g/L lactate and 30 g/L sucrose)	Constant feed concentration - NF3 membrane - pH 5.5 - ΔP=5-13 bar	3.1	94 (sucrose) 32 (lactate)	11	Sikder *et al.*, 2012

Table 2.4 (continued).

Feed composition	Operating conditions	Results			References
		J_V ($x10^{-5}$ m^3 m^{-2} s^{-1})	Retention (%)	SF_{max}	
Mixture solution (0.3 M succinate, 0.1 M lactate, 0.1 M formate and 0.1 M acetate)	Constant feed concentration - NF45 membrane - pH 7.0 - ΔP=3.5-20.5 bar	0.3	87 (succinate) 20 (lactate) -58 (formate) -17 (acetate)	-	Kang and Chang, 2005
	Diafiltration - NF45 membrane - pH 7.0 - ΔP=13.7 bar - 36 h	≈ 0.3	78 (succinate) -18 (lactate) -98 (formate) -55 (acetate)	-	

2.8.6 Crystallization

The principle in this method is that carboxylic acids have varied distribution between dissociated and undissociated forms at varied pH, and the undissociated carboxylic acid has different solubility.

After removal of cell biomass and organic impurities by centrifugation and activated carbon absorption, respectively, a direct vacuum distillation-crystallization was used for succinic acid recovery from broth by Luque *et al*. (2009). The pH of the aqueous broth was adjusted to 4.2 by addition of hydrochloric acid before vacuum distillation. Some volatile by-product carboxylic acids, such as acetic, formic acids, pyruvate acids in broth were removed under vacuum distillation at 60°C. The followed crystallization of succinic acid was carried out at 4°C. When used with a simulated broth, the highest succinic acid yield and purity were 75% and 97%, respectively. However, when this method was applied in actual fermentation broth produced by *A. succinogenes* ATCC55618, the yield and purity of succinic acid crystals were only 28% and 45%, respectively (Luque *et al.*, 2009; Sze *et al*., 2010) (Table 2.5).

Crystallization method was also studied by Li *et al*. (2010b). They found that the solubility of succinic acid was only 3% at 4°C and pH 2.0 while the other acid by-products, such as lactic acid, acetic acid, and formic acid, were still fully water miscible. In their study, crystallization of succinic acid from broth was carried out at 4°C and pH < 2. While acidic by-products remained in the solution, succinic acid could be selectively crystallized. By this one-step recovery method, succinic acid yield and purity were 70% and 90%, respectively (Table 2.5). As one of the oldest but effective processes for the preparation of succinic acid crystals, crystallization process could be used usually as the final purification step. Direct crystallization might provide the

desired product (in solid or crystal form) without the need for many unit operations. However, the product yield is low because much succinate is still residual in the broth and the low-purity product cannot be used as a monomer for polymerization.

Table 2.5 Feed compositions, the operating conditions for separation, the yield and purity of organic acids obtained after recovery using crystallization.

Feed composition	Operating conditions	Results		References
		Yield (%)	Purity (%)	
Synthetic fermentation broth (50 g/L succinate, 5 g/L pyruvate, 5 g/L formate and 5 g/L acetate	Direct vacuum distillation and crystallization (pH 2.0 and 4.0°C)	75	97	Luque *et al*., 2009
Real fermentation broth (70.6 g/L succinate, 2.3 g/L pyruvate, 0.3 g/L formate and 2.8 g/L acetate)	Direct vacuum distillation and crystallization (pH 2.0 and 4.0°C)	28	45	Sze *et al*., 2010
Real fermentation broth (97.8 g/L succinate, 23.5 g/L formate, 17.4 g/L acetate and 5.1 g/L lactate)	Direct crystallization (pH 2.0 and 4.0°C)	70	90	Li *et al*., 2010b

2.8.7 Discussion

The difficulties in developing an efficient process to separate succinic acid from fermentation broths are associated with the complexity of the fermentation broth. The common succinate recovery method is based on precipitation. For example, calcium hydroxide can be used to control the fermentation pH and precipitate succinate. In this process, a large amount of gypsum is accumulated, which is commercially useless (McKinlay *et al.*, 2007). In order to reduce this problem, the design of alternative production schemes has recently been investigated. Reactive extraction, ion exchange, crystallization, electrodialysis and nanofiltration were proposed to replace precipitation. However, as far as the reduction of the environmental impact is concerned, the use of reactive extraction or ion exchange for organic acid recovery from fermentation media still remains problematic. Under acidic conditions, the solubility of succinic acid is low and thus the crystallization method could be used for the separation of succinate. However, this process requires recrystallization step to make the final product suitable for commercial use. Therefore, membrane operations, like electrodialysis and nanofiltration for instance, appear very attractive since the generation of useless by-products can be significantly reduced. It was reported by many research groups that some NF membranes show lower retention of monovalent anions such as acetate than that of divalent anions such as succinate (Pontalier *et al*., 1997; Schaep *et al*., 2001; Kang and Chang, 2005). NF is a novel membrane process that is often more capital and energy efficient when compared with the chemical separation processes (Li *et al*., 2006). Therefore, in this work, it was chosen to investigate the succinate separation from fermentation broth by using a NF process.

2.9 Nanofiltration

NF is a membrane process that can be used for separating organic acids from fermentation broth. The separation mechanism of NF is based upon steric-hindrance (size) and charge effects. After a brief introduction into the fundamentals of membranes and membrane process, the NF process characteristics are also mentioned. Finally, the mass transfer mechanisms of NF process are discussed.

2.9.1 General concerns

Membrane filtration can be categorized into four major pressure-driven membrane processes: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO). MF is principally used for separation of micron-sized species that are usually particles or large macromolecules. UF is used for the separation of macromolecules (e.g. proteins) and NF and RO are used for low-molecular weight solutes (e.g. salts or organic solutes). NF is a pressure driven membrane separation process. The driving force is pressure difference between the feed (retentate) and the filtrate (permeate) sides at the separation layer of the membrane (Wang *et al*., 2002).

MF and UF membranes have well-defined pores and the separation is mainly fixed by size exclusion. For NF and RO membranes, which have smaller pores and the suggested separation mechanisms are size exclusion similar to MF and UF, or solution diffusion. In the solution-diffusion mechanism, species are absorbed into the membrane, which diffuses through the membrane structure, and are then desorbed. The relative rates of the adsorption desorption, and diffusion of the species controls the separation (Sablani *et al.*, 2007). Moreover, the electrostatic repulsion interaction between the charged solute and the fixed charge on membrane surface was also identified to play a role (Wang *et al*., 1997).

NF membranes were characterized by effective pore diameters ranging about 1 to a few nanometers, and by molecular weight cut-off between reverse osmosis membranes (dense structure) and ultrafiltration membranes (porous structure). Because of its selectivity, one or several components of a dissolved mixture are retained by the membrane despite the driving force, while water and substances with a molecular weight about 200-2000 Daltons are able to pass through. Because NF membranes also have selectivity with respect to the charge of the dissolved components, monovalent ions can pass through the membrane while divalent ions can be rejected (Schäfer *et al*., 2005).

2.9.2 NF process characteristic parameters

Before going further into the mass transfer mechanisms of NF, two parameters need to be introduced here which are permeate flux and retention coefficient. Moreover, the efficiency of NF process can be expressed using several parameters e.g. separation factor, purity, and product recovery (yield).

2.9.2.1 Permeate flux

In membrane processes, the pressure applied across the membrane which is a driving force pushing solute toward the membrane to another side is called "transmembrane pressure". The volumetric flux rate of permeate per unit area of membrane is the *permeate flux*, usually denoted by J_V,

$$J_v = \frac{1}{A} \cdot \frac{dV}{dt} \tag{2.2}$$

In which V is the total volume that has permeated through the membrane at time t, A is the effective area of the membrane (m^2), the units of permeate flux are $m^3.m^{-2}.s^{-1}$. The permeate flux (J_v) is calculated by measuring the

quantity of permeate collected during a certain time and dividing it by the effective membrane area for filtration.

Furthermore, other parameter that is usually determined before doing each experiment is the water flux (J_w). It can be used to calculate the membrane permeability, L_{p0}, which give an image of the membrane.

$$L_{p0} = \frac{J_w}{\Delta P} \tag{2.3}$$

where L_{p0} is the water permeability ($L.m^{-2}.h^{-1}.bar^{-1}$), J_w is the water flux ($L.m^{-2}.h^{-1}$) and ΔP is the pressure difference (bar).

2.9.2.2 Retention coefficient

A measure of the solute transfer across the membrane is conventionally expressed in term of retention coefficient, R_{obs}. R_{obs} is a measurement of the membrane selectivity towards a solute. It can be calculated as shown below:

$$R_{obs} = 1 - \frac{c_p}{c_f} \tag{2.4}$$

where R_{obs} is the observed retention of the solute, c_p is the concentration of the solute in the permeate and c_f is the concentration of the solute in the feed.

A retention coefficient equal to 1 indicates that the solute is totally retained, on the other hand, a retention equal to zero means that the solute permeates freely through the membrane.

2.9.2.3 Separation factor

An alternative parameter that is used to express the separation efficiency of NF beside the retention coefficient is the separation factor, *SF*. This factor indicates the variation of succinate and acetate composition in the retentate

compared with those in the feed. SF values than one like those obtained in this study indicate that succinate is enriched in the retentate compared to the feed. In this work, since separation of succinate from acetate is the main target, *SF* is calculated as:

$$Separation\ factor = \frac{C_{p,acetate}\ /C_{p,succinate}}{C_{f,acetate}\ /C_{f,succinate}} = \frac{1-R_{obs,acetate}}{1-R_{obs,succinate}} \quad (2.5)$$

2.9.2.4 Yield and purity

Moreover, the succinate yield and purity can be used to characterize the membrane separation performance. The succinate yield is defined as the ratio of the succinate concentration in the retentate over the total amount of succinate in the feed solution.

$$\%\ Yield = \frac{V_r C_{r(succinate)}}{V_f C_{f(succinate)}} \times 100\% \quad (2.6)$$

where V_f is the feed volume.

The succinate purity is defined as the succinate concentration compared with the sum of succinate and acetate concentration in the retentate.

$$\%\ Purity = \frac{C_{r(succinate)}}{C_{r(succinate)} + C_{r(acetate)}} \times 100\% \quad (2.7)$$

2.9.3 Mass transfer mechanisms

The retention of neutral solutes, e.g. glucose and lactose etc., in NF can be explained by size effects which are governed by solute and membrane pore size (Wang *et al*., 1997). The large solute molecule is retained or held in feed side (retentate) while the smaller one passes through the membrane to filtrate side (permeate) easier as shown in Fig. 2.9a. The smaller solutes predominantly transport across NF membrane by convection (is carried along with the fluid) in the membrane pores due to pressure difference. On the other hand, both of the solutes can pass

through the membrane more easily when they are filtrated with the more open membrane (wider membrane pore size) as shown in Fig. 2.9b. For instance, Pontalier *et al*. (1997) studied solute transfer mechanisms of glucose and lactose with 400Da membrane in single-solute solutions. They reported that the retention of glucose was 68% while that of lactose was 99%. Wang *et al*. (2002) studied the mass transfer mechanism of glucose and sucrose in single-solute solutions with 300Da NF membrane. They found that the retention of glucose was about 80% and sucrose retention was almost 100%. It can be said that the size of lactose is bigger than that of glucose so that the glucose pass through the membrane easier than lactose. In addition, Sjöman *et al.* (2006) studied the separation mechanism of xylose and glucose in binary-solute solutions with 150-300Da NF. The results showed that the retention of xylose was 80% and glucose was 90%. It was due to the xylose pass through the membrane easier than glucose.

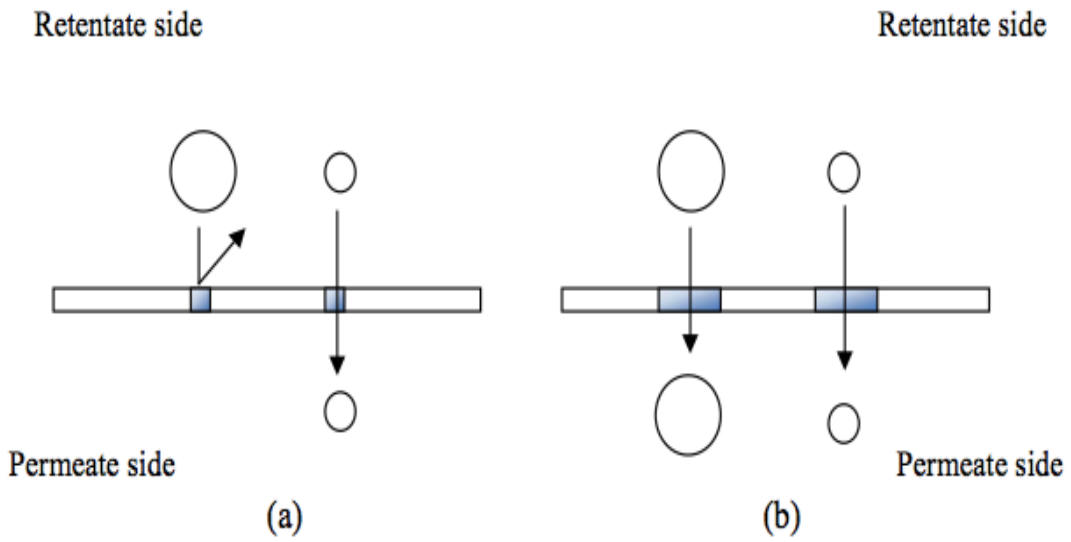

Figure 2.9 Mass transfer mechanisms of two different size solutes through a NF membrane. (a) "tight membrane" (b) "open membrane" (Schäfer *et al*., 2005).

Mass transfer mechanism of charged solutes e.g. succinate ion, lactate ion, magnesium ion, and chloride ion etc., can be explained by combination of size and charge effects. Indeed, Schaep *et al*. (1998) failed to describe mass transfer mechanisms of charged solute by only charge effect. The charge effect was an influence of electrostatic interaction (repulsion between the same sign of charges and attraction between opposite sign of charges) between the solutes and the fixed charges on the membrane surface. The co-ions (same sign of charge as fixed membrane charges) were repelled by membrane surface, while the counter-ion (opposite sign of charge to the fixed charge of membrane) can pass through it. However, the co-ions can pass through the membrane as well as the counter-ion because of electroneutrality (charges in both retentate and permeate have to be balanced). In the same way co-ions were retained in retentate, counter-ions need to also stay in retentate to neutralize repelled co-ions and thus salt retention occurs.

Variation of feed concentration can affect the retention of monovalent ion whereas it was less effective on retention of divalent ions. This can be explained by considering in the mass transfer mechanism of ionic solutes permeation through NF membrane as shown in Fig. 2.10. Fig. 2.10a shows ionic solutes cannot permeate through NF membrane at low feed concentration. As the monovalent counter-ions and co-ions always accompany each other, they cannot pass through the membrane separately. If co-ions are repelled by fixed charges of membrane, both cannot pass through the membrane. Divalent ions were totally retained because their size was larger than that of pore and/or stronger electrostatic repulsion effect e.g. Na_2SO_4 and $MgCl_2$ were completely retained by NF40 membrane whatever the feed concentration

(Schaep *et al*. 1998). Fig. 2.10b illustrates ionic solutes at high feed concentration can pass through the membrane, more easily than those at low feed concentration. The retention of monovalent salts such as NaCl, KCl and sodium lactate, generally decreases with increasing the salt concentration (Bargeman *et al*., 2005). For instance, the sodium lactate retention decreases from 80% to 25% for increasing sodium lactate concentration from 0.1 M to 1 M, respectively (Bouchoux *et al*., 2005). The decrease of salt retention in presence of high feed concentration can be explained as followed. At the low salt concentrations, electrostatic repulsions were predominant so that high salt retentions were obtained. As the feed concentration increases, electrostatic interactions become weaker and thus the retention decreases. This is known as the 'screening effect' (Kang *et al*., 2004; Kang and Chang, 2005). The membrane surface being screened by counter-ions, the repulsion between co-ions and fixed charges on membrane surface becomes smaller causing the retention decline as shown in Fig. 2.10(b). Ideally, at a sufficient salt concentration, electrostatic interactions are negligible so that the retention of charge solutes is mainly fixed by size effects.

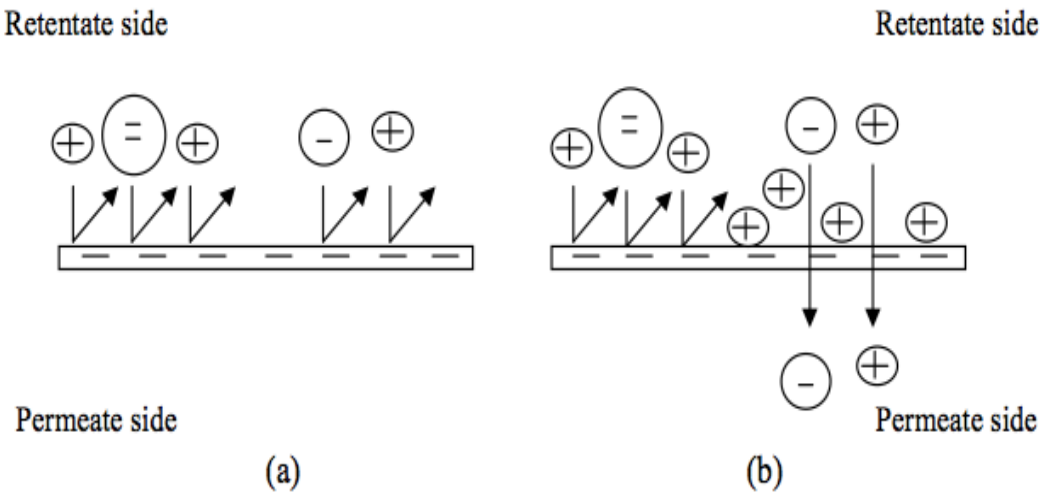

Figure 2.10 Mass transfer mechanisms of charge solute in NF. (a) low concentration (b) high concentration (Schäfer *et al*., 2005).

According to the definition of retention coefficient (Eq. 2.4) a negative value can be obtained when the solute concentration in the permeate is higher than that in the retentate. It was reported by many research groups that numerous NF membrane show lower retention of monovalent anion such as chloride ion, formate, acetate and lactate than divalent anions such sulfate, succinate (Pontalier *et al*., 1997; Kang and Chang, 2005; Umpuch *et al.*, 2010). Especially, in case of mixed-salt solutions with mono and divalent anions, NF membrane can exhibit negative retention of monovalent anions (Garcia-Aleman *et al.*, 2004). Kang and Chang (2005) reported the negative retention of monovalent anions such as formate, lactate and acetate was obtained in the presence of divalent anions such as succinate. In addition, Umpuch *et al.* (2010) also observed a negative retention of monovalent anion in binary-solute solution containing lactate and sulphate.

Fig. 2.11 shows schematic diagram of solute transport though NF membrane in such condition. In these circumstances a large ion can be excluded from transport by a like charged membrane (e.g. SO_4^{2-}) this results in a higher than the concentrations of counter- ions (Na^+) in the permeate. Consequently, the smaller membrane permeable co-ions (Cl^-) were drawn across the membrane to neutralize the charge imbalance. At this stage the concentration of these smaller ions can be greater than in the feed giving negative retention (Mandale and Jones, 2008). Bowen and Mukhtar (1996) carried out such experiments when evaluating NF transport models, clearly illustrating the negative retention of the chloride ion.

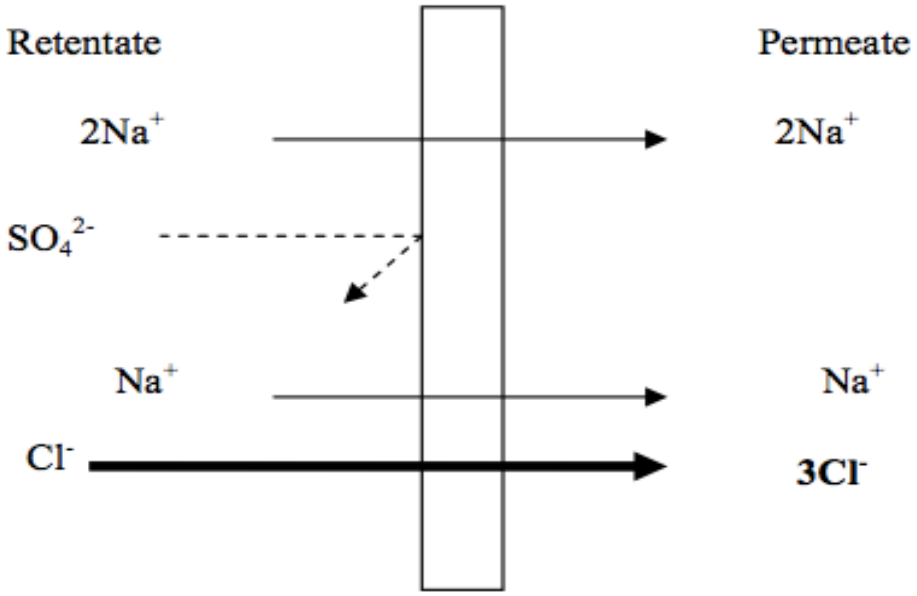

Figure 2.11 Schematic diagram of two salts transport through a NF membrane (Umpuch, 2010).

In this work, nanofiltration was investigated with a mixed salts solution containing succinate and acetate. Then negative retention of acetate could be obtained in the presence of succinate. Kang and Chang (2005) and Umpuch *et al.* (2010) suggested that the major factor influencing the negative retention of monovalent anion was the ratio of the concentration of divalent anion to monovalent anion. Moreover, negative values of the retention for monovalent anion are generally obtained at low permeate flux (Umpuch *et al.*, 2010). Therefore, to understand the influence of various parameters (concentration ratio between divalent anion and monovalent anion, retention of the various co-ions, permeate flux) on the retention of acetate (R_{Ace-}), we can simulate the acetate retention in the presence of succinate (R_{Suc2-}) using Eq. 2.8 (mass balance equations were derived as given in detail in Appendix C).

$$R_{Ace-} = R_{K+}[1 + 2\alpha(1 - \beta)] \tag{2.8}$$

where R_{Ace-} is the acetate retention, R_{K+} is the potassium retention and α is defined as the ratio of succinate to acetate concentrations in the retentate:

$$\alpha = \frac{[Suc^{2-}]r}{[Ace^{-}]r} \tag{2.9}$$

where $[Suc^{2-}]r$ is the succinate concentration in the retentate, $[Ace^{-}]r$ is the acetate concentration in the retentate and β is the ratio of succinate retention to potassium retention:

$$\beta = \frac{R_{Suc2-}}{R_{K+}} \tag{2.10}$$

where R_{Suc2-} is the succinate retention.

In addition, the retentions of succinate and acetate are affected by the solvent flux. At low solvent flux, the retention ratio succinate and potassium increase β with J_v. The difference between the succinate and potassium retentions increase. Consequently, acetate retention becomes lower and reaches negative values (Fig. 2.12). On the other hand, the retention ratio (β) of succinate retention to potassium retention decreases with J_v to reach a constant value at higher solvent flux. The difference between succinate and potassium retentions decreases and becomes constant. Then, the acetate retention increases and reaches positive values.

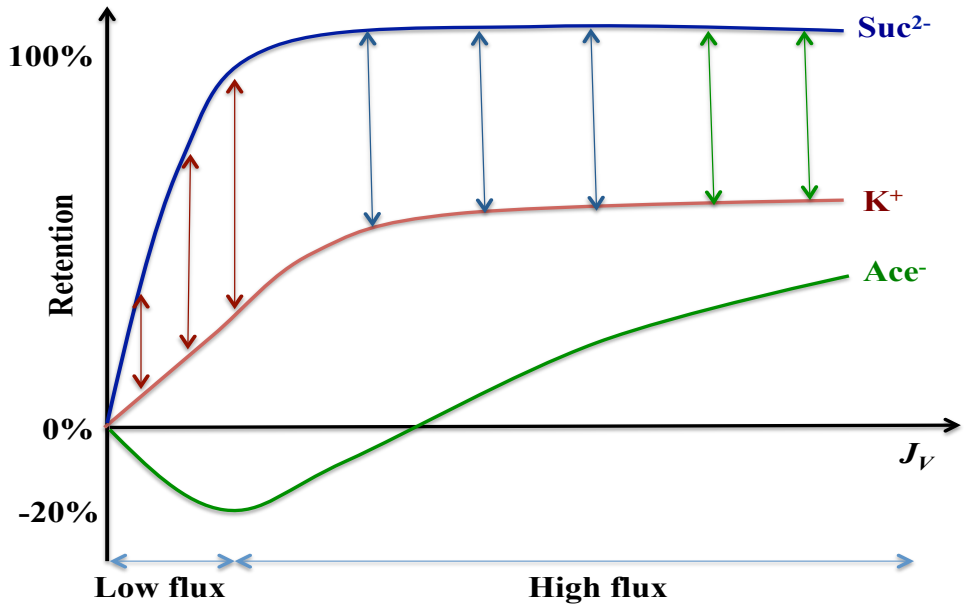

Figure 2.12 Retention of succinate, acetate and potassium as a function of permeate flux - general trend.

2.9.4 Selectivity of NF

NF offers the possibility to separate monosaccharide and disaccharide. Goulas *et al*. (2002) found that DS-5DL membrane has retention of 77% and 99% for fructose and sucrose, respectively, in the binary mixture. NF gives the possibility to separate monovalent and divalent ions. Low retention of monovalent ions (at high feed concentration) and very high retention of divalent ions in a mixture solution can be obtained. Pontalier *et al*. (1997) studied selective retention of NaCl/Na_2SO_4 in binary-solute solution with a 400Da membrane. The both salts contain the same counter-ion and different co-ions. It was observed that the retention of Cl^- decreases with increasing the salt concentration (either NaCl or Na_2SO_4). SO_4^{2-} was almost completely retained with all salt concentrations while Na^+ can pass through the membrane easily, resulting an excess of positive charge on the permeate side. This excess generates an electrostatic force which increases anion transfer, particularly of

Cl^-, because SO_4^{2-} cannot cross the membrane. However, Na^+, which was the counter-ions, was retained because anions and cations cannot permeate independently, but permeate through the membrane while maintaining electroneutrality (Wang *et al*., 2002). The difference of mass transfer mechanism between divalent co-ions, which are completely retained, and monovalent co-ions, which were allowed to pass through the membrane, contributes to the selectivity. Kang and Chang (2005) reported a divalent anion such as succinate was more retained than monovalent anions such as formate, lactate and acetate. Therefore, it can be concluded that it is possible to separate monovalent ion from divalent ion in mixture solution using NF.

The separation of monovalent ion and disaccharide sugar such as lactose separation was also investigated (Li and Shahbazi, 2006). It was found that at high salt concentration, lactose is completely retained by DS-5DK membrane while sodium lactate is enriched in the permeate and thus the purification achieved.

In addition, Bouchoux *et al*. (2005) investigated the separation between glucose and sodium lactate. It was revealed that glucose retention strongly decreased with increasing sodium lactate concentration. Thus the retention of both solutes became very close to each other making the separation impossible. Moreover, Bouchoux *et al*. (2005) also reported that the presence of glucose does not affect the retention of sodium lactate. NF cannot separate monosaccharide from lactate monovalent ion in these conditions. However, Umpuch *et al.* (2010) found that the different lactate and glucose retentions increase when adding Na_2SO_4. Therefore, it can be noted that the separation of monovalent ion and monosaccharide with NF is achievable by addition of a strongly retained anion e.g. large size and high charge.

2.9.5 Conclusion

Due to these interesting separation properties, NF begins to be used in a wide range of applications in the food industry. According to the numerous works published, the use of NF as a downstream operation in organic acids production processes is going to be a large and new application field of this technology (Timmer *et al*., 1994; Moresi *et al*., 2002)

Organic acids (acetic, lactic, succinic acid) are mainly produced by fermentation. The fermentation generates a broth containing organic acid salts and impurities like salts, residual sugars or other organic acid salts. Different operations of separation and purification are then required in order to recover organic acids from fermentation broth. The integration of NF in the process can be investigated at different stages depending on the composition of fermentation broth. On one hand, NF can be used to remove residual sugars such as glucose or sucrose from organic salts. On the other hand, NF can be applied to separate divalent anions like succinate from monovalent anions like acetate, formate and lactate. It was shown that NF is an appropriate method for the downstream processing of succinate fermentation broths.

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CHAPTER III

IMPROVED XYLOSE UPTAKE AND UTILIZATION IN ENGINEERED *Escherichia coli* KJ122 FOR SUCCINATE PRODUCTION

3.1 Introduction

Lignocellulosic biomass has significant potential to serve as a sustainable raw material for the production of renewable fuels and chemicals (Chundawat *et al*., 2011). Xylose is a major sugar of hemicellulose in lignocellulosic biomass, and most of microorganisms are unable to utilize it as a carbon source (Hahn-Hagerdal *et al*., 2007). Therefore, the improvement in xylose utilization in microorganism is would be beneficial for bio-based succinate production.

Succinate is a member of the C4-dicarboxylic acid family. It has attracted much interest because it has been used as a precursor of many industrially important chemicals in the biopolymer, food, chemical, and pharmaceutical industries (Delhomme *et al*., 2009; McKinlay *et al*., 2007). Traditionally, succinic acid is produced commercially by catalytic hydrogenation of petrochemical derived maleic acid or maleic anhydride. Due to increasing global demands for oil and the emergence of environmental consequences from excessive using fossil fuels, fermentative production of succinic acid from renewable biomass by anaerobic bacteria has become more attractive economically (Willke and Vorlop, 2004).

Most studies of succinate fermentations by engineered *E. coli* used glucose as the feedstock (Lin *et al*., 2005; Andersson *et al.*, 2007; Kwon *et al.*, 2007; Jantama *et al*., 2008; Zhang, *et al*., 2009), and further research is needed to accomplish the efficient conversion of renewable lignocellulosic materials to succinate. Poor utilization of xylose, the main component of hemicellulose in plants, is a major problem for hydrolysate fermentation by engineered *E. coli* (Wang *et al.*, 2013).

Xylose can be transported into the cell by two different inducible permeases in *E. coli*. One is an ABC transporter coded by the *xylF*, *xylG* and *xylH* genes. The second is a proton/xylose symporter coded by *xylE* (Sumiya *et al*., 1995; Gonzalez *et al*., 2002). In *E. coli*, xylose is transported mainly by the ABC transporter, which is driven by ATP (Hasona *et al*., 2004; Andersson *et al*., 2007). During this process, one molecule of xylose requires one ATP for its transportation and other ATP is needed for phosphorylation of xylose, whereas the conversion from xylose to succinate only yield 1.67 ATP per xylose during anaerobic fermentation in *E. coli* strains (Andersson *et al*., 2007; Liu *et al*., 2012). Therefore, the ATP production is not enough to convert xylose to succinate. To improve the utilization efficiency of xylose, it is necessary to enhance ATP supply in engineered *E. coli*.

Recently, *E. coli* KJ122 was originally developed to ferment glucose from starch into succinate (Jantama *et al*., 2008; Zhang *et al*., 2009). However, this strain performs poorly on xylose caused by an ATP deficiency is observed under anaerobic conditions (Wang *et al.*, 2013; Hasona *et al.*, 2004). Therefore, this study investigated the improving xylose consumption in *E.coli* KJ122 under anaerobic conditions by a combined strategy of metabolic engineering and metabolic evolution.

3.2 Objectives

The objective of this work was to improve xylose uptake and utilization in *E. coli* KJ122 by using metabolic engineering and metabolic evolution.

1) To investigate the effect of *xylFGH* and *xylE* genes deletion in *E. coli* KJ122 on succinate production from pure xylose under anaerobic conditions.

2) To select the mutant strains of *E. coli* KJ12201 (Δ*xylFGH*) that exhibited an efficient production of succinate from xylose by using metabolic evolution technique.

3) To produce succinate in fed-batch fermentation by an evolved *E. coli* KJ12201 using xylose as carbon substrate.

3.3 Materials and methods

3.3.1 Strains, media and growth conditions

All chromosomal modifications were made in *E. coli* KJ122. All the bacterial strains, plasmids, and primers used in this study are listed in Table 3.1 and Table 3.2. Luria Bertani (LB) broth containing the following components per liter of broth: 10 g peptone, 5 g yeast extract and 5 g sodium chloride, was used for *xylFGH* and *xylE* genes deletion of succinate-producing strain, KJ122. Cultures were also maintained on solid media (20 g/L agar). Ampicillin (50 μg/mL) and kanamycin (50 μg/mL) were added to the medium for selecting the positive clones. A slightly modified low salts medium, AM1 (Martinez *et al*., 2007) supplemented with 1 mM betaine, was used as a fermentation medium (Table 3.3).

Table 3.1 *E. coli* strains and plasmids used in this study.

	Relevant characteristics	**Sources**
***E. coli* Strains**		
KJ122	*E. coli* ATCC 8739 (*ΔldhA, ΔadhE, ΔackA, Δ(focA-pflB), ΔmgsA, ΔpoxB, ΔtdcDE, ΔcitF, ΔaspC, ΔsfcA, pck*, ptsI*)*	Jantama *et al*., 2008
KJ12201	KJ122 Δ*xylFGH*	This study
KJ12202	KJ122 Δ*xylE*	This study
KJ12203	KJ122 Δ*xylFGH* Δ*xylE*	This study
Plasmids		
pKD4	*bla* FRT-*kan*-FRT	Datsenko, 2000
pKD46	*bla γ β exo* (Red recombinase), temperature-conditional replicon	Datsenko, 2000
pFT-A	*bla flp* temperature-conditional replicon and FLP recombinase	Posfai *et al*., 1997

* spontaneous mutation

Table 3.2 Primers used in this study.

	Relevant characteristics	**Sources**
Primers		
XylFGH	Forward: 5’ATGAAAATAAAGAACATTCTACTCACCCTTTGCACCTCACTCCTGGTGTAGGCTGGAGCTGCTTC 3’	This study
	Reverse: 5’TCAAGAACGGCGTTTGGTTGCGGAGTCCATCCATACTGCCAGCAACATATGAATATCCTCCTTAG 3’	This study
XylE	Forward: 5'ATGAATACCCAGTATAATTCCAGTTATATATTTTCGATTACCTTAGTGTAGGCTGGAGCTGCTTC 3'	This study
	Reverse: 5’TTGCAGCGTACCAGTTTGTTGTGTTTTCTTCGTTTCCGGTTCCCACATATGAATATCCTCCTTAG 3’	This study

20 bp underlined is *kan* cassette

Table 3.3 Composition of AM1 mineral low salts medium (excluding carbon source).

Component	**Concentration (mmol/L)**
$(NH_4)_2HPO_4$	19.92
$NH_4H_2PO_4$	7.56
Total PO_4	27.48
Total N	47.93
[a]Total K	1.00
$MgSO_4.7H_2O$	1.50
Betaine-HCl	1.00
Trace Elements	(µmol/L)[b]
-$FeCl_3.6H_2O$	8.88
-$CoCl_2.6H_2O$	1.26
-$CuCl_2.2H_2O$	0.88
-$ZnCl_2$	2.20
-$Na_2MoO_4.2H_2O$	1.24
-H_3BO_3	1.21
-$MnCl_2.4H_2O$	2.25
Total Salts	4.1 g/L

[a]KOH was used to neutralize betaine-HCl stock.

[b]Trace metal stock (1000X) was prepared in 120 mM HCl.

3.3.2 Genetic engineering techniques

3.3.2.1 DNA amplification by Polymerase Chain Reaction (PCR)

The standard PCR reaction was performed using 10x PCR Master Mix solutions (Qiagen, Valencia, CA) in a PCR reaction of 50 µL. Twenty five micro-liters of master mix containing 10 mM of each dNTP (dATP, dGTP, dCTP and dTTP), PCR reaction buffer (20 mM Tris-HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 1% (v/v) Triton® X-100, 1 mg/mL nucleasefree BSA, and *Taq* polymerase enzyme), 40 pmole of each primer (forward and reverse strand primers), and 50 ng of either plasmid or chromosomal DNA template and distilled water, were added to the mixture. The reaction was performed in automated Flexcycler PCR machine (Analytikjena, Germany). The PCR condition was shown in Table 3.4. After the amplification reaction was finished, an aliquot of the PCR reaction mixture was examined on 1.0% (w/v) agarose gel electrophoresis.

Table 3.4 PCR parameters for the amplification of specific genes. The extension time is depended on the length of the genes (1 kb/min).

PCR Profile to amplify gene				
Step	Period	Temperature (°C)	Time	Number of cycles
1	Pre-denaturing	95	5 min	1
2	Denaturing	95	30 sec	
	Annealing	55	30 sec	35
	Extension	72	3.3 min	
3	Extra-extension	72	10 min	1

3.3.2.2 Agarose gel electrophoresis of DNA

To analyze the size of DNA fragments, the PCR products and DNA fragments was subjected to agarose gel electrophoresis. The appropriate amount of agarose powder was dissolved in 0.5x TBE buffer [89 mM Tris-HCl, 89 mM boric acid, 25 mM EDTA pH 8.0] or 1x TAE buffer [40 mM Tris-HCl, 40 mM acetic acid, 25 mM EDTA pH 8.0] under boiling temperature to ensure the homogeneity of the gel solution. Five microliters of loading dye [0.1% (w/v) bromophenol blue, 40% (w/v) Ficoll and 5 mM EDTA)] were added and mixed well to the DNA samples before loading into the wells of the solidified gel. The electrophoresis was performed at a constant voltage, 100 V, for 45 hour. After completion of electrophoresis, the gel was stained with 2 μg/mL ethidium bromide for 2 to 4 minutes and destained in distilled water for 10 min. The DNA bands were visualized under UV light and photographed by a gel documentation system.

3.3.2.3 Preparation of *E. coli* KJ122 competent cells by $CaCl_2$ method

A single colony (diameter of about 2-3 mm) of *E. coli* KJ122 was inoculated into 3 ml of LB broth and incubated at 37°C for overnight. Cells were diluted to 1:100 in LB medium and incubated at 37°C with shaking until the OD_{550} was 0.3-0.5. The culture was centrifuged at 4,000 rpm, 4°C for 10 min. The pellet was re-suspended and washed in 5 ml of ice-cold $CaCl_2$ for 3 times. After washing the cell, the white cell pellet was re-suspended in 2 ml of ice-cold $CaCl_2$ and placed on ice for 1 h. Glycerol was added into the cell suspension at 15% (v/v) final concentration then 200 µl aliquots were stored at -80°C.

3.3.2.4 Preparation of *E. coli* KJ122 competent cells by electro-transformation method

A single colony (diameter of about 2-3 mm) of *E. coli* KJ122 harboring *Red* recombinase (pKD46) was inoculated into 3 ml of LB broth and incubated at 30°C for overnight. Cells were diluted to 1:100 in LB medium with 50 µg/mL ampicillin and 5% (w/v) L-arabinose, and incubated at 30°C with viscous shaking until the OD_{550} reached 0.3-0.5. The culture was centrifuged at 4,000 rpm, 4°C for 10 min. The pellet was re-suspended and washed in 5 ml of sterile ice-cold water for 4 times. After washing the cell, the white cell pellet was re-suspended in 1 ml of sterile ice-cold water. Eighty microliters of aliquot were dispensed into electroporation cuvette.

3.3.2.5 Transformation of plasmids into *E. coli* KJ122 by heat shock method

One microliter of plasmid was mixed gently with 200 µl of *E. coli* KJ122 competent cells and placed on ice for 30 min. The cells were heat-shocked at 42°C for 90 sec and incubated on ice for additional 5 min. The transformed cells were mixed with 800 µl of LB broth in 15 ml tube and incubated at 37°C for 1.5 h. Two hundreds microliters of transformed cells were then plated on LB agar plates containing ampicillin (50 µg/mL), and incubated overnight at 30°C.

3.3.2.6 Transformation of *E. coli* KJ122 by electroporation

Linearized DNA, 100 ng-10 µg (in 5-10 µl of sterile water), was mixed with electroporated competent cells, and the mixture was transferred to an ice-cold 0.4 cm electroporation cuvette. The cuvette was incubated on ice for 5 min. The cells were pulsed by using electroporation (Bio-Rad MicroPulser^{TM}, USA) under the conditions used with *E. coli* (2,500 V, pulse length 5 ms). Then 1 ml of 1 M ice-cold LB broth was added to the cuvette immediately and the solution was transferred to a sterile 15 ml tube. The tube was incubated at 30°C with 150 rpm shaking for 2 h. Transformed cells, 200 µl, was spread on LB agar plates containing suitable antibiotics depending on antibiotic resistant genes harbored in the DNA fragments, and incubated overnight at 37°C.

3.3.2.7 Deletion of *xylFGH* and *xylE* genes in *E. coli* KJ122

Plasmids and primers used in this study are summarized in Table 3.1 and Table 3.2. Methods for chromosomal deletions, integrations, and removal of antibiotic resistance genes have been previously

described (Datsenko and Wanner, 2000; Grabar *et al*., 2006; Posfai *et al*., 1997; Zhou *et al*., 2006). Sense primers contain sequences corresponding to the N-terminus of each targeted gene followed by 20 bp (underlined) corresponding to the FRT-*kan*-FRT cassette. Anti-sense primers contain sequences corresponding to the C-terminal region of each targeted gene (boldface type) followed by 20 bp (underlined) corresponding to the cassette. The FRT-*kan*-FRT cassette was amplified by PCR by using these primers and pKD4 as the template. Amplified DNA fragments were electroporated into *E. coli* KJ122 harboring *Red* recombinase (pKD46). In resulting recombinants, the FRT-*kan*-FRT cassette replaced the deleted region of the target gene by homologous recombination (double-crossover event). After integration, recombinants were transformed with pFT-A and grown at 30°C. During growth in LB medium with 50 μg/mL chlortetracycline, FLP recombinase was induced and in turn excises the DNA bracketed by concurrently facing FRT sites (selectable marker and replicon) from the chromosome. After growth at 42°C to eliminate pFT-A, only a single FRT should remain in the chromosome. Chromosomal deletions and integrations were verified by testing for antibiotic markers, PCR analysis, and analysis of fermentation products.

3.3.3 Metabolic evolution

Metabolic evolution was performed by serial transfers in pH-controlled mini-fermentors (Jantama *et al.,* 2008). *E. coli* KJ12201 was sub-cultured in AM1 medium containing 10% (w/v) xylose (Fig. 3.1). Starting at initial OD_{550} nm of 0.1, cells was grown at 37°C, pH 7 and 200 rpm. The culture was rapidly transferred in fresh AM1 medium when the OD_{550} nm of the culture approached the range of 1.00 to 2.00. The transfers were performed until no further improvement in the growth rate

was observed. After 14 serial transfers, broth was spreaded on LB agar with 1% xylose. The selected colonies were tested individually for xylose utilization. KJ12201-14T showed the highest growth rate and highest succinate productivity.

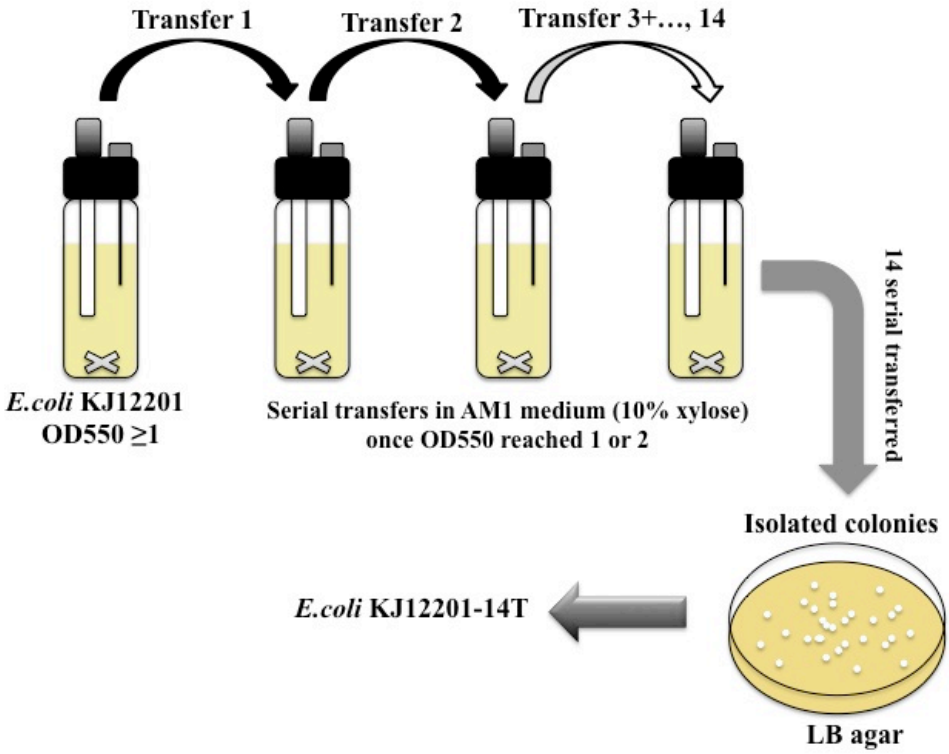

Figure 3.1 Metabolic evolution of *E. coli* KJ12201 in AM1 medium with 10% xylose.

3.3.4 Fermentation

3.3.4.1 Fermentation in mini-fermenters

The inocula were prepared by inoculating seed into AM1 medium containing 2% (w/v) sugar. All of the inocula were grown at 37 °C, 200 rpm for 16 h. Fermentations were inoculated at OD_{550} of 0.1 into AM1 medium. Batch fermentations were performed with AM1 medium (Martinez *et al.*, 2007) supplemented with 1 mM betaine, 100 mM $KHCO_3$, and sugar (as indicated) as a

carbon source. The cells were cultured in mini-fermenters (500 ml vessels) containing 300 ml media, under anaerobic condition at 37 °C, pH 7 and 200 rpm. Fermentations were run with xylose or a mixture of glucose/xylose at either 5% (w/v) or 10% (w/v) total sugar. The pH was kept constant with 6 M KOH and 3 M K_2CO_3 (Jantama *et al*., 2008) automatic additions. No antibiotics were included during the growth of seed preparations or in the fermentation broth. All the fermentations were performed triplicate (average and standard deviations are shown in the tables and plots).

3.3.4.2 Fed-batch fermentation in 2 L bioreactor

The fed-batch fermentation was performed in a 2L bioreactor with 1L initial working volume containing 5% xylose. The xylose (250 g/L) was intermittently fed into the bioreactor. The residual xylose was maintained between 5 and 30 g/L. The bioreactor was controlled at 37°C and 200 rpm. The pH was controlled at 7.0 by automatically adding 6 M KOH and 3 M K_2CO_3. AM1 was used as a fermentation medium. The cultivation conditions in the fed-batch were the same as those in batch experiments. The experiments were performed in triplicate.

3.3.5 Analytical methods

Samples were removed during fermentation for the measurement of cell mass, organic acids, and sugars. Cell mass were estimated from the optical density at 550 nm (1.00 OD_{550} = 0.33 mg of cell dry weight/ml) with a Spekol® 1500 spectrophotometer (Analytikjena, Germany). Organic acids and sugars were determined by using high performance liquid chromatography, HPLC, (Agilent Technology 1200 series, Germany) equipped with refractive index detectors with a Bio-Rad Aminex HPX-87H ion exclusion column. The column and detector

temperatures were kept constant at 45°C. The mobile phase used in the HPLC system is 4 mM sulfuric acid at a flow rate of 0.4 ml/min. The cultures were centrifuged at 13,500 rpm (Wisespin®) for 4 min to separate cells and supernatant. The supernatant was filtered through 0.2 μm filter membrane before injecting to HPLC. Ten microliters of injection volume were automatically analyzed.

3.4 Results

3.4.1 Influence of *xyl* transporter deletions on succinate production in *E. coli* KJ122

E. coli KJ122 was developed to produce succinate by using metabolic engineering and metabolic evolution. The *E. coli* KJ122 produced a high titers of succinate from glucose (Jantama *et al*., 2008) but poor growth and low succinate productivity were observed when this strain was cultured on xylose (Wang *et al*., 2013). This problem was confirmed using xylose and served as a starting point for strain improvement. *E. coli* KJ122 exhibited a lag phase of 48 h with 10% xylose. Reducing the xylose concentration to 5%, the lag phase was decreased from 48 h to 24 h (Fig. 3.2). However, xylose was still remained after 120 h. After fermentation, 25.89±0.89 g/L and 30.99±0.52 g/L succinate were produced from 5% and 10% xylose, respectively. In addition, this result was consistent with the succinate yield and productivity of KJ122 in both 5% xylose (0.72±0.26 g/g and 0.21±0.01 g/L.h) and 10% xylose (0.67±0.02 g/g and 0.26±0.01 g/L.h), respectively (Table 3.5). Acetate was also increased during cell growth and succinate production. At 120 h, the acetate concentrations were about 7.86±0.07 g/L and 8.46±0.37 g/L in the broth containing of 5% and 10 % xylose, respectively. Since conversion of xylose to succinate in KJ122

generates only one ATP during glycolysis, but requires two ATP for xylose utilization when xylose is transported through *xylFGH* genes. Thus, growth and xylose metabolism in KJ122 could be limited due to insufficient ATP supply.

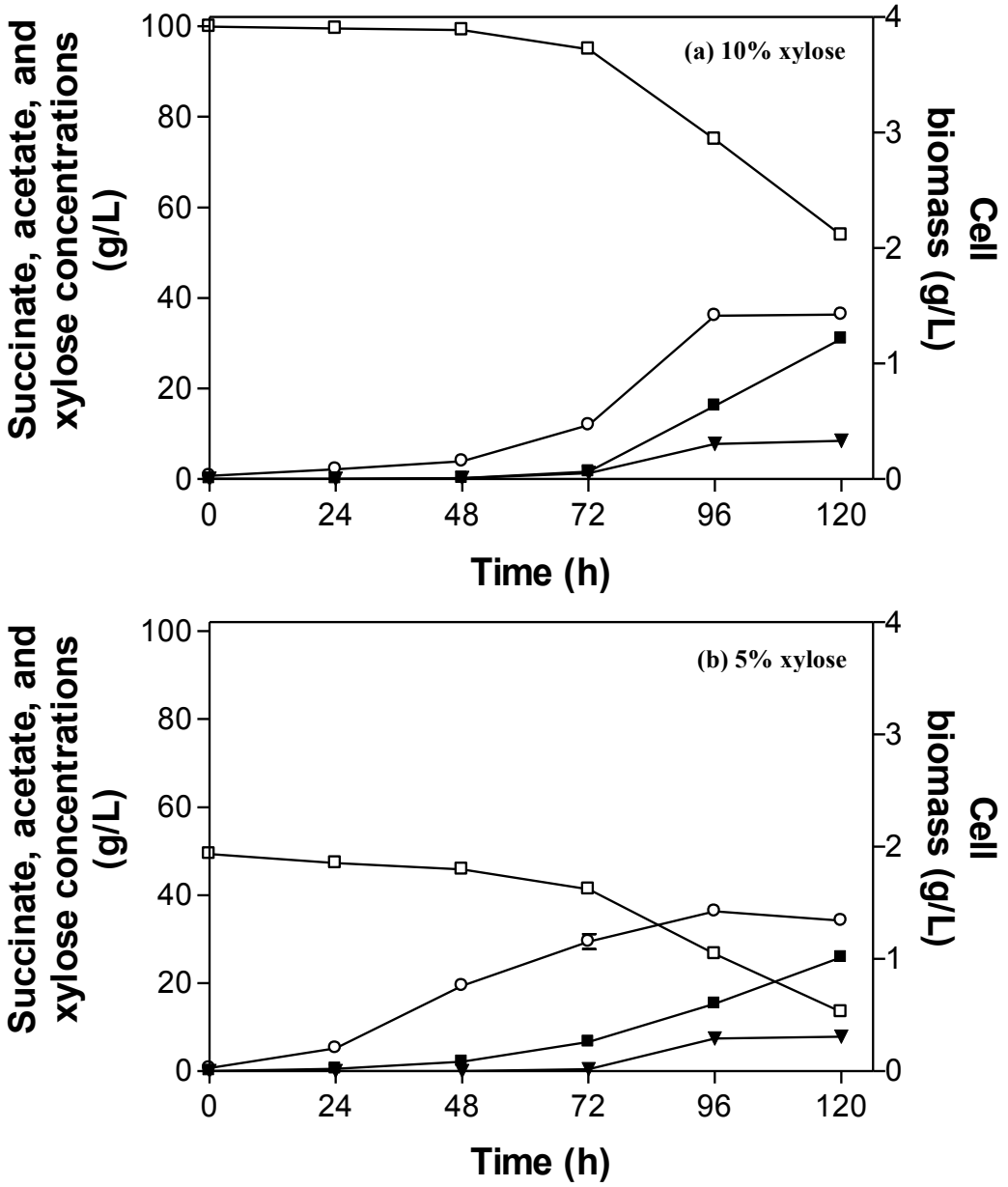

Figure 3.2 Succinate production from xylose by *E.coli* KJ122 in AM1 medium. (a). Fermentation of 10% xylose; (b). Fermentation of 5% xylose. Symbols for all: xylose (open square), succinate (filled square), acetate (filled triangle), biomass (open circle).

In order to enhance the ATP supply for xylose metabolism in KJ122, genes encoding ATP-dependent xylose transporter (encoded by *xylF*, *xylG* and *xylH*) were deleted. KJ12201 (*E.coli* KJ122 *ΔxylFGH*) completed the fermentation of 5% xylose within 72 h but the remaining xylose was still about 19.5 g/L when performing the fermentation in 10% xylose even though the fermentation time was prolonged (Fig. 3.3). At the end of fermentation, KJ12201 produced succinate at 37.43±0.67 g/L and 70.76±3.39 g/L from 5% and 10% xylose, respectively. Also, acetate was detected at 10.57±0.42 g/L and 9.34±0.74 g/L from 5% and 10% xylose, respectively. In addition, strain KJ12201 utilized xylose for higher succinate production with yields of 0.77±0.02 g/g and 0.87±0.03 g/g from 5% and 10% xylose, respectively, and the productivity of 0.31±0.01 g/L.h and 0.59±0.02 g/L.h from 5% and 10% xylose, respectively as compared with KJ122 (Table 3.5). Moreover, strain KJ12201 exhibited an increase in the cell biomass and xylose consumption (Table 3.5). Utrilla *et al.* (2012), also reported that the deletion of *xylFGH* genes in *E. coli* JOU1 improved growth rate and D-lactate productivity. The lack of *xylFGH* genes in the mutant stain resulted in a conserve ATP supply for xylose metabolism.

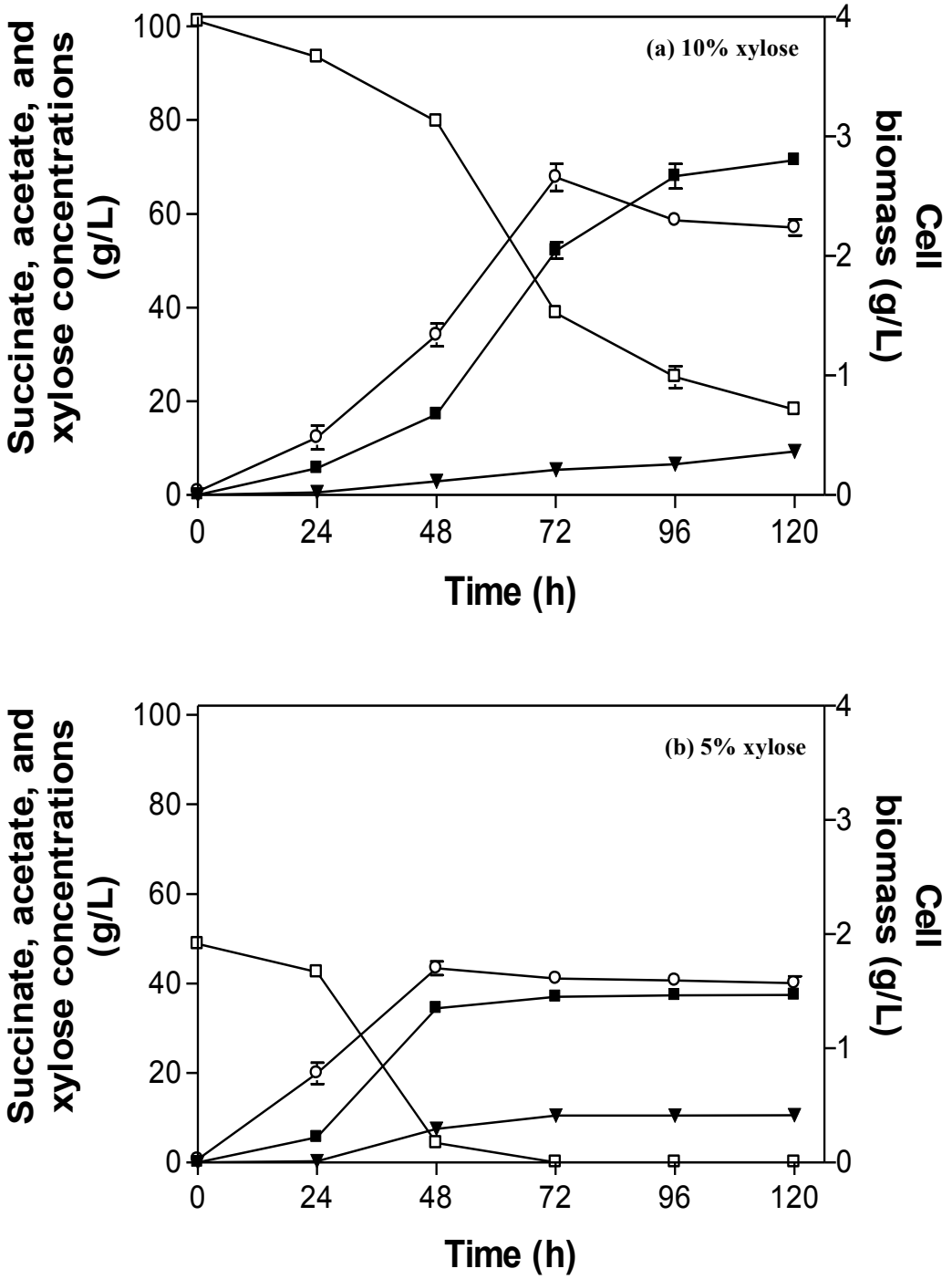

Figure 3.3 Succinate production from xylose by *E.coli* KJ12201 (KJ122 *ΔxylFGH*) in AM1 medium. (a). Fermentation of 10% xylose; (b). Fermentation of 5% xylose. Symbols for all: xylose (open square), succinate (filled square), acetate (filled triangle), biomass (open circle).

To investigate the role of *xylE* in xylose transport, *xylE* was deleted from the parent strain (KJ122). Strain KJ12202 (KJ122 *ΔxylE*) performs poorly on 10% xylose (Fig. 3.4a), however, reducing the xylose concentration from 10% to 5% improved xylose consumption (Fig. 3.4b). At the end of fermentation time (120 h), succinate at the concentration of 23.80±0.59 g/L and 11.46±0.17 g/L were detected in 5% and 10% xylose fermentation, respectively. In addition, strain KJ12202 produced succinate yield at 0.61±0.01 g/g and 0.71±0.02 g/g, respectively, and productivity at 0.20±0.01 g/L.h and 0.10±0.01 g/L.h, respectively. Acetate was observed as a major by-product at the concentration of 12.84±1.06 g/L and 3.97±0.12 g/L in 5% and 10% xylose, respectively. KJ12202 showed a 68% and 80% decrease in xylose consumption at 10% xylose compared with KJ122 and KJ12201, respectively, due to the shortage supply of ATP required by xylFGH transporter. As the insufficient ATP supply during xylose fermentation in KJ12202, cells enhance the formation of acetate to increase ATP yield (Andersson *et al.*, 2007). As a result, the succinate yield and productivity decreased in this mutant strain. Therefore, KJ12202 exhibited a higher concentration of acetate compared with KJ122 and KJ12202 when the strain was grown on 5% xylose.

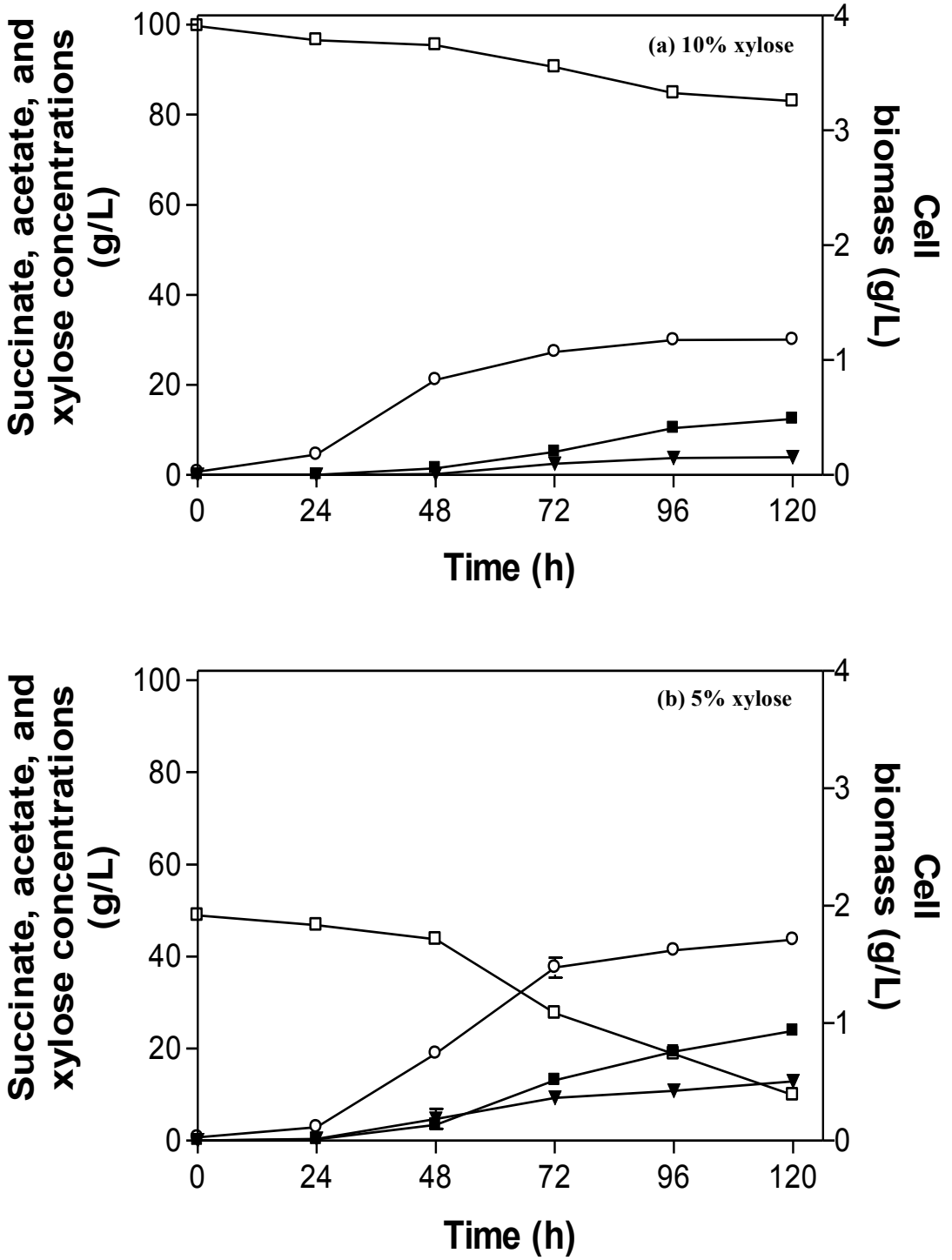

Figure 3.4 Succinate production from xylose by *E.coli* KJ12202 (KJ122 *ΔxylE*) in AM1 medium. (a). Fermentation of 10% xylose; (b). Fermentation of 5% xylose. Symbols for all: xylose (open square), succinate (filled square), acetate (filled triangle), biomass (open circle).

Furthermore, *xylFGH* and *xylE* genes were deleted from KJ122. The deletions of *xylFGH* and *xylE* in KJ122 resulted inhibited poor growth on xylose (Fig.3.5 and Table 3.5). Also, lower cell biomass was observed in KJ12203 (KJ122 Δ*xylFGH* Δ*xylE*). This result indicated that xylose is poorly transported in this mutant strain due to lack of native xylose transporters (*xylFGH* and *xylE*). Utrilla *et al.* (2012) also reported the deletions of *xylFGH* and *xylE* in *E. coli* CL3 (D-lactate producing strains) resulted a 26% reduction in growth rate. Moreover, Khankal *et al.* (2008) revealed that the deletion of *xylG* and *xylE* in *E.coli* W3110 exhibited a decrease in xylose consumption and xylitol titer.

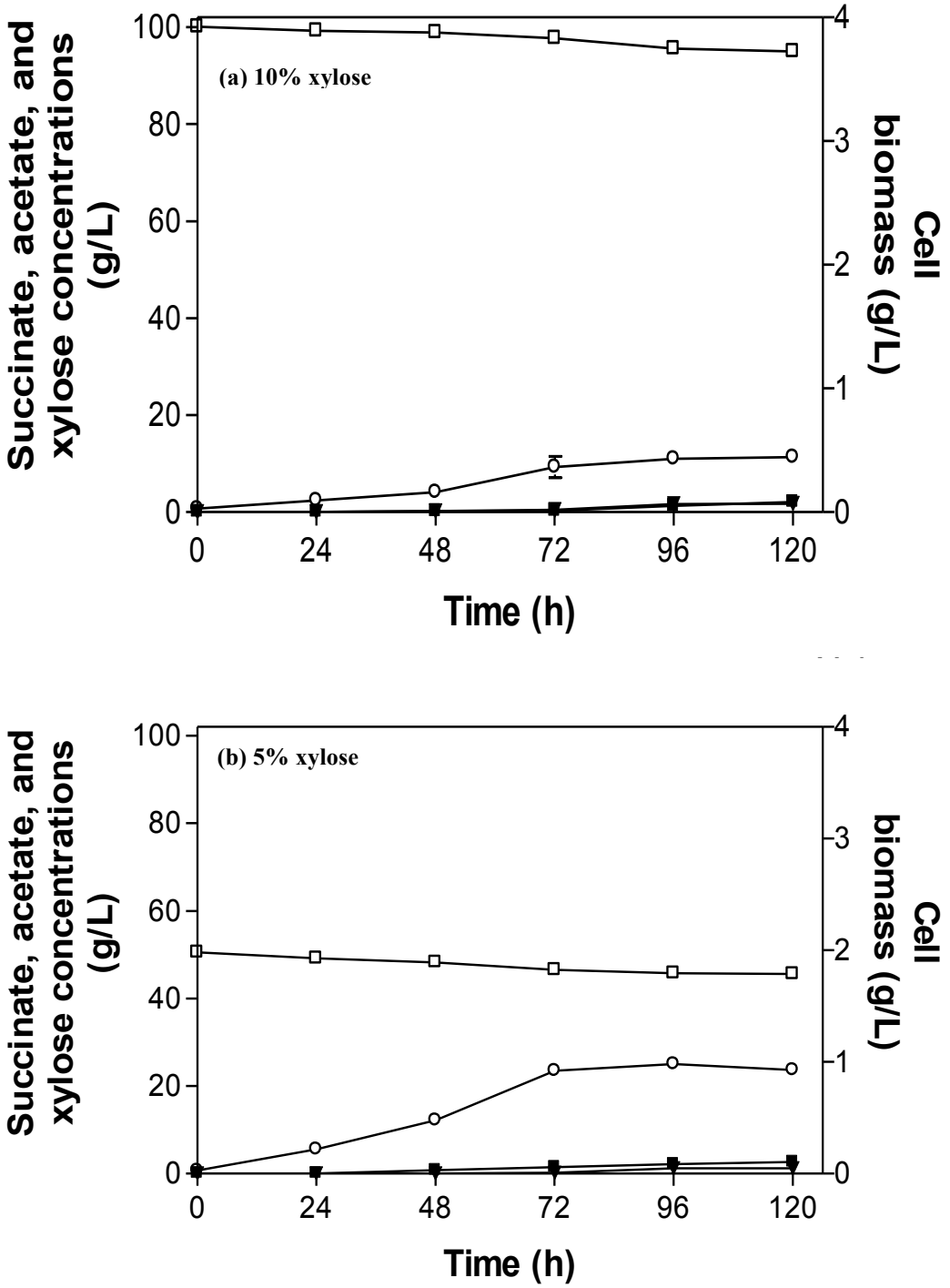

Figure 3.5 Succinate production from xylose by *E.coli* KJ12203 (KJ122 *ΔxylFGH ΔxylE*) in AM1 medium. (a). Fermentation of 10% xylose; (b). Fermentation of 5% xylose. Symbols for all: xylose (open square), succinate (filled square), acetate (filled triangle), biomass (open circle).

Table 3.5 Kinetic parameters of *E. coli* KJ122, KJ12201, KJ12202 and KJ12203 in AM1 medium containing xylose in mini-fermenters.

Strains	**Xylose (%)**	**KJ122**	**KJ12201**	**KJ12202**	**KJ12203**
Maximum CDW (g/L)	5	$1.43\pm0.03^{d,\ \alpha}$	$1.61\pm0.08^{\beta}$	$1.71\pm0.01^{\gamma}$	$0.93\pm0.02^{\delta}$
	10	$1.42\pm0.01^{\alpha}$	$2.66\pm0.19^{\beta}$	$1.18\pm0.01^{\gamma}$	$0.44\pm0.05^{\delta}$
Xylose consumption (g/L)	5	$35.89\pm0.31^{\alpha}$	$48.82\pm0.96^{\beta}$	$39.02\pm0.07^{\gamma}$	$4.93\pm0.70^{\delta}$
	10	$46.00\pm0.38^{\alpha}$	$81.51\pm0.81^{\beta}$	$16.62\pm0.38^{\gamma}$	$5.03\pm0.63^{\delta}$
Succinate (g/L)	5	$25.89\pm0.89^{\alpha}$	$37.43\pm0.67^{\beta}$	$23.80\pm0.59^{\gamma}$	$2.66\pm0.78^{\delta}$
	10	$30.99\pm0.52^{\alpha}$	$70.76\pm3.39^{\beta}$	$11.46\pm0.17^{\gamma}$	$2.06\pm0.10^{\delta}$
Succinate yield[a] (g/g)	5	$0.72\pm0.26^{\alpha}$	$0.77\pm0.02^{\alpha}$	$0.61\pm0.01^{\gamma}$	$0.54\pm0.01^{\gamma}$
	10	$0.67\pm0.02^{\alpha}$	$0.87\pm0.03^{\beta}$	$0.71\pm0.02^{\gamma}$	$0.41\pm0.05^{\delta}$
Succinate productivity[b] (g/L.h)	5	$0.21\pm0.01^{\alpha}$	$0.31\pm0.01^{\beta}$	$0.20\pm0.01^{\gamma}$	$0.02\pm0.00^{\delta}$
	10	$0.26\pm0.01^{\alpha}$	$0.58\pm0.02^{\beta}$	$0.10\pm0.01^{\gamma}$	$0.017\pm0.00^{\delta}$
Specific succinate productivity[c] (g/g.h)	5	$0.16\pm0.00^{\alpha}$	$0.20\pm0.12^{\beta}$	$0.11\pm0.00^{\gamma}$	$0.02\pm0.07^{\delta}$
	10	$0.18\pm0.00^{\alpha}$	$0.19\pm0.01^{\beta}$	$0.09\pm0.00^{\gamma}$	$0.04\pm0.01^{\delta}$

Table 3.5 (continued).

Strains	Xylose (%)	KJ122	KJ12201	KJ12202	KJ12203
Acetate (g/L)	5	$10.57\pm0.42^{\alpha}$	$10.57\pm0.42^{\beta}$	$12.84\pm1.06^{\gamma}$	$1.23\pm0.05^{\delta}$
	10	$8.39\pm0.51^{\alpha}$	$9.34\pm0.74^{\beta}$	$3.97\pm0.12^{\gamma}$	$1.77\pm0.22^{\delta}$

[a]The succinate yield was calculated as gram(s) of succinate produced divided by gram(s) of xylose consumed.

[b]The succinate productivity was calculated as succinate concentration produced divided by overall incubation time.

[c]The succinate specific productivity was calculated as succinate productivity divided by cell dry weight.

[d]Values bearing different Greek symbols are significantly different ($P<0.05$) among strains between column.

3.4.2 Metabolism of xylose in *E. coli* KJ12201

Xylose is abundant in lignocellulosic biomass. The fermentation of xylose is still a key problem in engineered *E.coli* for conversion of xylose to high value chemicals such as succinate. KJ122 has been engineered to produce succinate at high yield and titer from glucose but showed a poor ability to metabolize xylose. The ATP requirement for xylose uptake is a limitation for xylose metabolism in *E. coli* KJ122 when *xylFGH* plays the main role in xylose transport. In this research, we hypothesized that the deletion of *xylFGH* genes could enhance the ATP supply and high succinate production from xylose in the strain KJ122.

Xylose was expected to permeate into cells by a low affinity proton symporter, which was a function of *xylE* with H^+ symport (Fig. 3.6). After xylose uptake into the cell, *xylA* (xylose isomerase) convert xylose to xylulose. Then, xylulose is phosphorylated to xylulose 5-phosphate by *xylB* (xylulokinase). Xylulose 5-phosphate is metabolized by the enzymes of the pentose phosphate pathway (PPP) before entering into the glycolysis pathway as fructose 6-phosphate and glyceraldehyde 3-phosphate. These are all the key enzymes functioning in the mutant strain (KJ12201). In addition, KJ122 is a metabolic engineered strain and it has some mutations of key enzymes such as phosphoenolpyruvate carboxykinase (PCK). PCK was recognized as a vital enzyme for caboxylation and conservation of energy in succinate production pathway (Zhang *et al*., 2009).

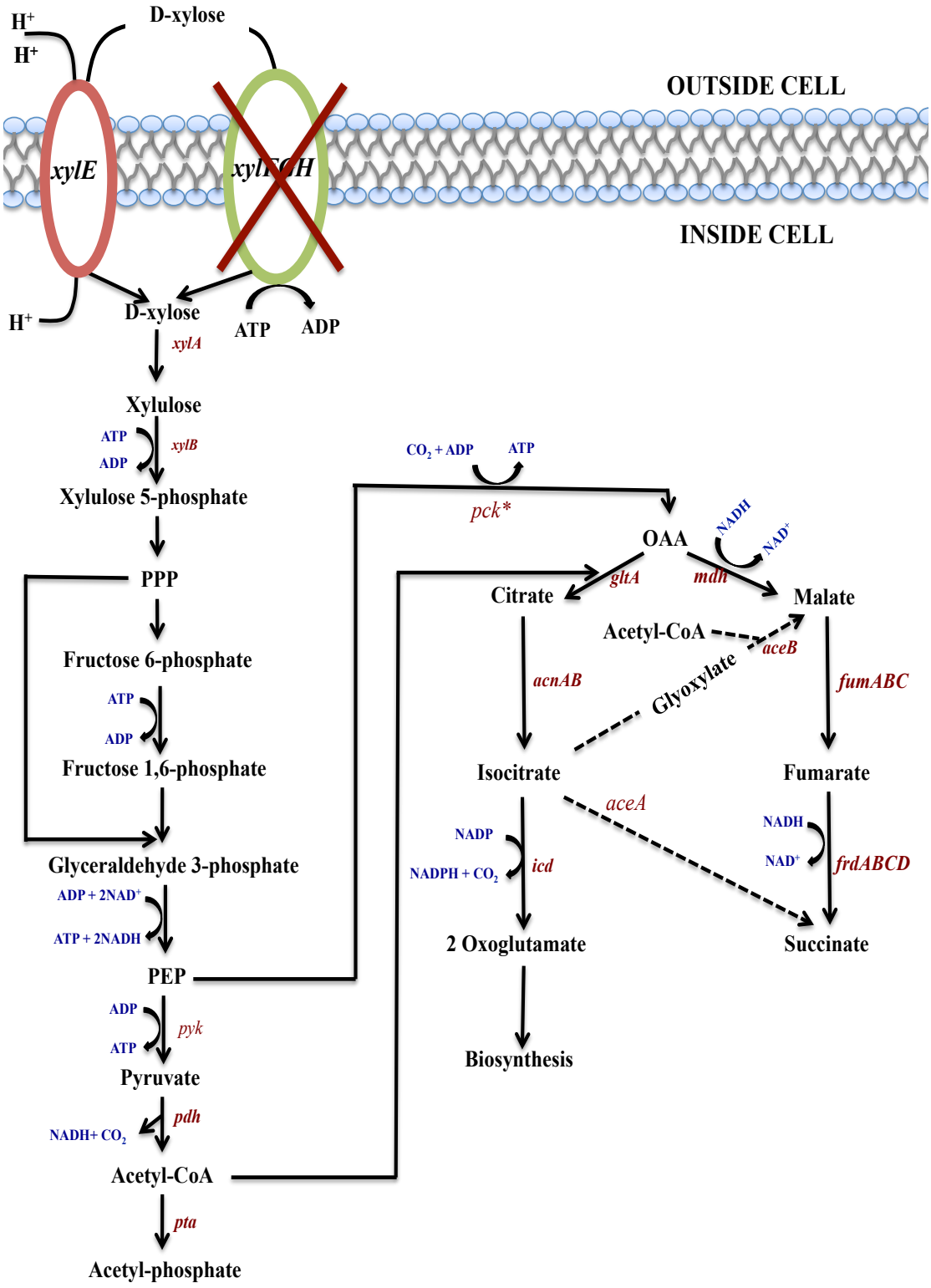

Figure 3.6 Proposed mechanism of xylose metabolism in KJ12201.

3.4.3 Metabolic evolution of *E. coli* KJ12201 to produce *E. coli* KJ12201-14T

Metabolic evolution has been applied to microorganisms for many fermentation products including ethanol (Yomano *et al.*, 2008), 2,3-butanediol (Jantama *et al.*, 2015), D-lactate (Zhou *et al.*, 2003; Utrilla *et al.*, 2012), and succinate (Jantama *et al*., 2008; Sawisit *et al.*, 2015). Under strong pressure such as high temperature, strong and sole substrate limitation, microorganisms have mutation occurred in DNA genes and then selection occurred in the phenotype which naturally change in proteins, especially, substrate specific proteins. This directs the evolution of new function. The target gene of evolution in all variants occurs as point mutations in the DNA sequence changing the function of enzymes. Many studies on the ability of the whole new metabolic pathway have been exposed for metabolic engineering *E. coli* in novel environments. Only microorganisms existing target enzyme can survive and enhance reactions in the new resource. This is a selection of strongly genes within microorganism for survival under strong pressure of selection (Jantama *et al*., 2008). This procedure was applied to *E. coli* KJ12201 for the fermentation of xylose to obtain the strain exhibiting high growth rate and succinate production.

The KJ12201 strain was sub-cultured into a newly fresh AM1 medium containing 10% xylose and transferred till the transfer number 20. The succinate concentration and biomass increased along with increasing in number transfer (Fig. 3.7a and b). During these transfers, accumulation of acetate was still detected (Fig. 3.7c). At the end of the metabolic evolution, the culture of KJ12201 produced higher titer of succinate (83.61 g/L) and improved xylose consumption, while high amount of acetate (11.85 g/L) was also observed. From these results, we decided to select the

best strain for the succinate production. The culture of KJ12201 from the transfer number of 14 was designated *E. coli* KJ12201-14T, and subjected as the representative strain for further experiments. The culture of KJ12201-14T produced succinate titer of 83.66±0.19 g/L (709±1.57 mM), with a succinate yield of 0.86±0.01 g/g (1.31±0.02 mol/mol) based on xylose metabolized. Unlike KJ122, strain KJ12201-14T grew on 10% xylose without a lag phase (Fig. 3.7). The maximum theoretical yield of succinate from xylose and CO_2 (excess) is 1.43 mol per mole xylose based on the following Eq. 3.1:

$$7\ C_5H_{10}O_5 + 5\ CO_2 \rightarrow 10\ C_4H_6O_4 + 5\ H_2O \qquad (3.1)$$

In addition, KJ12201-14T produced higher levels of biomass and improved xylose consumption as compared with KJ122 and KJ12201 (Table 3.6). These results demonstrated that the combination of gene deletions and metabolic evolution resulted in significant changes in xylose metabolism in *E. coli* KJ12201-14T.

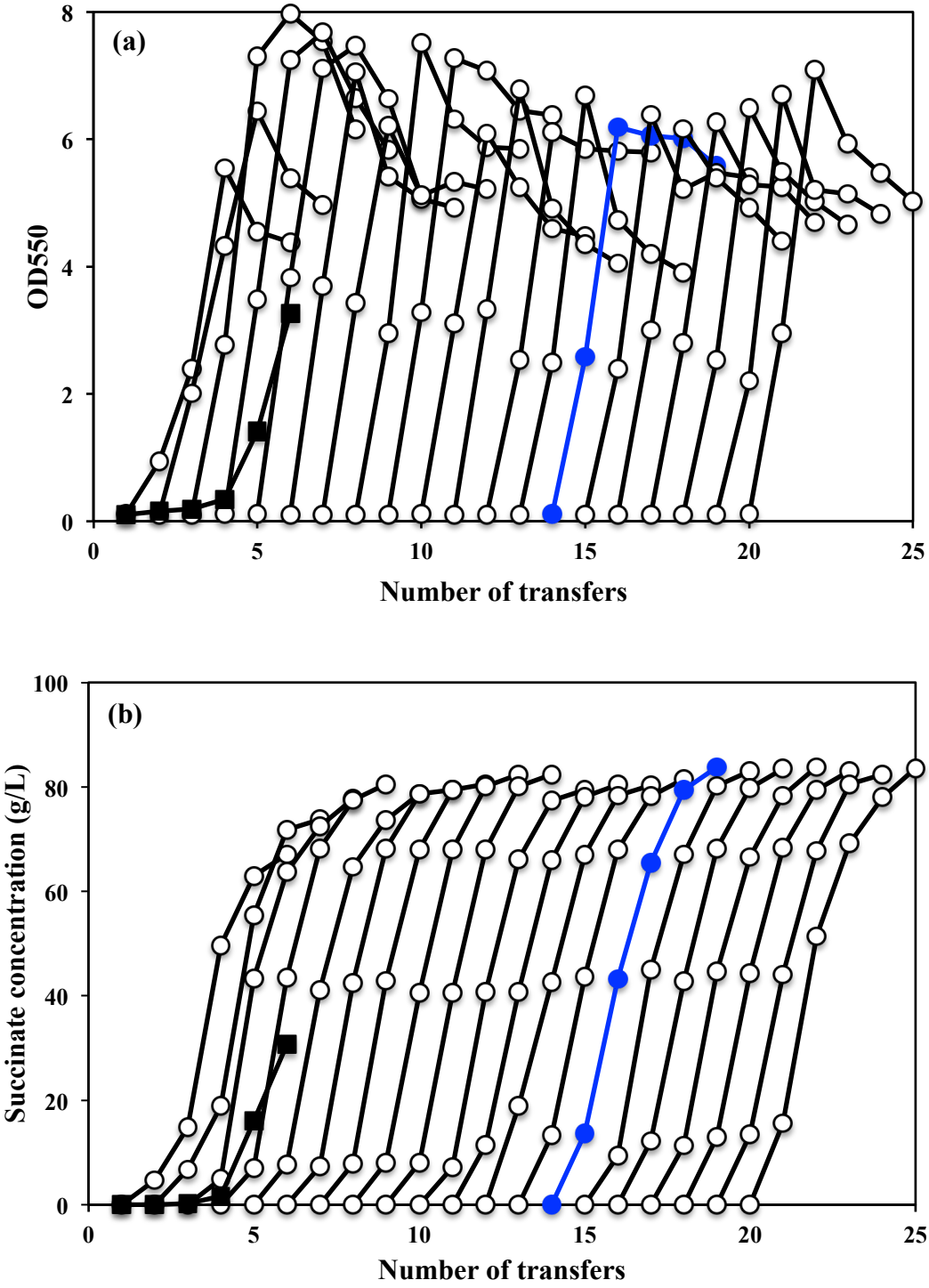

Figure 3.7 Metabolic evolution of *E.coli* KJ12201 in AM1 medium containing 10% xylose. (a). Cell biomass; (b). Succinate; (c). Acetate. Symbols for all: KJ122 (filled square), KJ12201 (open circle), Blue circle and line indicate the source for isolation of KJ12201-14T.

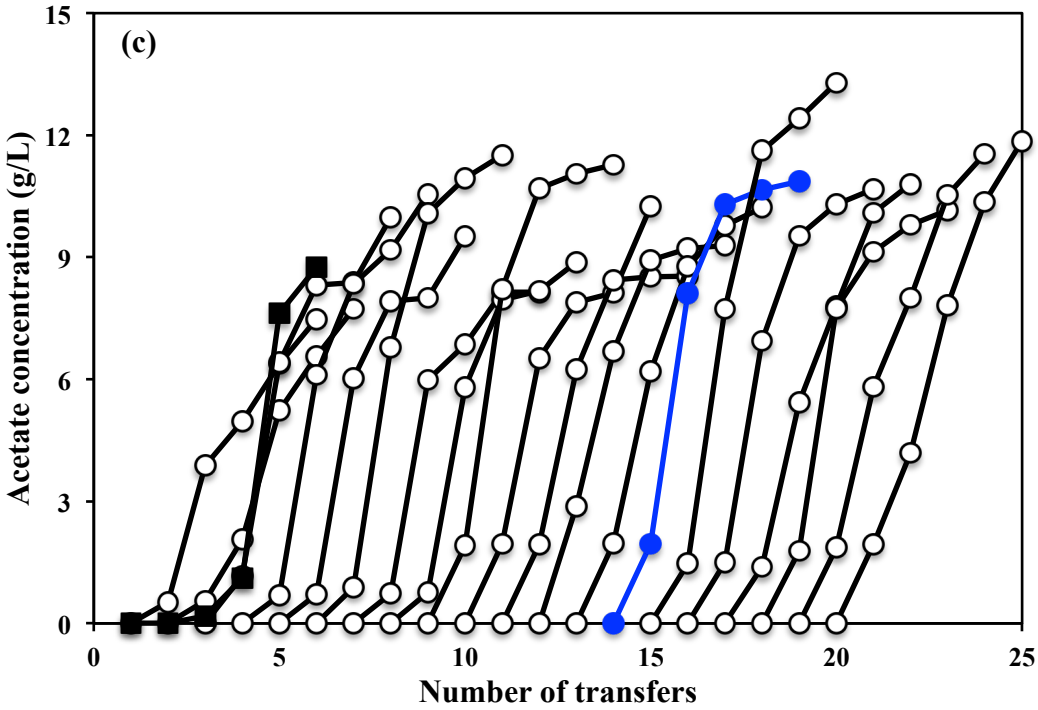

Figure 3.7 (continued).

Table 3.6 Kinetic parameters of *E.coli* KJ122, KJ12201 and KJ12201-14T in AM1 medium containing 10% xylose.

Strains	**KJ122**	**KJ12201**	**KJ12201-14T**
Maximum CDW (g/L)	1.42±0.01[d, α]	2.66±0.19[β]	2.98±0.43[γ]
Xylose consumption (g/L)	46.00±0.38[α]	81.51±0.81[β]	97.36±1.28[γ]
Succinate (g/L)	30.99±0.52[α]	70.76±3.39[β]	83.66±0.19[γ]
Succinate yield[a] (g/g)	0.67±0.02[α]	0.87±0.03[β]	0.86±0.01[β]
Succinate productivity[b] (g/L.h)	0.26±0.01[α]	0.59±0.02[β]	0.70±0.01[γ]
Specific succinate productivity[c] (g/g.h)	0.18±0.00[α]	0.26±0.01[β]	0.32±0.06[β]
Acetate (g/L)	8.46±0.37[α]	9.34±0.74[α]	10.78±0.10[β]

[a]The succinate yield was calculated as gram(s) of succinate produced divided by gram(s) of xylose consumed.

[b]The succinate productivity was calculated as succinate concentration produced divided by overall incubation time.

[c]The succinate specific productivity was calculated as succinate productivity divided by cell dry weight.

[d]Values bearing different Greek symbols are significantly different ($P<0.05$) among strains between column.

3.4.4 Comparison of *E. coli* KJ12201 and KJ12201-14T for succinate production in fed-batch fermentation.

Fed-batch fermentation is a batch process fed continuously or sequentially with substrate. Fed-batch processes were introduced in order to avoid substrate inhibition resulting in high osmotic pressure at high sugar concentration (Ozmichi and Kargi, 2007). Fed-batch operation offers special advantages over batch

and continuous operation by eliminating substrate inhibition as a result of slow feeding of highly concentrated substrate solution. Fermentation is started with relatively low substrate concentration at a low volume. As the substrate is consumed, it is replaced by addition of a concentrated substrate solution at a low rate. In addition, fed-batch process is keeping the substrate concentration in the reaction below the toxic level (Qureshi and Blaschek, 2001).

Fed-batch fermentation for succinate production was carried out in 2L bioreactor with an initial volume of 1.0 L containing 50 g/L xylose. KJ12201 strain was initial grown in batch mode. At the late exponential phase of batch fermentation, 100 ml of 250 g/L xylose was intermittently fed into the bioreactor to maintain xylose concentration in the range of 5-30 g/L. The results showed that the xylose concentration dramatically utilized from 50 g/L to 5 g/L and the biomass was increased (1.51±0.11 g/L) (Fig. 3.8a) during the first 60 h. At the same time, succinate at concentration of 37.23±0.89 g/L was detected. After 60 and 90 h, xylose was fed into the bioreactor. The succinate titer continuously increased up to the concentration of 69.94±0.58 g/L at 120 h while the cell entered the stationary phase (Fig. 3.8a). At the end of fermentation, acetate was observed as a major by-product at the concentration of 18.08±0.79 g/L (Table 3.7). The yield and overall productivity of succinate production were 0.79±0.01 g/g and 0.58±0.01 g/L.h, respectively (Table 3.7). However, the fed-batch cannot greatly enhance succinate production over batch process in the case of KJ12201.

Based on the above results, strain KJ12201 was improved in growth and cell biomass by several sub-culturing under strong pressure conditions or metabolic evolution. The clone passing through metabolic evolution process was

selected and designated *E.coli* KJ12201-14T. Therefore, this strain was also performed in a fed-batch fermentation and compared the results with a KJ12201, on the succinate production, cell growth and xylose metabolism. KJ12201-14T fermented xylose within 84 h (Fig. 3.8b). KJ12201-14T could consume 98.89±0.58 g/L xylose and the highest succinate production reached 84.55±0.67 g/L at 84 h of fermentation time. The succinate yield (0.86±0.01 g/g) and productivity (1.01±0.01 g/L.h) were improved compared with those obtained in KJ12201. Also, the maximum biomass concentration in KJ12201-14T (3.35±0.32 g/L) was higher than that in KJ12201 (1.69±0.22 g/L). However, the succinate specific productivity of KJ12201 (0.39±0.01) and KJ12201-14T (0.39±0.02) were not significantly different (Table 3.7). Furthermore, acetate production was lower in KJ12201-14T (14.41±0.28) as compared with KJ12201 (18.08±0.79). These results showed that KJ12201-14T has an ability to produce succinate from pure xylose compared with other engineered *E. coli* strains (Table 3.8). In conclusion, fed-batch fermentation was applied to improve growth and succinate productivity. Therefore, it would be feasible for industrial succinate production from lignocellulosic biomass employed in the future and could be applied for other similar fermentation processes.

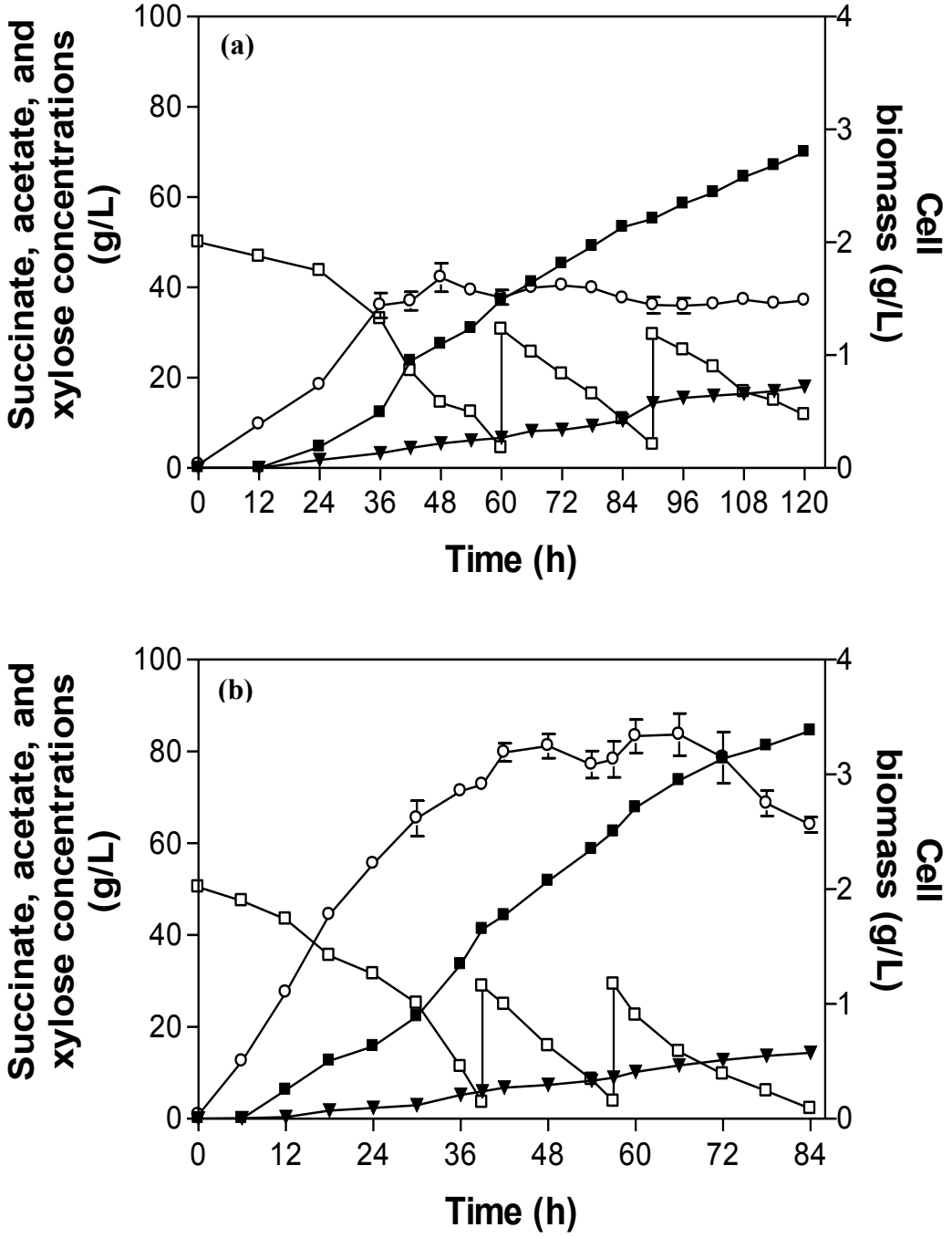

Figure 3.8 Fed-batch fermentation profile of KJ12201 and KJ12201-14T for succinate production in AM1 medium containing xylose. (a). Succinate production by KJ12201; (b). Succinate production by KJ12201-14T. Symbols for all: xylose (open square), succinate (filled square), acetate (filled triangle), biomass (open circle).

Table 3.7 Kinetic parameters of *E.coli* KJ12201 and KJ12201-14T in fed-batch fermentation.

Strains	**KJ12201**	**KJ12201-14T**
Maximum CDW (g/L)	1.69±0.22	3.35±0.32
Xylose consumption (g/L)	89.01±0.94	98.89±0.58
Succinate (g/L)	69.94±0.58	84.55±0.67
Succinate yield[a] (g/g)	0.79±0.01	0.86±0.01
Succinate productivity[b] (g/L.h)	0.58±0.01	1.01±0.01
Specific succinate productivity[c] (g/g.h)	0.39±0.01	0.39±0.02
Acetate (g/L)	18.08±0.79	14.41±0.28

[a]The succinate yield was calculated as gram(s) of succinate produced divided by gram(s) of xylose consumed.

[b]The succinate productivity was calculated as succinate concentration produced divided by overall incubation time.

[c]The succinate specific productivity was calculated as succinate productivity divided by cell dry weight.

Table 3.8 Comparison of succinate production in various media containing pure xylose by engineered *E. coli* strains.

Strains	**Media/Mode of process**	**Succinate**			**References**
		Titer (g /L)	**Yield[a] (g/g)**	**Productivity[b] (g/L.h)**	
KJ12201-14T (*E.coli* KJ122 Δ*xylFGH*), 14th serials transfer in 10% xylose	100 g/L xylose/AM1/batch fermentation 100 g/L xylose/AM1/fed-batch fermentation	83.75 84.35	0.85 0.85	0.70 1.01	This study
AS1600a (*E. coli* KJ122), 16th serials transfer in 10% xylose	100 g/L xylose/AM1/batch fermentation	84.26	0.88	0.70	Sawisit *et al*., 2015
E. coli DC115(*ΔldhA*, *ΔpflB*, *ΔptsG*) selected by the atmospheric and room-temperature plasma mutation system combining with a 15th serials transfer in 1.5% xylose	20 g/L xylose/LB medium supplemented with chemically defined medium/simple bath fermentation	12.10	0.67	0.17	Jiang *et al*., 2014
E. coli BA305 (*ΔldhA*, *ΔpflB*, *Δppc*) and overexpression of ATP-forming (PEPCK)	40 g/L xylose /LB medium supplemented with chemically defined medium/repetitive fermentation	24.00	0.98	Not reported	Liang *et al*., 2014

Table 3.8 (continued).

Strains	Media/Mode of process	Succinate			References
		Titer (g /L)	Yield[a] (g/g)	Productivity[b] (g/L.h)	
E. coli BA305, *ΔldhA*, *ΔpflB*, *Δppc*, *ΔptsG* and overexpression of ATP-forming (PEPCK)	20 g/L xylose/complex medium supplemented with LB/simple batch fermentation	5.20	0.72	0.05	Liu *et al*., 2013
E. coli BA204, *ΔldhA*, *ΔpflB*, *Δppc* and overexpression of ATP-forming (PEPCK)	20 g/ L xylose/complex medium supplemented with LB/dual-phase fermentation	9.58	0.87	0.60	Liu *et al*., 2012
E. coli AFP184, *ΔldhA*, *ΔpflB* and *ΔptsG*	≈100 g/L xylose/complex medium supplemented with 0.4 g/L corn steep liquor (50% solid)/dual-phase fermentation	25.00	0.50	0.78	Andersson *et al*. 2007

[a]The succinate yield was calculated as gram(s) of succinate produced divided by gram(s) of xylose consumed.
[b]The succinate productivity was calculated as succinate concentration produced divided by overall incubation time.

3.5 Discussion

Fermentation of xylose is different from that of glucose because the ATP supply during xylose fermentation is lower than that during glucose fermentation in *E. coli* (Hasona *et al*., 2004). In *E. coli* KJ122, the net yield of ATP per xylose is 0 mol/mol while the net yield of ATP per glucose is 1 mol/mol during anaerobic fermentation. Then, the limitations of xylose metabolism in *E. coli* KJ122 is due to the low ATP supply. An improved strain was obtained by deleting the *xylFGH* genes, designated KJ12201.

The mutant strain significantly improved in xylose consumption and succinate production as results from *xylFGH* deletion. The result suggests that the deletion of *xylFGH* resulted in more ATP supply to xylose metabolism. In addition, we speculate that the mutant strain had increased flux through OAA via PCK activity, resulting in high yield of ATP. In addition, *xylE* gene is positively controlled by cAMP and CRP proteins (Gonzalez *et al*., 2002). Meanwhile, strain KJ12202 showed an increase of cAMP and CRP catabolic repression proteins even when growing on xylose. Therefore, the high xylose consumption resulted in high succinate yield in KJ12202. Despite the major improvements, the succinate productivity using xylose as carbon source was only 80% of the productivity displayed on glucose. Therefore, succinate productivity and growth could be improved by metabolic evolution. The 14^{th} transfer-isolated strain (from 20^{th} generation), named KJ12201-14T, exhibited 2.7 and 1.2-fold improvement in succinate productivity (0.70±0.02 g/L.h) as compared with KJ122 and KJ12201, respectively. This indicated that the spontaneous mutations, which occurred during metabolic evolution, caused the activation of succinate production routes and the increase in biomass yield. Moreover, it is generally known that in the presence of oxygen, the PDH complex oxidatively-decarboxylates pyruvate to acetyl-

CoA with the conservation of reductant as NADH (de Graef *et al*., 1999). Since KJ122 is unable to produce acetyl-CoA due to *pflB* mutation, the PDH activity activated to certain, to compensate the lack of *pflB* activity, even in the absence of oxygen. The NADH produced from PDH activity provided more reducing equivalents to reduce fumarate to succinate resulting in a greater molar succinate yield than 1 mol/mol xylose used. Utrilla *et al.* (2012) also reported the increase in PDH observed in *E. coli* JU15 when cells were cultured on xylose for lactate production. Moreover, they found that the *xylA* and transketolase 2 were overexpressed during xylose fermentation in the mutant strain. However, the slight increase in transketolase 2 has no significant changes in gene expression were observed in the PP pathway for xylose fermentation in engineered *E. coli* strains (Tao *et al*., 2001; Utrilla *et al*., 2012). In addition, Utrilla *et al.* (2012) reported that the mutation in the *gatC* is responsible for xylose transport in *E. coli* JU15. Additionally, Sawisit *et al.* (2015) found that the mutation in *galP* in *E. coli* AS1600a improved xylose metabolism.

3.6 Conclusion

The *E. coli* KJ12201-14T (KJ122 Δ*xylFGH*) strain significantly improved the xylose consumption and succinate production yield (up to 0.85 g/g) compared with *E. coli* KJ122. This mutant also exhibited high succinate productivity (1.01±0.01 g/L.h) during fed-batch fermentation. Succinate production by the KJ12201-14T strain was performed in AM1 medium with pure xylose under aerobic conditions. The use of mineral salts medium offers advantages over complex supplements by reducing the cost of media and product purification. However, optimized parameters affecting succinate biosynthesis by KJ12201-14T need to be further investigated to achieve higher and more efficient strategies for succinate production from lignocellulose hydrolysates on an industrial scale.

3.7 References

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CHAPTER IV

PURIFICATION OF SUCCINATE BY NANOFILTRATION

4.1 Introduction

Current trend is to move from primary carbohydrate resources to more complex ones, like lignocellulosic materials as a bio renewable feedstock, to produce biofuels or chemical building blocks, like organic acids. This evolution requires significant modifications at different stages in the process, including fermentation and downstream processes, among which membrane operation can play an important role (Abels *et al*., 2013). Previous work has also demonstrated that nanofiltration can be used for the purification of lactic acid from fermentation broth (Bouchoux *et al.*, 2005; Umpuch *et al.*, 2010).

Succinic acid is an important building block for the synthesis of high added-value products such as biopolymers, pharmaceuticals or food additives. Currently, succinic acid has been mainly supplied by petrochemical process through the conversion of maleic anhydride. Unlike chemical process, succinic acid fermentation is based on renewable resources. Therefore, the succinic acid fermentation has more benefits over the chemical process (Sauer *et al.*, 2008).

Recently, *E. coli* KJ122 was engineered to ferment pure glucose streams from starch into high titers of succinate (Jantama *et al*., 2008). However, fermentation broth does not only contain succinate but also some impurities like remaining carbon

sources, salts and other organic acids. *E. coli* KJ122 can produce about 0.35-0.7 M succinate from 5%-10% glucose, respectively. Furthermore, 0.05-0.1 M acetate were produced from 5%-10% glucose, respectivly, as a major by-product (Jantama *et al.*, 2008). Thus, the product recovery is an important step in succinate production from fermentation.

In this work, nanofiltration was investigated as a purification method in the production of succinate from fermentation. The experiments were carried out with synthetic fermentation broth of increasing complexity containing, succinate salt and different impurities like salts, glucose or other organic acids. The influence of the operating conditions (pH, pressure) as well as of the fermentation broth composition on the NF performances were studied. The mechanisms governing the transfer of the solutes through the membrane were investigated in order to determine the best conditions for achieving the purification of succinate from fermentation broth. The effectiveness of NF in a diafiltration mode was also demonstrated for the removal of impurities from succinate.

4.2 Objectives

The objective of this work was to investigate the separation and purification of succinate from fermentation broth by using nanofiltration.

1) To understand mechanisms governing the mass transfer of solutes through NF membranes focusing on the role of operating conditions (pH, pressure) as well as the fermemtation broth compositions.

2) To obtain an efficient NF process in the recovery and purification of succinate from fermentation broth.

4.3 Materials and methods

4.3.1 Chemicals and fluids

The neutral solute used in the experiments was glucose from Acros Organics. Three types of potassium salts were used: potassium acetate from VWR chemicals PROLABO®, potassium chloride from Acros Organics and potassium phosphate from Ajax Finechem. One type of sodium salt was sodium chloride from VWR chemicals PROLABO®. Three organic acids were used: succinic acid and pyruvic acid from Alfa Aesar® (Johnson Matthey Company) and acetic acid from Acros Organics. The relevant characteristics of the solutes used in the NF experiments are listed in Table 4.1. These organic compounds were typically representative solutes found in succiniate fermentation broth based on *E.coli* KJ122 strain. As shown in the Table 4.1, the differences of these organic compounds are their sizes as well as their pK_A. The feed concentration of the model solution used was determined in accordance with the final composition of succinic acid fermentation of glucose. The compositions of feed solutions (single and mixed solutions) used for constant feed concentration NF are listed in Table 4.2. The feed composition of synthetic fermentation broth used in diafiltration and concentration mode is presented in Table 4.3. Synthetic broths were prepared in ultra-pure water. The feed compositions indicated above were combined to make synthetic broths of increasing complexity, i.e. single, binary, ternary and quaternary solutions. The composition and concentration of synthetic fermentation broth were formulated on the basis of raw fermentation broth obtained by *E. coli* KJ122 (Jantama *et al.,* 2008). All feed solutions were prepared with ultra-pure water. The pH was adjusted by adding potassium hydroxide (EMSURE®, Merck Millipore).

Table 4.1 Relevant characteristics of the solutes used.

Solutes	M_W (g.mol^{-1})	Diffusion coefficient, D_s (x10^{-9} m^2.s^{-1})	Stokes radius[e], r_s (nm)	pK_A
Glucose	180.16	0.69[a]	0.365	-
Na^+	22.99	1.33[a]	0.184	-
K^+	39.1	1.957[b]	0.124	-
Cl^-	35.45	2.032[b]	0.12	-
Ace^-	59.05	1.295[c]	0.187	4.76
Pyr^-	87.06	1.233[c]	0.198	2.5
Suc^{2-}	116.09	0.94[c]	0.295	4.2 ; 5.6
PO_4^{3-}	94.97	0.99[d]	0.247	2.15 ; 7.09 ; 12.32

[a] see Weast *et al.,* 1986

[b] see Wang *et al*., 2005

[c] see Liu *et al.*, 2004

[d] see Crank, 1976

[e] Calculated from the Stokes-Einsten relation $r_s = k_B T/6\pi\mu_0 D_s$, with k_B=1.3807x10^{-23} J/K, μ_0 = 8.937x10^{-4} Pa.s and T=298.15 K

Table 4.2 Feed composition of single and mixed solutions used for constant feed concentration experiments. K_2Suc: potassium succinate, Na_2Suc: sodium succinate, KPyr: potassium pyruvate, KAce: potassium acetate, KCl: potassium chloride, NaCl: sodium chloride.

Solutions	**Compositions**	**Analytical method**
Single solutions	- 0.1 M glucose, 0.1 to 0.7 M K_2Suc pH 7.0	Refractometer
	- 0.1 M K_2Suc pH 2.2 to 7.6	Refractometer
	- 0.1 M KPyr pH 2.0 and pH 7.0	and
	- 0.1 M KAce pH 2.09 and pH 7.0	Conductimeter
	- 0.1 M Na_2Suc pH 7.0	
	- 0.1 M KCl pH 7.0	
	- 0.1 M NaCl pH 7.0	
Binary solutions	- 0.1 M KAce + 0.7 M K_2Suc pH 4.2 to 7.6	HPLC (Shodex)
	- 0.1 M KAce + 0.1 to 0.7 M K_2Suc pH 7.0	and Conductimeter
	- 0.7 M K_2Suc + 0.1 M KAce pH 7.0 (Dilution factor 1)	
	- 0.35 M K_2Suc+ 0.05 M KAce pH 7.0 (Dilution factor 2)	

Table 4.2 (continued).

Solutions	**Compositions**	**Analytical method**
Binary solutions	- 0.175 M K_2Suc+ 0.025 M KAce pH 7.0 (Dilution factor 4)	HPLC (Shodex) and Conductimeter
	-0.117 M K_2Suc + 0.017 M KAce pH 7.0 (Dilution factor 6)	
	- 0.0875 M K_2Suc + 0.0125 M KAce pH 7.0 (Dilution factor 8)	
	- 0.07 M K_2Suc + 0.01 M KAce pH 7.0 (Dilution factor 10)	Conductimeter and HPLC (Dionex)
	- 0.35 M K_2Suc + 0.065 M KAce pH 7.0	
Ternary solutions	- 0.35 M K_2Suc + 0.065 M KAce 0.027 M glucose pH 7.0	Conductimeter and HPLC (Shodex and Dionex)
	- 0.35 M K_2Suc + 0.065 M KAce + 0.0045 M KCl pH 7.0	
	- 0.35 M K_2Suc + 0.065 M KAce + + 0.017 M K_3PO_4 pH 7.0	

Table 4.2 (continued).

Solutions	Compositions	Analytical method
Quaternary solutions	- 0.35 M K_2Suc + 0.065 M KAce + 0.0045 M KCl + 0.017 M K_3PO_4 + 0.027 M glucose pH 7.0 - 0.175 M K_2Suc + 0.065 M KAce + 0.0045 M KCl + 0.017 M K_3PO_4 + 0.027 M glucose pH 7.0 (Dilution factor 2)	Conductimeter and HPLC (Shodex and Dionex)

Table 4.3 The compositions of synthetic fermentation broth used for diafiltration mode and concentration mode.

Experiments	Compositions	Analytical method
Diafiltration mode	- 0.175 M K_2Suc + 0.065 M KAce + 0.0045 M KCl + 0.017 M K_3PO_4 + 0.027 M glucose pH 7.0 (Dilution factor 2)	Conductimeter and HPLC (Shodex and Dionex)
Concentration mode	- 0.164 M K_2Suc + 0.0008 M KAce + 0.0043 M K_3PO_4 + 0.0054 M glucose pH 7.0	Conductimeter and HPLC (Shodex and Dionex)

4.3.2 Membranes

In this study four membranes were evaluated, three nanofiltration and one reverse osmosis membranes: NF45 (Filmtec, Mineapolis, MN), Desal 5 DK, Desal HL (GE Osmonic, USA) and XLE (Filmtec, Minneapolis, MN). All four membranes are flat sheet composite membranes**.** The selective layer is polyamide. These membranes are negatively charged at pH above 3 for XLE, at pH above 4 for NF45 and Desal HL and at pH above 5.5 for Desal 5 DK. Average molecular weight cut-off of NF45, Desal DK and Desal HL are about 150-300 $g.mol^{-1}$. XLE membrane has an average molecular weight cut-off about ~100 $g.mol^{-1}$. The maximum operating temperatures of the four membranes are 45°C for NF45 and XLE, and 50°C for Desal 5 DK and Desal HL. These characteristics of the membranes are presented in Table 4.4.

Table 4.4 Characteristics of the membranes used.

Parameter	**NF45**	**Desal 5 DK**	**Desal HL**	**XLE**
Manufacturer	Filmtec	GE Osmonic	GE Osmonic	Filmtec
Active layer	Polyamide	Polyamide	Polyamide	Polyamide
Maximum operating pressure (Bar)	55[a]	40[a]	40[b]	41[e]
Maximum operating temperature (°C)	45	50	50	45
pH resistance	2-10[a]	3-10[a]	3-9[b]	2-11[e]
Molecular weight Cut-off ($g.mol^{-1}$)	150-300	150-300	150-300	~100
Isoelectric point (IEP)	4[c]	4[d]	5.5[b]	3[e]
Water flux ($L.m^{-2}.h^{-1}$) at 20 bar and 25°C	46.6[f]	24.0[f]	93.0[f]	44.4[f]

[a] Rasanen *et al*. (2002).

[b]Hussain *et al.* (2007).

[c]Tanninen and Nystrom (2002).

[d]Mullett *et al.* (2014).

[e]Diop *et al.* (2011).

[f]Obtained in this work.

4.3.3 Analytical methods

The analytical methods used in this study are presented in Table 4.2 and Table 4.3. In single solutions, glucose and organic salts concentrations were determined using a refractometer (Atago RX-5000, Tokyo, Japan). Conductimeter (LF318 WTW, Germany) was also used for determining the conductivity of organic salts.

For binary solution (succinate/acetate), the concentration of succinate and acetate were determined by high-performance liquid chromatography (HPLC, Jasco, France) using a Shodex SUGAR SH1011 column (Showa Denko, Kawasaki, Japan). The details of the HPLC conditions are given in Table 4.5. The samples were diluted to 20-50 folds before injection.

Table 4.5 Characteristics of the high-performance liquid chromatography analysis.

HPLC, Jasco	
Column	Shodex SH1011
Temperature	50°C
Mobile phase	H_2SO_4 (10 mM)
Flow rate	1 mL.min^{-1}
Pump	PU2089plus
Auto sampler	AS2055plus
Injection volume	20 µL
Refractive index detector	RI-2031plus

For mixed solutions, HPLC (Jasco, France) was used to determine concentration of uncharged solute (glucose). The charged solutes such as succinate, acetate, chloride, phosphate and potassium concentrations were determined by ion chromatography (IEC, Dionex, France). The characteristics of the ion

chromatography system are listed in Table 4.6. The samples were diluted about 25-200 folds by ultra-pure water before injection.

Table 4.6 Characteristics of the ion chromatography analysis.

Dionex ICS 3000	
Ion type	Anion, cation
Column	IonPac™ AS11, IonPac™ CS12
Temperature	30°C
Mobile phase	NaOH: 5 mM (95%) + 100 mM (5%); $CH_4O_3SO_3$: 20 mM (100%)
Flow rate	1 $mL.min^{-1}$
Pump	GP40
Auto sampler	AS50
Injection volume	25μL
Suppresser	ASRS 4mm (137mA) CSRS 4mm (59mA)
Conductivity detector	CD20

4.3.4 NF experimental set-up

The NF cell was purchased from Osmonics (Sepa ® CF Membrane Cell). As shown in Fig. 4.1, the NF membrane sheet was placed in the NF cell, inside which, a permeate carrier was placed on top of the membrane, feed spacer below the membrane, and O-ring was used to seal the assembly. The active side of the membrane faced the feed spacer. The permeate carrier and the feed spacer were pre-wetted with ultra-pure water and placed in the cell body. The feed, permeate, and concentrate lines were then connected. The surface area of the flat sheet membrane was 138 cm^2.

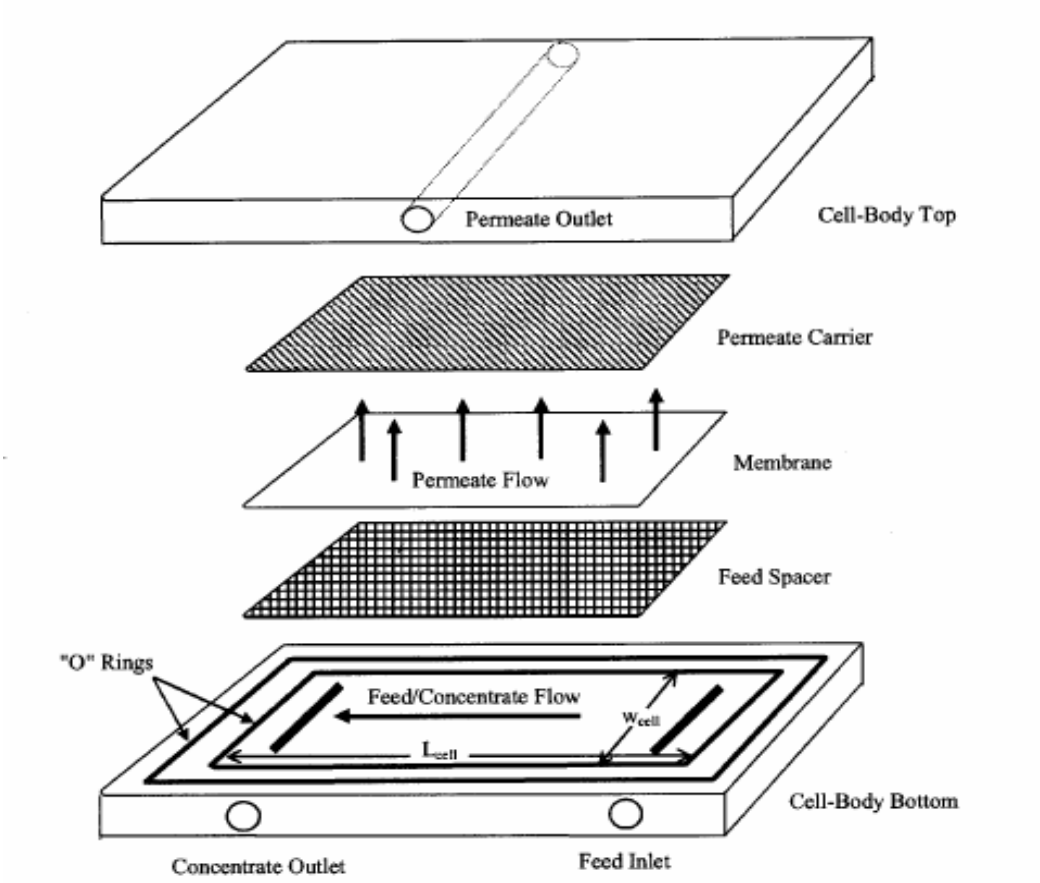


Figure 4.1 Schematic diagram of the membrane cell set up (Falls, 2002).

4.3.5 NF experimental procedure

In this study, nanofiltration system was set up as Fig. 4.2. Synthetic broths were prepared in ultra-pure water. The feed composition indicated above was combined to make synthetic broths of increasing complexity, i.e. single, binary, ternary and quaternary solutions. The composition and concentration of each solution in the synthetic fermentation broth were formulated on the basis of raw fermentation broth obtained by metabolically engineered *E. coli* KJ122 (Jantama *et al.,* 2008). The feed solution was contained in a 5 L feed vessel. The temperature of the feed vessel was controlled at 25 ±0.5^{o}C by using circulating thermostat bath. A high-pressure pump was used to pass the feed solution through the cross flow NF membrane

module. Transmembrane pressure was controlled by a back pressure valve, which was installed on the retentate outlet. The pressure was monitored through two digital manometers located on the inlet and outlet of the membrane module. The feed flow rate was controlled at 400 $L.h^{-1}$. The influence of the pressure 2, 4, 6, 8, 10, 14, 18 and 20 bars and the pH 2 to 7.6 was investigated. After each pressure adjustment, the sample was collected when permeate volume was greater than 20 ml. The samples were weighted to determine the permeate flux and solute concentrations were determined. The conductivity and pH of samples were measured during operation.

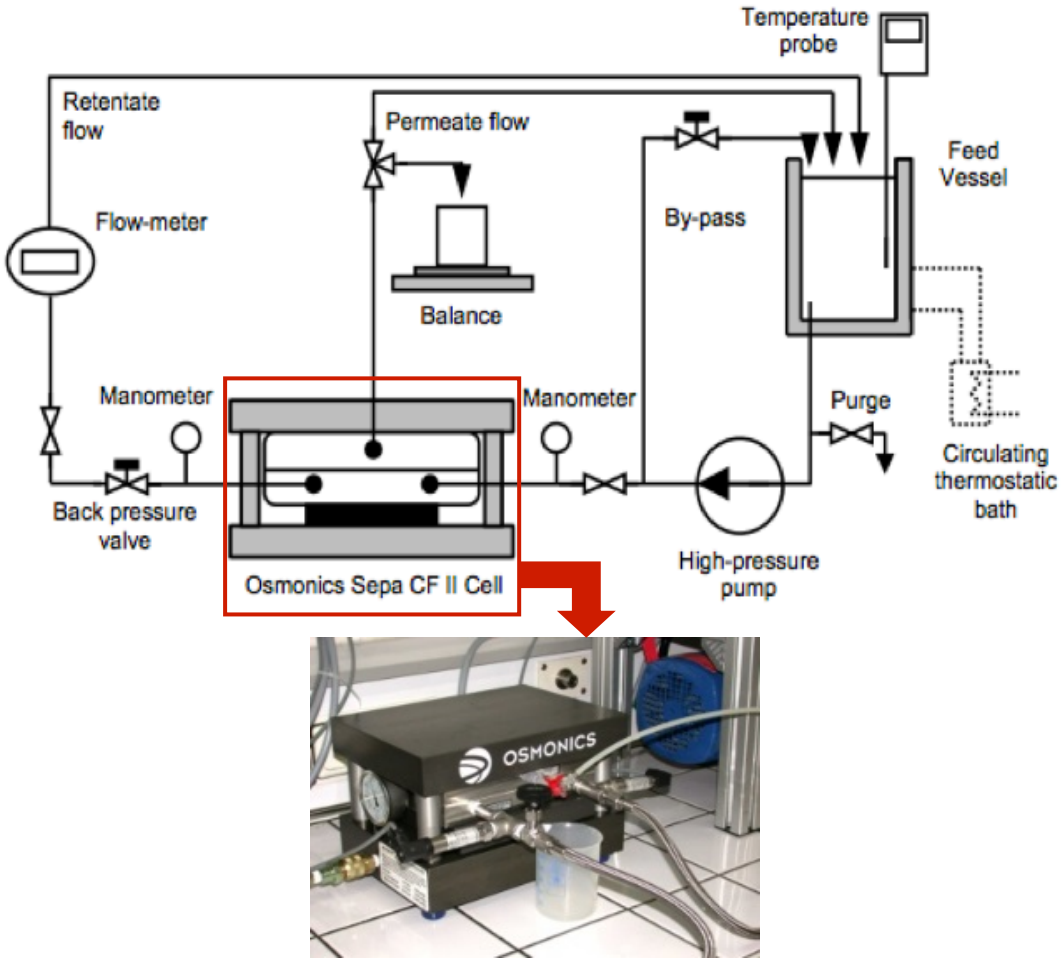


Figure 4.2 Flow schematic diagram of the nanofiltration system (Bouchoux *et al*., 2005).

4.3.6 Membrane pretreatment and cleaning

In this work, the virgin membrane was pre-compacted by ultra-pure water at a constant temperature of 25±0.5°C, flow rate 400 $L.h^{-1}$ and 20 bars until the water permeation flux was constant (1.5 h). At the end of each experiment, the membrane was cleaned by RO water until the conductivity of water in the feed tank was below 20 $\mu S.cm^{-1}$. After that, the membrane was continually cleaned with ultra-pure water until the conductivity of water in the feed tank was below 5 $\mu S.cm^{-1}$. The cleaning steps were operated at 25±0.5°C, 10 bar and flow rate 150 $L.h^{-1}$.

4.3.7 Water permeability

The water permeability is always measured prior to and after each experiment by circulating ultra-pure water in order to monitor a change in the membrane properties during the previous experiment. The mean hydraulic permeability L_{p0} is then calculated from the slope of the plot of J_W versus ΔP, which is in accordance with equation 4.1.

$$L_{p0} = \frac{J_W}{\Delta P} \tag{4.1}$$

where L_{p0} is the water permeability ($L.m^{-2}.h^{-1}.bar^{-1}$), J_w is the water flux ($L.m^{-2}.h^{-1}$) and ΔP is the applied pressure (bar).

The water flux was measured for increasing pressures from 2 to 20 bar. A membrane sample presenting a visible mechanical damage or an abnormally high water flux (more than 20% difference between two consecutive permeability measurements) was replaced by a new one.

The initial water permeabilities of Desal 5 DK, Desal HL and NF45 and XLE membranes are presented in Table 4.7. The initial water permeability was

measured before doing the first experiment. The Desal HL exhibited the highest initial water permeability of 5.0 x $10^{-6}m^3.s^{-1}.m^{-2}.bar^{-1}$ whereas Desal 5 DK exhibited the lowest one of 1.3 x $10^{-6}m^3.s^{-1}.m^{-2}.bar^{-1}$. The initial water permeabilities of NF45 and XLE membranes were about 2.5 x $10^{-6}m^3.s^{-1}.m^{-2}.bar^{-1}$ and 2.5 x $10^{-6}m^3.s^{-1}.m^{-2}.bar^{-1}$, respectively.

Table 4.7 Initial water permeabilities of Desal 5 DK, Desal HL, NF45 and XLE membranes (25 °C and 4-20 bars).

Membrane	**Pure water permeability L_{po} (x $10^{-6}m^3.s^{-1}.m^{-2}.bar^{-1}$)**	**R-squared**
Desal 5 DK	1.3	0.971
Desal HL	5.0	0.956
NF45	2.5	0.989
XLE	2.5	0.998

In addition, Table 4.8 shows the initial hydraulic permeabilities of each piece of NF45 membrane used in this work. One can state that the water permeability varies about 2.5 to 2.8 x $10^{-6}m^3.s^{-1}.m^{-2}.bar^{-1}$ and has average value as 2.6 x $10^{-6}m^3.s^{-1}.m^{-2}.bar^{-1}$. R-square values obtained from the linear plot J_V versus ΔP were higher than 0.989 and had average values as 0.994 ± 0.003. The slight deviation of the initial water permeabilities of each membrane guarantees the repeatability of the experiments carried out with the different membrane samples.

Table 4.8 Initial pure water permeabilities of NF45 membrane samples used in this work (25°C and 4-20 bars).

Number of membrane	**Pure water permeability L_{po} (x 10^{-6} $m^3.s^{-1}.m^{-2}.bar^{-1}$)**	**R-squared**
1	2.5	0.989
2	2.6	0.994
3	2.7	0.998
4	2.8	0.992
5	2.6	0.995
Average	2.6±0.011	0.994±0.003

4.3.8 Retention coefficient

In NF experiments, the observed retention, R_{obs}, of each component is usually defined as:

$$R_{obs} = 1 - \frac{C_p}{C_f} \tag{4.2}$$

where C_p and C_f are the permeate and retentate (or feed) solute concentrations (M) respectively.

The retention coefficient equal to 1 indicates that the solute is totally retained, whereas a value of zero means that the solute is completely transfered through the membrane.

4.3.9 Experimental procedure at constant feed concentration mode

The investigation of the mass transfer has been carried out in constant concentration mode, with both retentate and permeate streams recycled back into the feed tank. Firstly, the feed solution (2 L) was circulated at the flow rate of 400 $L.h^{-1}$ and at applied pressure of 2 bar during 30 min in order to homogenize the feed solution. Then, various pressures have been applied between 2 and 20 bar. For each pressure, a permeate sample was collected and weighed in order to determine the permeation flux as well as the solute concentration in the permeate to calculate the retention of the solutes.

The glucose retention has been also measured in order to check the membrane characteristics. This was systematically performed after the membrane pretreatment step for any new membrane and then randomly once a month with a solution of glucose at a concentration of 0.1 M.

4.3.10 Experimental procedure for the purification step

The purification step of the synthetic fermentation broth was operated using a two-step process. The first one is a nanofiltration step carried out in a diafiltration mode. Diafiltration is a dilution process that involves removal of non-retained impurities and the recovery of retained target species, like succinate based on their size and chrges by using NF membrane in order to obtain pure succinate. In that case, the permeate is not recycled back to the retentate tank and the retentate volume is maintained constant by adding ultra-pure water. The initial retentate volume is fixed at 2L. The diafiltration has been carried out at 20 bar during 26 h. The permeation flux as well as the solutes concentrations were measured every 30 min. The diafiltration mode using the NF membrane has been followed by a concentration

step using a RO membrane in order to increase the succinate concentration in the purified synthetic fermentation broth. In that case, only retentate is recycled back to the feed tank whereas the permeate is collected in the permeate tank. This concentration step has been also carried out at 20 bar using the XLE reverse osmosis membrane. Starting with 2 L of the diafiltrated synthetic fermentation broth, the operation was carried out during 2.5 h. The permeation flux as well as the solutes concentrations were measured every 30 min.

The schematic diagram of diafiltration mode is shown in Fig. 4.3. The mass balance equation can be represented by

$$C_r = C_0 \exp[-V^*(1 - R_{obs})] \tag{4.3}$$

and

$$V^* = \frac{V_p}{V_0} \tag{4.4}$$

where c_0 is the initial concentration of solute (M), c_r is the concentration of solute in retentate (M) and V^* is the number of diavolumes. A diavolume was defined as the total ultra-pure water volume, V_p (L) added during diafiltration divided by the initial retentate volume, V_0 (L).

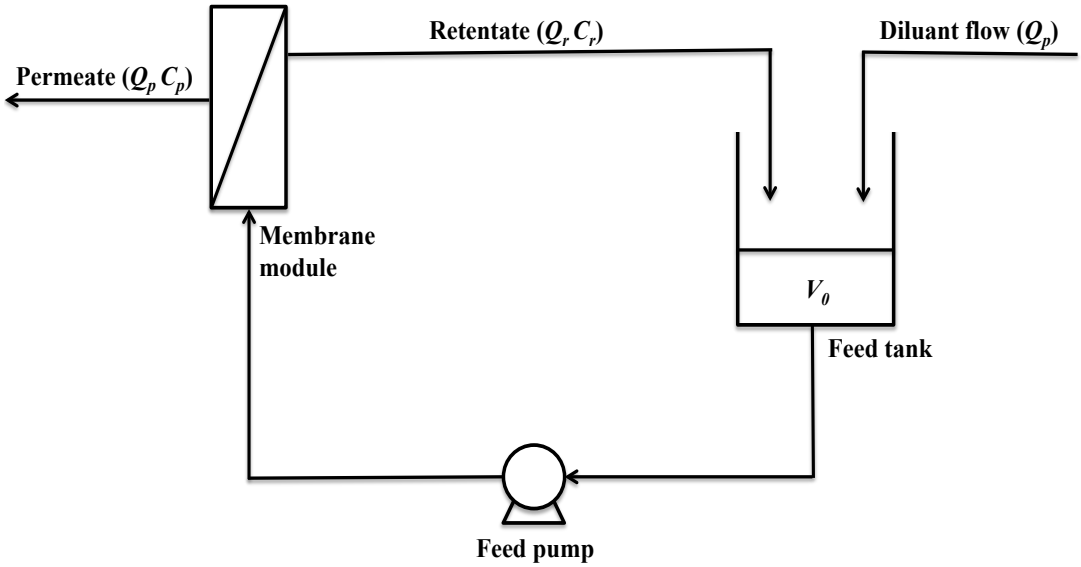


Figure 4.3 Schematic diagram of diafiltration mode.

Additionally, Fig. 4.4 shows the schematic diagram for concentration mode. The mass balance equation can be written in the following way:

$$\left(\frac{V_0}{V_r}\right)^{R_{obs}} = \frac{C_r}{C_0} \tag{4.5}$$

where V_r is the retentate volume (L).

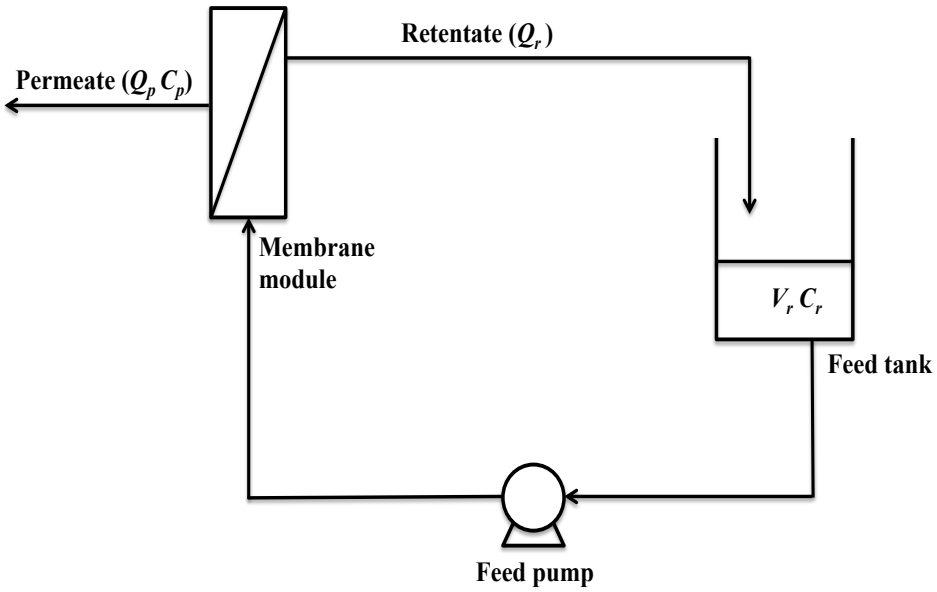


Figure 4.4 Schematic diagram of concentration mode.

4.3.11 Separation factor and purification performances

In order to estimate the succinate/acetate separation efficiency, one can also use the separation factor, SF, which is expressed by the solute concentration ratio in the permeate divided by the concentration ratio in the retentate. The separation factor can be also calculated from the succinate and acetate retentions as:

$$SF = \frac{(C_{Ace}/C_{Suc})_p}{(C_{Ace}/C_{Suc})_r} = \frac{1-R_{obs,Ace}}{1-R_{obs,Suc}} \quad (4.6)$$

SF values higher than 1, like those obtained in this work, mean that the succinate is enriched in the NF retentate, which is the target specie, compared to the feed.

The process performances can be also evaluated according to the succinate yield in the retentate, defined as the succinate concentration in the retentate compared to the feed solution was also estimated:

$$\%\, Yield = \frac{C_{r,Suc}\, V_r}{C_{f,Suc}\, V_f} \times 100 \quad (4.7)$$

where V_f and V_r are the feed and retentate volumes (L), respectively.

Finally, the succinate purity, defined as the ratio of the succinate concentration to the sum of succinate and acetate concentration in the retentate.

$$\%\, Purity = \frac{C_{r,Suc}}{C_{r,Suc}+C_{r,Ace}} \times 100 \quad (4.8)$$

4.4 Results and discussions

Experiments were first carried out with synthetic single-solute solutions as well as binary-solute solutions containing succinate and acetate. The influence of the operating conditions (pH, pressure) as well as the broth composition on the nanofiltration performances were investigated. Then, according to the knowledge of

the mechanisms governing the mass transfer of the solutes through the membranes the best conditions to be used to purify the succinate have been evaluated regarding the purity of succinate.

4.4.1 Preliminary experiments

Many research groups have reported that NF membranes show a higher retention of divalent anions such as succinate than monovalent anions such as acetate. Especially, in the mixed solution with monovalent and divalent anions, some NF membranes showed negative retention of monovalent ions. (Schaep *et al.*, 2001; Kang *et al.*, 2005; Umpuch *et al.*, 2010). Then, nanofiltration could be applied to recover succinate and remove the impurities such as acetate. As succinate is divalent anion having a larger size than acetate, the first objective of this study was to select the suitable NF membranes for the separation of succinate from acetate in the fermentation broth by combination between charge and size effects. In this experiments, three NF membranes, NF45, Desal HL and Desal 5 DK were first chosen for comparing the process performance. All three NF membranes are negatively charged at pH above 4 and average molecular weight cut-off of are about 150-300 g. mol^{-1}. All three NF membranes were tested with uncharged solutions such as glucose for comparing a permeate flux and glucose retention.

In order to compare the permeate flux, glucose retention and succinate retention, three NF membranes, NF45, Desal HL and Desal 5 DK, were tested with pure water, glucose and succinate solutions. Firstly, the experiments with pure water and 0.1 M glucose solution were carried out at various pressure differences. The variations of the permeate flux in water and glucose solutions versus the applied pressure are depicted in Fig. 4.5. As expected, one can observe that all the three

membranes show an increase of the permeate flux for increasing pressures. The permeate flux in presence of glucose increases in the order: Desal HL > NF45 > Desal 5 DK. The permeate flux of Desal HL is higher than that of NF45 and Desal 5 DK.

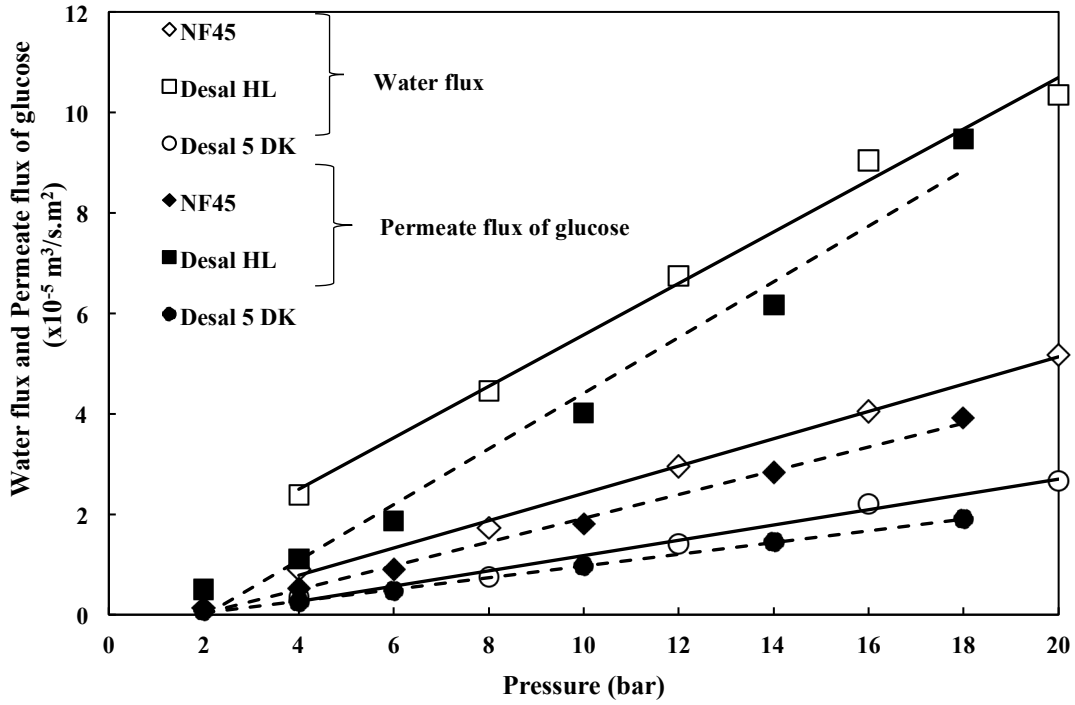


Figure 4.5 Permeate flux for water (empty symbols) and glucose solutions (full symbols) of three NF membranes as a function of the pressure. NF45 (◇,◆), Desal HL (□,■) and Desal 5 DK (○,●).

Then, the glucose retention was also determined. The variations of glucose retentions as a function of the permeate flux for the three NF membranes are shown in Fig. 4.6. As expected, the glucose retention of all three membranes increases with the permeate flux. It is observed that Desal 5 DK and NF45 membranes show a high glucose retention (>90%) for the highest permeate fluxes. Glucose retention by Desal HL is lower than those obtained for the other two membranes. The glucose retention of Desal 5 DK membrane is slightly higher than that of NF45 membrane for

a permeate flux lower than J_v = 1×10^{-5} $m^3.m^{-2}s^{-1}$ (ΔP < 8 bar) and the glucose retention for both membranes showed no difference for higher permeation fluxes (ΔP > 10 bar) (Fig. 4.6).

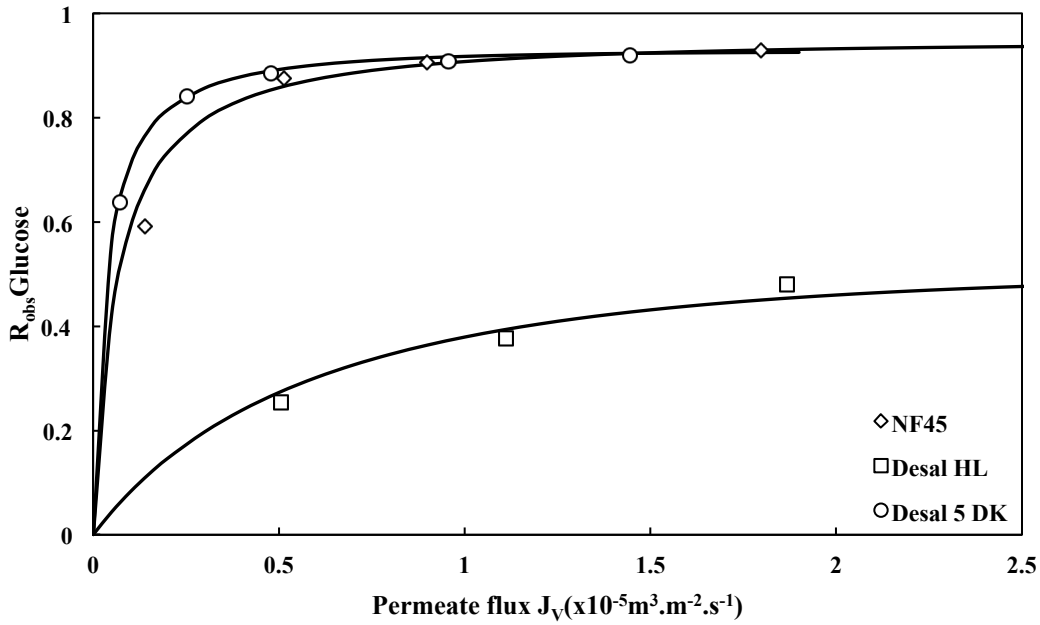


Figure 4.6 Observed retention of glucose as a function of permeate flux - 0.1 M glucose solutions - NF45 (◇), Desal HL (□) and Desal 5 DK (○) membranes. The lines were fitted by Eq. B.1 (See Appendix A).

The retention and permeate flux of a synthetic solution containing 0.7 M succinate was measured with the three NF membranes since previous studies have reported that *E. coli* KJ122 produced about 0.7 M succinate from 10 % glucose. Fig. 4.7 illustrates the variations of the permeate flux versus the operating pressure. As expected, the permeate fluxes of succinate with Desal 5 HL, NF45 and Desal 5 DK increase with the applied pressure. It was also observed that the permeate flux in presence of succinate decreases according to the sequence: Desal HL>NF45>Desal 5

DK. In addition, the permeate flux is lower than that of pure water and glucose solutions because of the higher osmotic pressure in presence of succinate.

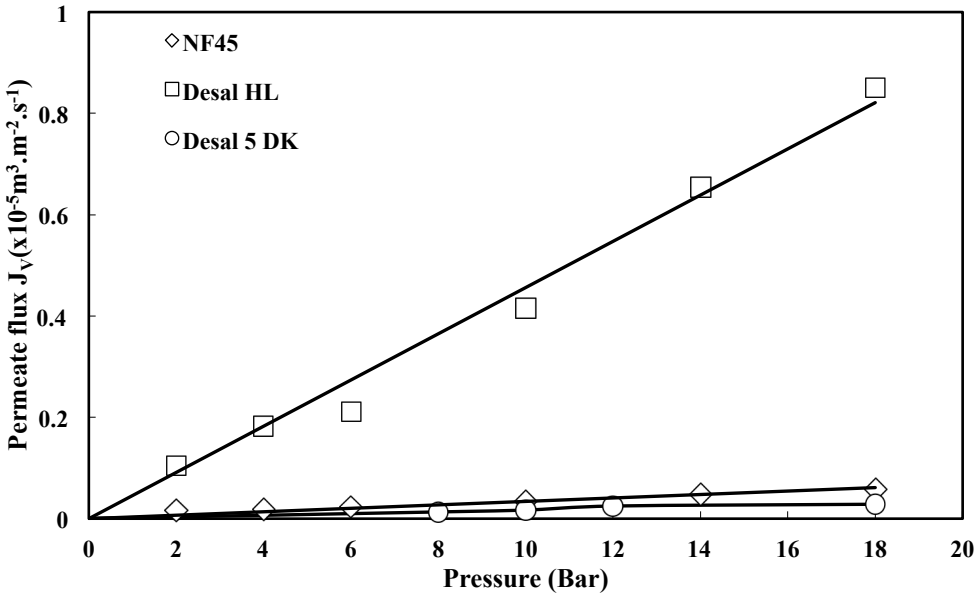


Figure 4.7 Effect of pressure on permeates flux - 0.7 M potassium succinate. NF45(◇), Desal HL(□) and Desal 5 DK(○) membranes at pH 7.

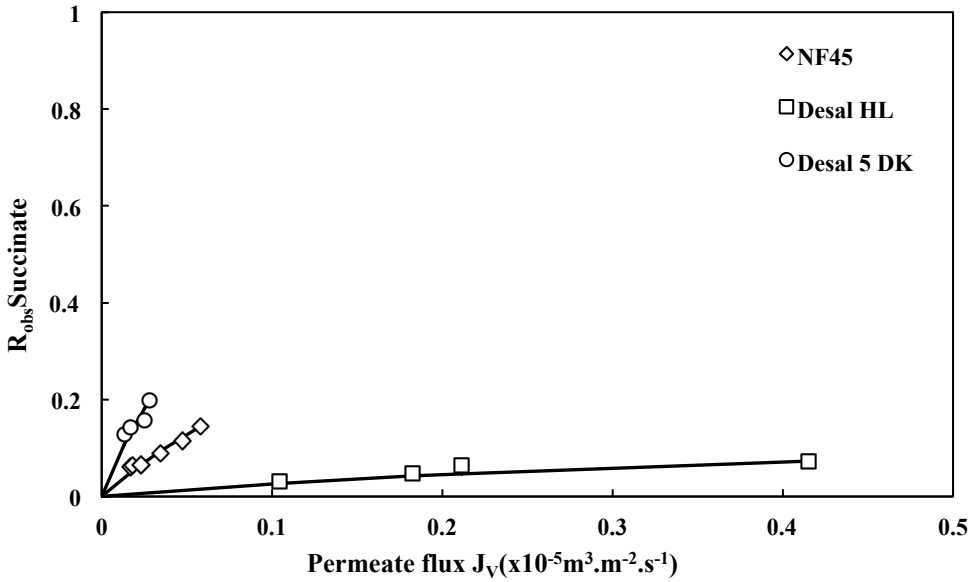


Figure 4.8 Observed retention of succinate as a function of permeate flux - 0.7 M potassium succinate - NF45 (◇), Desal HL (□) and Desal 5 DK (○) membranes at pH 7. The lines were fitted by Eq. A.1 (See Appendix A).

In addition, the retention of succinate for the three membranes increases with the permeate flux. The retention of succinate is lower than 20% among all the three NF membranes. Nevertheless, Desal 5 DK showed a higher retention of succinate than that of NF45 and Desal HL (Fig. 4.8).

Discussion

The permeate flux in presence of glucose is lower than that measured in water due to the osmotic pressure of the glucose solution. In addition, the glucose retention of three membranes was measured. As the retention of uncharged solute such as glucose only results from size effect, glucose is retained because the size of glucose might be much larger than that of membrane pore. In addition, the observation is analogous to the results reported by Umpuch *et al*., (2010), Bouchoux *et al*., (2005), Wang *et al*., (2002a), Wang *et al*., (2005), and Vellenga and Tragardh (1998). Based on the experimental results, it can be concluded that Desal HL membrane is the loosest membrane and Desal 5 DK membrane is the tightest membrane.

Since the succinate is the target product in this work then all three membrances were tested with 0.7 M succinate. The transfer of a charged solute such as succinate depends on the combination of size and charge effects. The retention of a charged solute is high at low concentration due to charge effect. However, it is mainly fixed by its size at high concentration (Kang and Cheng, 2005; Umpuch *et al*., 2010) where charge effects are weak. Therefore, the succinate retention of three membranes was lower than 20% for the three membranes at 0.7 M of succinate.

The aim of this part was to select a NF membrane for the separation of succinate from fermentation broth. All three NF membranes, NF45, Desal HL and

Desal 5 DK were tested with glucose solution. A high-flux membrane with a high glucose retention would be expected to retain succinate while acetate would be removed. Thus, the NF45 membrane was selected for the separation of succinate from fermentation broth because it shows a high retention of glucose with a permeate flux comparable to that of the other two membranes.

4.4.2 Mass transfer in single-solute solution

4.4.2.1 Succinate transfer

This section investigates the mass transfer of succinate through the NF45 membrane. The experiments were carried out with single-solute solutions. The influence of the feed concentration as well as the feed solution pH on succinate retention are discussed. The influence of the counter-ion such as Na^+ and K^+ is also evaluated.

4.4.2.1.1 Influence of the feed concentration

The retention of a charged solute depends on its concentration, and the retention results from the combination of size and charge effects. It was previously shown that the retention of succinate at 0.7 M was low. Therefore, the influence of succinate concentration on its retention by NF45 membrane was investigated.

Firstly, the influence of the succinate concentration on the retention of succinate was investigated at pH 7 which is close to the value of the real fermentation broth. The experiments were carried out with single-solute solutions containing 0.1-0.7 M succinate. The observed retention of succinate versus permeate flux at different concentrations is presented in Fig. 4.9.

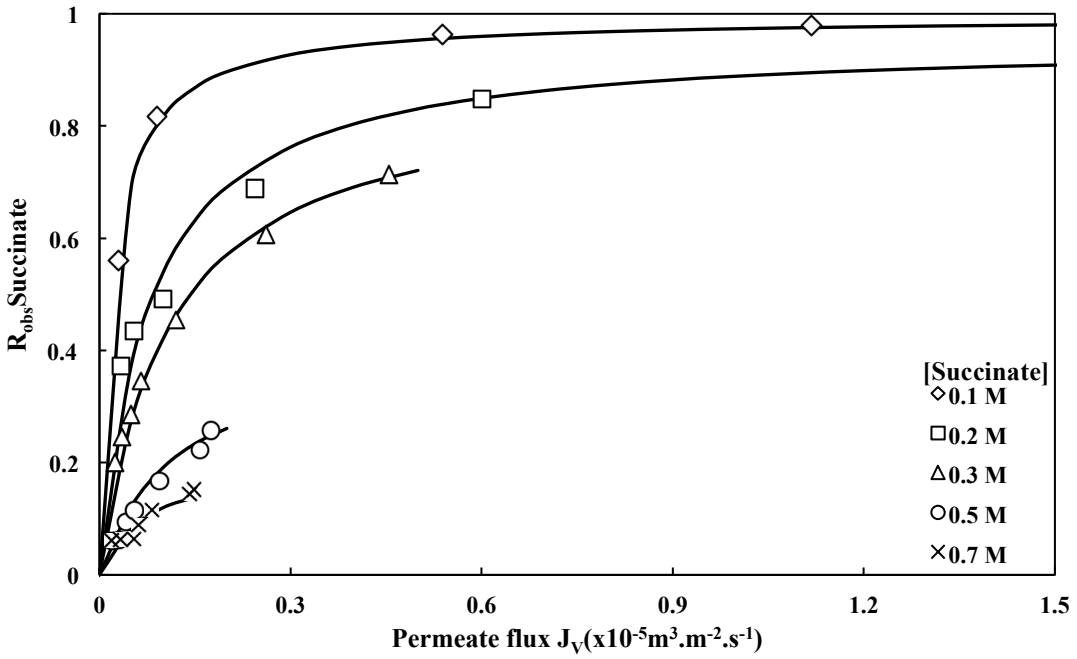


Figure 4.9 Observed retention of succinate as a function of permeate flux - NF45 membrane at pH 7 - influence of succinate concentration: 0.1 M (◇), 0.2 M (□), 0.3 M (△), 0.5 M (○) and 0.7 M (x). The lines were fitted by Eq. A.1 (See Appendix A).

From the results, it is observed that the retention of succinate increases with the permeate flux. The decline of the permeate flux through the membrane at high feed concentration is due to the effect of the osmotic pressure of succinate in feed solution. In addition, a continuous decrease of retention from 96 % to 15 % is observed for increasing concentrations from 0.1 to 0.7 M.

Discussion

Generally, the retention of a charged solute such as succinate is affected by electrostatic interactions and size effect. The succinate retention in single-solute solution is high at low salt concentration since the

electrostatic interactions are dominant. However, the retention decreases for increasing salt concentrations since the electrostatic repulsion become weaker and the retention is mainly governed by size effect. This phenomenon is called the screening effect (Kang *et al.*, 2004). In this case, the negative charge of membrane is screened up by its counter-ions. Then, the repulsion between co-ions and membrane surface becomes small, causing succinate permeate through the membrane more easily resulting in a decrease of the succinate retention. Similar results were also obtained by Labbez *et al*. (2002), Mazzoni *et al*. (2007), Bellona and Drewes, (2005), Kang *et al*. (2004), Bouchoux *et al*. (2005), Kang and Chang, (2005) and Umpuch *et al*. (2010). Furthermore, the retention of succinate at J_V = 0.2 x $10^{-5}m^3.m^{-2}.s^{-1}$ for different feed solutions is shown in Table 4.9. One can observe that at pH 7.0, the succinate retention increases with decreasing succinate concentration due to the strong electrostatic repulsion. However, the low retention observed at low concentration like 0.1 M and pH about 2.2 since the succinate is totally neutral form at pH 2.2 thus the retention is mainly fixed by size effect. Similarly, the transfer of succinate is governed by its size at high succinate concentration. Then, it was indicated that the succinate retention is close to the retention of neutal form (succinic acid) when more concentrated solutions (>0.7 M) are filtrated.

Table 4.9 Retention of succinate at J_V= 0.2 x$10^{-5}$$m^3$.$m^{-2}$.$s^{-1}$ for different feed solutions.

Feed solutions	Retention (%)
0.1 M succinate pH 7.0	90
0.2 M succinate pH 7.0	69
0.3 M succinate pH 7.0	58
0.5 M succinate pH 7.0	26
0.7 M succinate pH 7.0	15
0.1 M succinic acid pH 2.2	5

Conclusion

In this experiment, the influence of the concentration on succinate retention was investigated in single-solute solutions. The retention of succinate depends on its concentration and decreases for increasing concentrations due to the screening effect. Finally, it was concluded that at low succinate concentrations, succinate retention is mainly governed by its charge, while at high concentrations it is mainly governed by its size.

4.4.2.1.2 Influence of the feed pH

It was previously shown that the mass transfer of succinate at low salt concentration depends on the electrostatic interactions. These interactions, which are fixed by the charge of the solute as well as that of the membrane, is thought to be varied according to the pH of the solution. Thus, the influence of the pH is investigated at low concentration (0.1 M) where the charge effects are still involved.

The influence of the pH on the retention of succinate is represented in Fig. 4.10. The retention of succinate increases with the permeate flux for all feed pH. From the results, it is also observed that the retention increases with increasing feed pH. More precisely, at J_V = 4.0 x10^{-5} $m^3.m^{-2}.s^{-1}$, the retention of succinate increases from 28 % to 98 %, when the pH increases from 2.2 to 7.6.

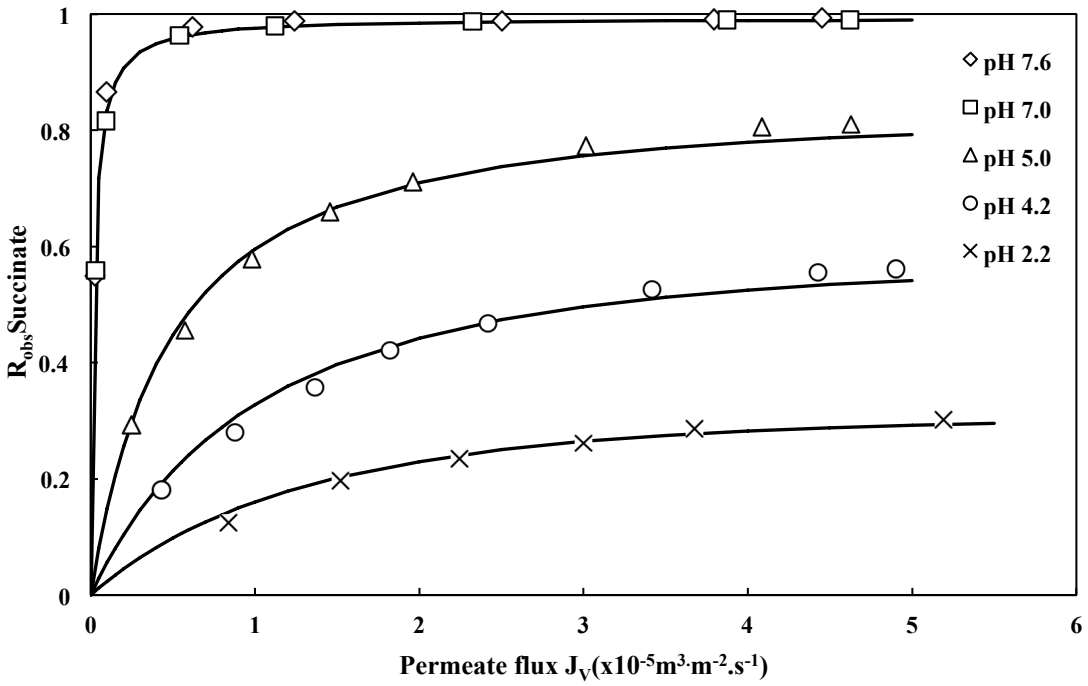


Figure 4.10 Observed retention of succinate and succinic acid as a function of the permeate flux at feed concentration 0.1 M - NF45 - influence of feed pH: pH 7.6 (◇), pH 7.0 (□), pH 5.0 (Δ), pH 4.2 (○) and pH 2.2 (×). The lines were fitted by Eq. A.1 (See Appendix A).

Discussion

The results show that the retention of succinate is strongly affected by the pH. The succinate retention increases with increasing the feed pH. Similar result has been reported by other researchers (Berg *et al.*, 1997; Freger *et al.*, 2000; Prochaska *et al.*, 2014). They found that the retention of acetic acid, lactic

acid, glutamic acid and fumaric acid increases with increasing pH from 3.0-7.0 due to more dissociated form of organic acids as well as more negatively charged membrane surface. Additionally, the high retention of amino acid such as L-glutamate (pK_{A1} = 2.17 and pK_{A2} = 9.13) was observed at pH 9.0 since the amino acid is mainly in a divalent form at pH 9.0 (Li *et al.*, 2003). In these conditions, one can observe that at low concentration, increasing pH resulted in a higher retention because of increasing electrostatic repulsions.

As succinic acid is a dicarboxylic acid, then it can exist in three forms i.e. neutral, monovalent and divalent. As already explained, the ionic fraction of each form can be obtained from the dissociation equilibrium constant. (See Appendix D). Succinate form varies with pH (pK_{A1} = 4.2 and pK_{A2} = 5.6). At pH 2.2, succinic acid is totally neutral. At pH 4.2, succinic acid is shared equally between the neutral and monovalent forms. At pH 5, it is mixed in the three forms, neutral, monovalent and divalent. At pH 5.6, it is a mixing between mono and divalent forms. At pH higher than 7, it is mainly in divalent form.

To demonstrate the role of the pH on the retention of succinate according to the ratio between its neutral and dissociated forms, we report the variation of the succinate retention versus the pH for various permeate fluxes in Fig. 4.11. The low retention observed at pH 2.2 corresponds to the retention of the neutral form (size effect). The retention increases with increasing feed pH from 2.2 to 5.0 since succinic acid becomes more monovalent form and the retention is fixed by a combination between size and charge effects. At pH above 7, the high retention is obtained according to the high fraction of divalent form. Furthermore, Fig. 4.12 shows that the curve representing the variation of the succinate retention versus the pH is a

S-shape curve which is completely similar to the variation of the ionic fraction of the divalent succinate form. From this result, it can be considered that at low succinate concentrations, the retention increases due to the increasing of divalent succinate ions.

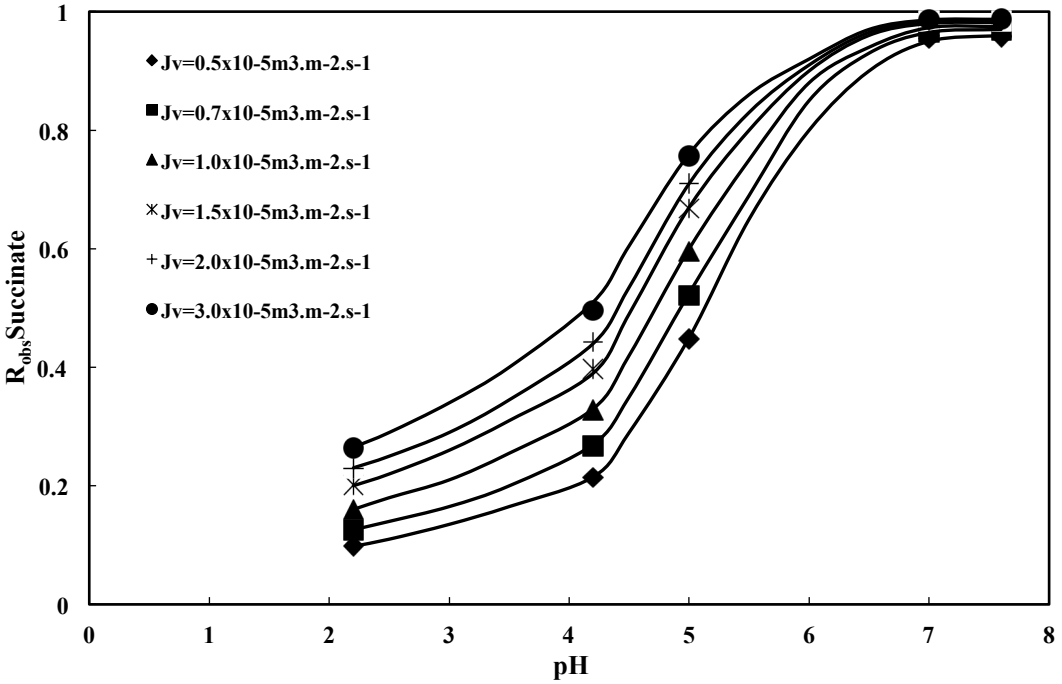


Figure 4.11 Observed retention of succinate as function of feed pH - influence of permeate flux (see legends) - NF45 - feed solutions containing 0.1 M at different pH values (pH 2.2-7.6).

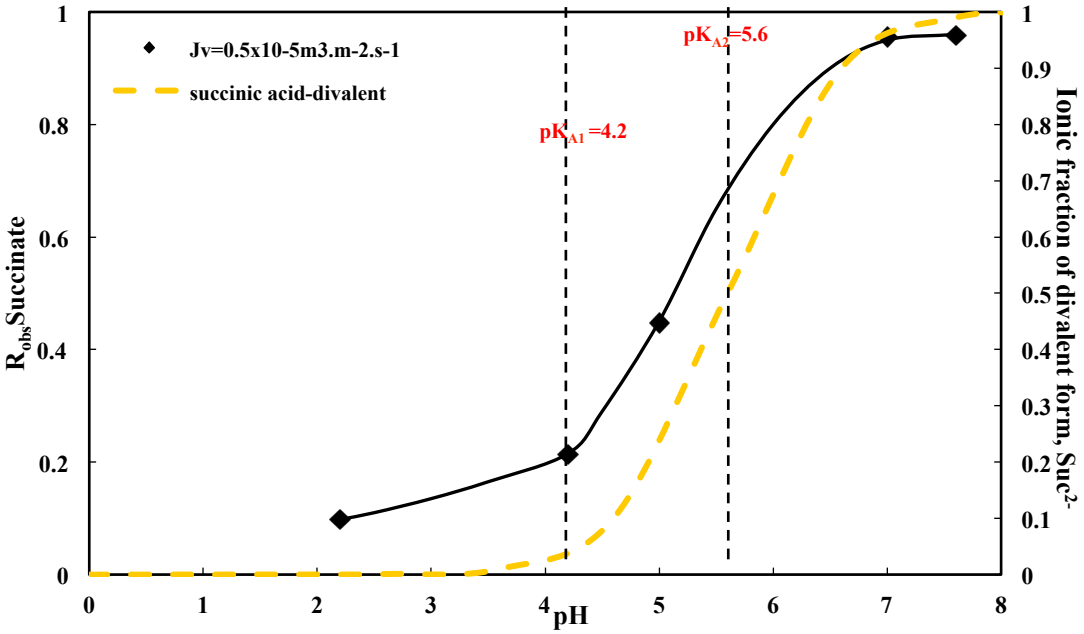


Figure 4.12 Observed succinate retention and ionic fraction of divalent succinate ions as function of feed pH - NF45 - feed solutions containing 0.1 M at different pH values (pH 2.2-7.6).

Conclusion

The influence of pH on the succinate retention was investigated in single-solute solutions. At low concentration (0.1 M), the succinate retention is strongly dependent on the pH. The retention increases with increasing the pH from 2.2 to 7.6 due to the electrostatic repulsions. In addition, a good correlation has been observed between succinate retention and its divalent ionic fraction. Indeed, the retention at high pH is mainly fixed by electrostatic repulsions then the succinate is retained. However, the succinate retention decreases at low pH since the succinate is totally neutral and its transfer is fixed by its size.

4.4.2.1.3 Influence of the counter-ion Na^+/K^+

In this work, succinic acid has been produced through fermentation by *E.coli* KJ122. To ensure the productivity of succinic acid, a suitable pH value for microorganism growth must be maintained at 7.0 by addition of a mixed solution of KOH and K_2CO_3. Thus, the purification process needed to obtain the succinic acid in a suitable form (potassium succinate). Previous work on succinate purification by NF, was made with a sodium succinate salt (Kang and Chang, 2005). Even if sodium ions and potassium ions have the same valence (monovalent cations), they have different size and diffusion coefficient as shown in Table 4.10. Then the influence of Na^+ and K^+ on succinate retention was investigated.

Table 4.10 Diffusion coefficient (D_s) and stokes radius (r_s) of ions.

Ions	Molecular weight (g/mol)	D_s ($10^{-9}m^2/s$)	r_s [c] (nm)	Valence
Na^+	23.0	1.333[a]	0.183	1
K^+	39.1	1.957[a]	0.124	1
Cl^-	35.45	2.032[a]	0.120	1
Suc^{2-}	116.09	0.94[b]	0.295	2

[a] see Wang *et al*., 2005

[b] see Liu *et al.*, 2004

[c] Calculated from the Stokes-Einsten relation $r_s = k_B T/6\pi\mu_0 D_s$, with k_B=1.3807x10^{-23} J/K, μ_0 = 8.937 x10^{-4} Pa.s and T=298.15 K

Fig. 4.13 shows the observed retentions of NF45 membrane in single-solute solutions (NaCl, KCl, Na_2Suc and K_2Suc) as functions of permeate flux, at a concentration of 0.1 M and pH 7.0. It was observed that the retention increased with increasing in permeate flux in all cases. The retentions of K_2Suc and Na_2Suc were

higher than that of KCl and NaCl. In addition, the retention of KCl was slightly lower than NaCl. The retentions of KCl and NaCl were about 58% and 65% at $J_V = 3.5 \times 10^{-5}$ $m^3.m^{-2}.s^{-1}$, respectively. At the same value of $J_V = 3.5 \times 10^{-5}$ $m^3.m^{-2}.s^{-1}$, the retentions of Na_2Suc and K_2Suc were about 99%.

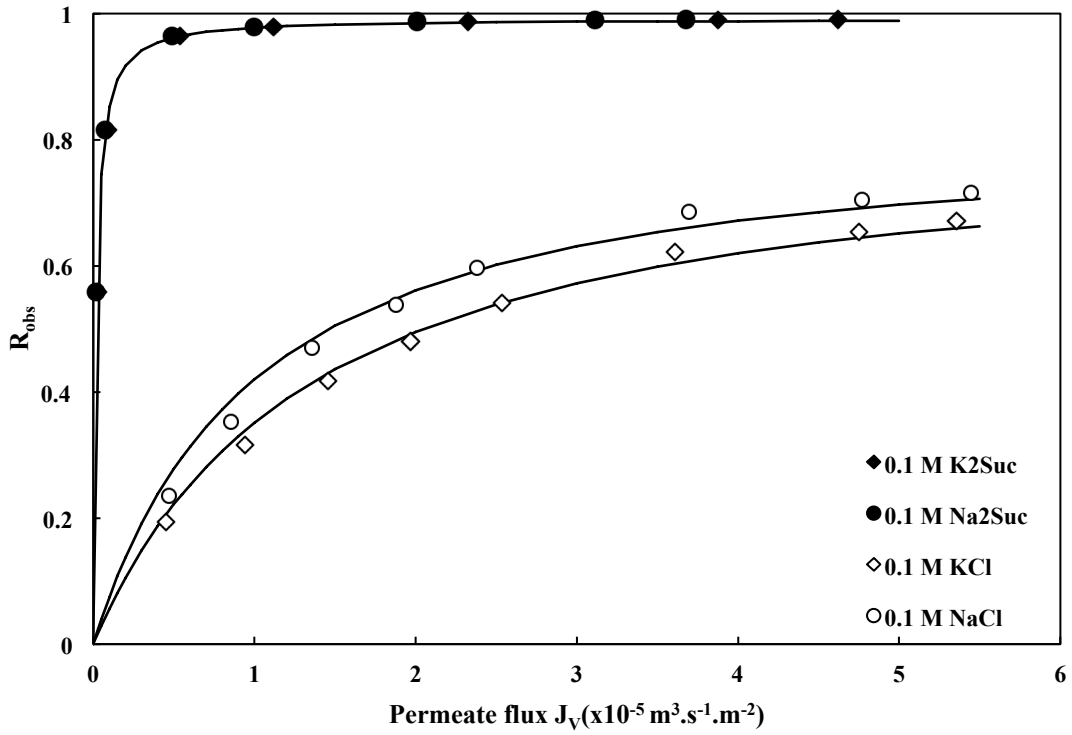


Figure 4.13 Observed retention of the succinate and chloride ions as a function of permeate flux: influence of the cation. 0.1 M K_2Suc (◆), Na_2Suc (●), KCl (◇) and NaCl (○) - NF45 membrane at pH 7.0. The lines were f itted by Eq. A.1 (See Appendix A).

Discussion

According to these results, one can observe that the retentions of Na_2Suc and K_2Suc are similar and higher than 99%. It might be due to the fact that the transfer of succinate is fixed by the fraction of the divalent form at pH 7 as already reported. A high succinate retention could be also affected by

counter-ions. Furthermore, the experiments were carried out with small anions like chloride to evaluate the impact of size and charge of anions on the transfer. It was found that the retention of succinate is higher than that of chloride ($R_{Na2Suc}>R_{NaCl}$ and $R_{K2Suc}>R_{KCl}$) due to the larger size and charge of succinate compared with chloride ions (Table 4.10). Similar results have been previously reported by Schaep *et al.* (1998) and Wang *et al.* (2005). They reported that a larger size and charge solute like sulphate (SO_4^{2-}) is more retained than chloride. Moreover, it was observed that the retention of KCl (K^+) is lower than that of NaCl (Na^+). As K^+ ion has a larger diffusion coefficient and a lower stokes radius than those of Na^+ (Table 4.10). Then, K^+ is less retained than Na^+ and thus results in a lower retention of KCl. In these conditions, one can conclude that the counter-ions like K^+ and Na^+ has influence on the transfer of small size and charge anions like chloride.

Conclusion

Based on the above results, the salt retention sequence for a NF45 membrane was: $R_{K2Suc}\approx R_{Na2Suc}>R_{NaCl}>R_{KCl}$. It can indicate that the retention of low valence and small size such as chloride is dependent on the diffusion coefficient and size of counter ions. On the contrary, the retention of high valence and large size solutes such as succinate is quite independent on the diffusion coefficient and size of counter ions since the retention is fixed by charge and size of succinate. Finally, it can be concluded that Na^+ and K^+ have no significant effect on succinate retention.

4.4.2.2 Transfer of different organic acids

E. coli KJ122 was developed for succinate production from glucose, acetate is produced as a major by-product and pyruvate is a minor by-product (Jantama *et al.*, 2008). pK_A of succinic acid, acetic acid and pyruvic acid are below pH 7 and above pH 2 as shown in Table 4.11. The comparison of succinate, pyruvate and acetate retentions were made at pH 7 and pH about 2. The experiments were carried out at low concentration (0.1M) for which the retention is influenced by the charge.

Fig. 4.14 illustrates the variation of the organic acids retention as function of the permeate flux for the two pH. The results indicate that the organic acids retention at pH 7 is higher than that at pH 2. At pH 7, succinate retention is higher than other two organic salts. The retentions are 97% for succinate, 81% for pyruvate and 80% for acetate at $J_V = 3.0 \times 10^{-5}$ $m^3.m^{-2}.s^{-1}$. At pH about 2, the retention sequence was pyruvic acid >succinic acid >acetic acid.

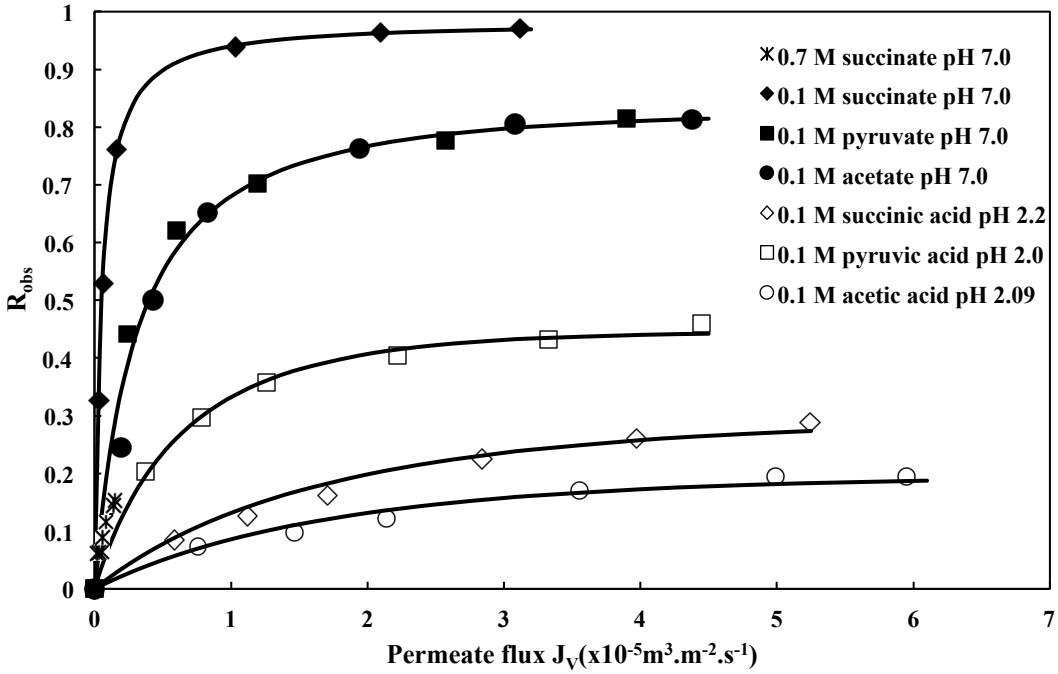


Figure 4.14 Observed retention of organic acids as a function of permeate flux - NF45 membrane - potassium salts; 0.1 M succinate pH 7.0 (◆), 0.1 M pyruvate pH 7.0 (■), 0.1 M acetate pH 7.0 (●), succinic acid pH 2.2 (◇), pyruvic acid pH 2.0 (□), acetic acid pH 2.09 (○) and 0.7 M succinate (x). The lines were fitted by Eq. A.1 (See Appendix A).

Discussion

Since the pK_A of the three organic acids (Table 4.11) are below pH 7, acetic acid and pyruvic acid are in monovalent form and succinic acid is mainly in divalent form, the electrostatic interactions between charged solute and charged membrane are dominant compared with the size effect. Then, the retention of organic salts (dissociated form) is higher than that in neutral form. At pH 7 the succinate retention is higher than the other two organic acid salts as expected from their charges (-2 for succinate and -1 for pyruvate and acetate) and sizes (succinate>pyruvate>acetate) as shown in Table 4.6. This result is similar to those

previously obtained with the same NF membrane (NF45) by Kang and Chang (2005). Since succinic acid and acetic acid are totally in neutral form at pH about 2, their transfer is mainly fixed by their size. Since acetic acid is smaller than succinic acid, its retention is lower. However, the retention of pyruvic acid at pH about 2 is higher than that succinic acid and acetic acid. This phenomenon could be explained by the ionic fraction of pyruvic acid as function of pH. Indeed, based on the pK_A of pyruvic acid (2.5), at pH 2, pyruvic acid is a mixed form between neutral and monovalent (Table 4.12). Thus, at pH 2, the retention of pyruvic acid is affected by its size as well as its charge.

Table 4.11 Characteristics of the solute used.

Organic salts	**Molecular weight (g/mol)**	**pK_A**	**Net charge**	***D_s*** [a] **(10^{-9} m^2/s)**	***r_s*** [b] **(nm)**
Succinate	116.09	pK_{A1} = 4.2 pK_{A2} = 5.6	2^-	0.94	0.295
Pyruvate	87.06	2.5	1^-	1.233	0.198
Acetate	59.05	4.76	1^-	1.295	0.187

[a] see Liu *et al.*, 2004

[b] Calculated from the Stokes-Einsten relation $r_s = k_B T/6\pi\mu_0 D_s$, with k_B=1.3807x10^{-23} J/K, μ_0 = 8.937x10^{-4} Pa.s and T=298.15 K

Table 4.12 The ionic fraction and organic acids retention at $J_V = 1x10^{-5}$ $m^3.m^{-2}.s^{-1}$ at pH 2 and pH 7.

pH	Ionic fraction (%)							Retention (%)		
	H_2Suc	$HSuc^-$	Suc^{2-}	HPyr	Pyr^-	HAce	Ace^-	Suc	Pyr	Ace
2	100	0	0	70	30	100	0	14	33	8
7	0	5	95	0	100	0.5	99.5	94	70	67

Additionally, the retention of the three organic acids is compared at $J_V = 1\ x10^{-5}$ $m^3.m^{-2}.s^{-1}$ for pH 2 and pH 7 as shown in Table 4.12. It was observed that the retention of the impurities such as pyruvate and acetate increases with increasing the pH from 2.0 to 7.0 similar to the retention of succinate since the fraction of monovalent form of both impurities increases. However, the retention of both impurities is not equal to 100 % whilst totally dissociated. It might be due to that pyruvate and acetate have low valence and smaller size than succinate. Then, the transfer of both impurities at pH 7 is not governed by only their charge but also depends on their size. On the other hand, at pH 7, the transfer of succinate is mainly fixed by the fraction of divalent form. The retention of succinate was close to 95%. Those of acetate and pyruvate were lower, 70 and 67 %, respectively.

Conclusion

The retention of succinate and impurities depends on the pH. At pH 2, the low retention of succinic acid and acetic acid are observed since they are in neutral form and the retention is fixed by their size. However, the transfer of pyruvic acid is fixed by combination between its charge and size. Then, the retention of pyruvic acid is higher than that of other two organic acids. At pH 7, the retention of

succinate is higher than that of both impurities due to their charge and size. Then, the separation between succinate and the impurities can be expected if it is carried out at pH 7.

4.4.3 Mass transfer in succinate/acetate mixtures

In the case of succinic acid fermentation from 10% glucose, *E.coli* KJ122 can produce about 0.7 M succinate with about 0.1 M acetate as the main impurity (Jantama *et al*., 2008). The transfer of these two organic acids through the membrane was investigated in order to explain different retentions according to the fermentation broth composition. Therefore, experiments were carried out with binary-solute solutions containing both succinate and acetate at different concentrations to simulate different fermentation broth composition. Also, the influence of pH, concentration ratio and dilution factor of feed solution on the retention of both organic salts was investigated.

4.4.3.1 Influence of the pH

Succinate and acetate have different charge and size as shown in Table 4.11 and the charge of both organic salts depends on the pH (Table 4.12). Then, the ratio of charged/uncharged solute of both acetate and succinate can be estimated by the relationship between pH and the pK_A.

$$\frac{[A^-]}{[\mathrm{HA}]} = 10^{[pH-pK_A]} \tag{4.6}$$

where $[A^-]$ is concentration of charged solute and $[HA]$ is concentration of uncharged solute.

The ratio between charged and uncharged solute is higher than one at pH above pK_A. Then, both acetate and succinate are mainly charged solute

(mono and divalent forms) and thus the transfer of organic salts is governed by their charge at low concentration. Consequently, the high retention is obtained at low concentration increasing pH above pK_A. On the contrary, at pH below pK_A, the ratio between charged/uncharged solute is lower than one. They shared between charged and uncharged solute. Then, the transfer of both acetate and succinate depends on the combination of size effect and electrostatic interaction and resulted in lower retention. However, it could be considered that the influence of pH on the organic acids retention is presented in only single-solute solutions and one knows that it can be different in a mixture. Then, the impact of pH on the transfer of succinate and acetate in binary-solute solutions was investigated. The experiments with binary-solute solutions containing 0.7 M succinate and 0.1 M acetate were performed at different feed pH.

The variations of the succinate and acetate retentions in binary-solute solutions versus the permeate flux at different feed pH (varying between 4.2 to 7.6) are plotted in Fig. 4.15. The results obtained in single solutions at pH 7 are also reported for comparison. The retention of succinate at pH 7.0 is almost the same as in single-solute solution. In contrast, the retention of acetate is lower than that in the single salt solution about 60%. The maximum retention of both organic acids is around 20% in all conditions.

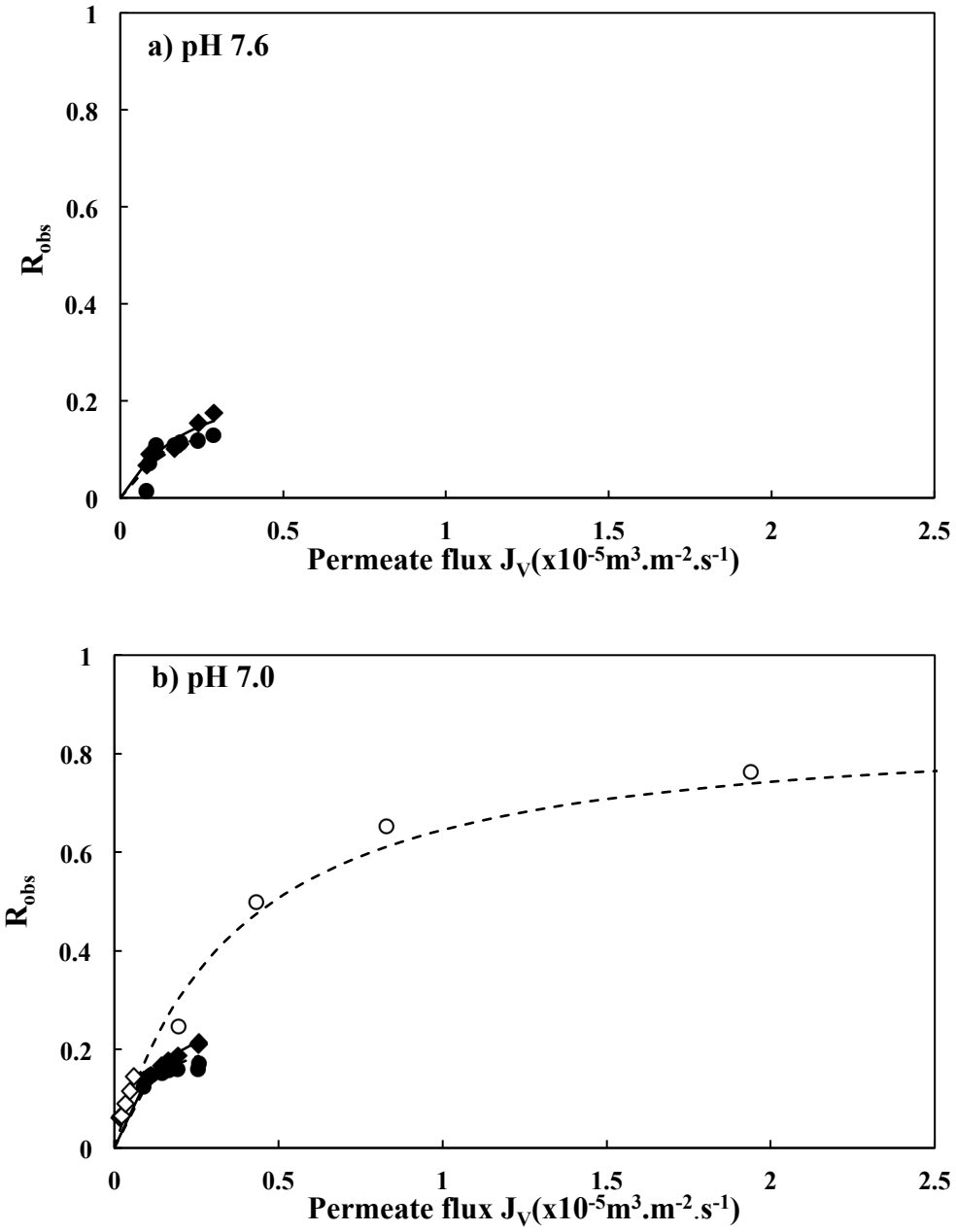
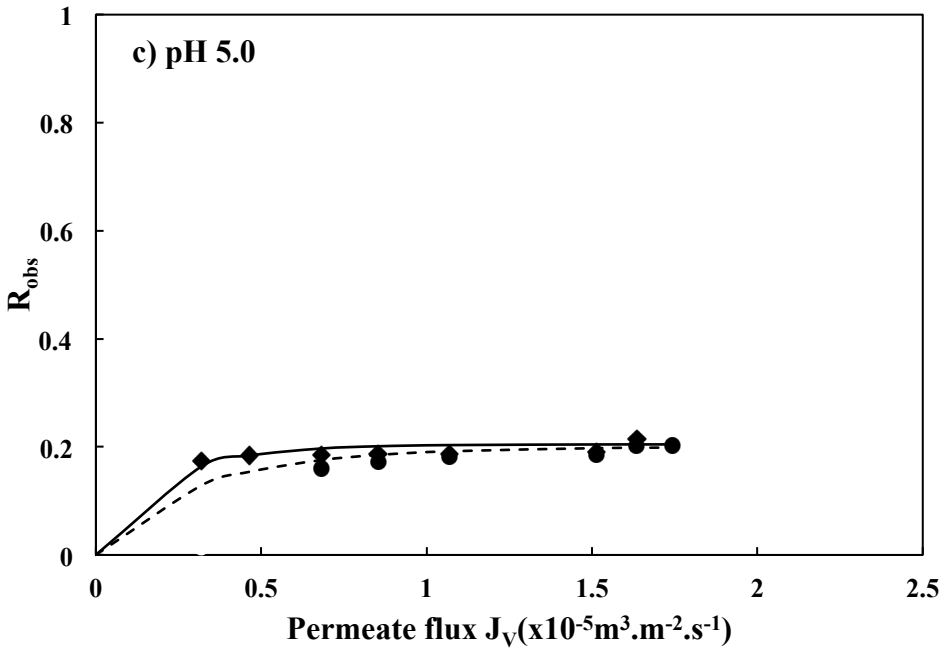


Figure 4.15 Observed retention of succinate and acetate in binary solute solutions versus permeate flux - influence of the pH (0.1 M acetate + 0.7 M succinate). Single solution: succinate (◇), acetate (○) and Binary solution: succinate (◆), acetate (●). The lines were fitted by Eq. A.1 (See Appendix A).



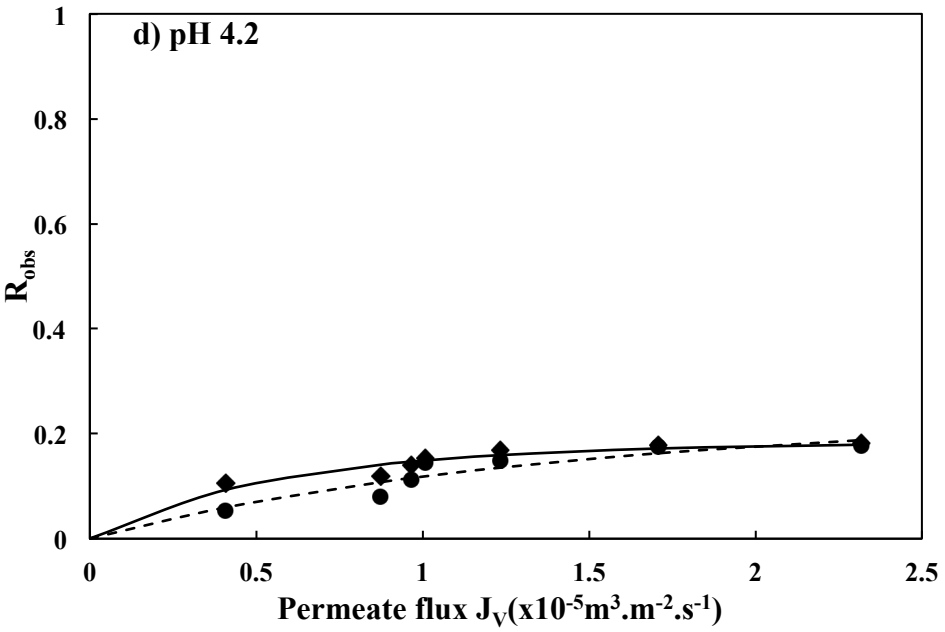


Figure 4.15 (continued).

Discussion

Succinic acid and acetic acid are carboxylic acids. Then, the ionic fraction of both organic acids varies with pH as shown in the Table 4.13. The charge of both organic acids increases with increasing the pH from 4.2 to 7.6. Succinic acid is totally in divalent form and acetic acid is in monovalent form. Then, the separation between succinate and acetate acid is mainly fixed by electrostatic repulsion at high pH and low concentration. However, at pH 7, the low retention of succinate is observed at high concentration (0.7 M) because of the screening effect that makes the electrostatic repulsions weaker as previously explained. The decrease in the retention of acetate in presence of high concentration of succinate is also due to the screening effect. Therefore, the retention of succinate is not different from acetate in all cases. Consequently, no separation is obtained. One can observe that the charges increase for increasing pH have no significant effect on separation since the transfer of both organic acids is governed by only their size due to the high salts concentration.

Table 4.13 Ionic fraction of each solute and each form for various pH investigated.

pH	Ionic fraction (%)				
	HAce	**Ace^-**	**H_2Suc**	**$HSuc^-$**	**Suc^{2-}**
4.2	75	25	50	50	0
5.0	40	60	15	70	15
7.0	0.5	99.5	0	5	95
7.6	0	100	0	1	99

Conclusion

The influence of the pH on the transfer of succinate and acetate is investigated in binary-solute solutions containing 0.7 M succinate and 0.1 M acetate. The results showed that the separation between succinate and acetate is not achievable

whatever the pH because of the strong decrease of acetate retention compared with that observed in single solution. Indeed, it was demonstrated that at a low concentration like 0.1 M, the retention of acetate is high and fixed by electrostatic replusions. But, in presence of a high succinate concentration, the electrostatic repulsions become weaker and thus gives a low acetate retention. Consequently, the retentions of succinate and acetate are not different in binary-solute solutions. It is also shown that the pH has no significant influence on the separation between succinate and acetate at such a high concentration.

4.4.3.2 Influence of the concentration ratio

Based on the above results with high salts concentrations, the retention of succinate and acetate was low and no separation can be expected due to negligible electrostatic repulsions (Fig. 4.15). As we know that electrostatic interactions are dominant at low succinate concentration and pH 7, then the retention is mainly fixed by charge effect. Since succinate is more retained than acetate at low concentration (Fig. 4.14), one can expect a separation by decreasing the succinate concentration. Therefore, experiments were conducted to investigate the influence of the concentration ratio between succinate and acetate in the feed on the retention. Decreasing concentrations of succinate from 0.7 M to 0.1 M were used for a constant concentration of acetate, 0.1 M.

The variations of the retention of succinate and acetate versus the permeate flux are plotted for different concentration ratios (succinate to acetate) in Fig. 4.16. As expected, the permeate flux increases with decreasing succinate concentrations. Moreover, it was observed that the retentions of succinate and acetate increase for decreasing concentration ratios from 7 to 1.

As previously discussed, Fig. 4.16a shows that the retentions of succinate and acetate in binary-solute solution that contains 0.7 M succinate and 0.1 M acetate are too close and lower than 20%. The retention of succinate in binary-solute solution is not different from that found in single-solute solution. On the other hand, the retention of acetate is lower than that in single-solute solution. Fig. 4.16b shows the variation of succinate and acetate in binary-solute solution containing 0.5 M succinate and 0.1 M acetate. The retention of succinate in binary-solute solution is similar to that observed in single-solute solution. However, the retention of acetate is much lower than that in single-solute solution. Moreover, negative retentions are observed. Fig. 4.16c illustrates the retention of succinate and acetate in binary-solute solution that contains 0.3 M succinate and 0.1 M acetate. Both retentions are lower than those observed in single-solute solution. However, the retention of succinate is higher than that of acetate. Finally, Fig. 4.16d shows that the retention of succinate is much higher than acetate in binary-solute solution that contains the mixture of 0.1 M succinate and 0.1 M acetate. The retentions of each organic acid are not different from that in single-solute solution.

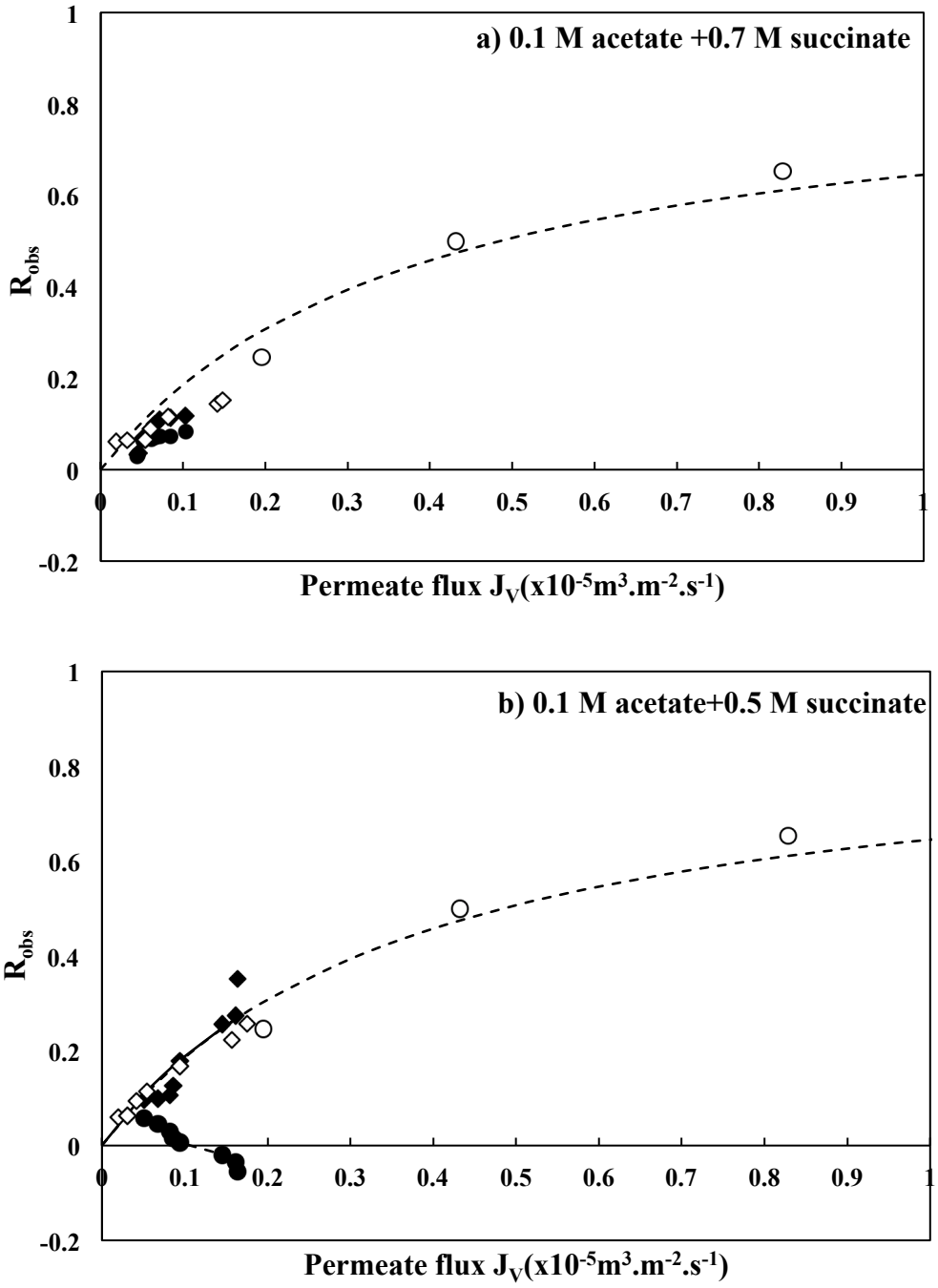


Figure 4.16 Observed retention of succinate and acetate versus permeate flux - feed pH 7, 0.1 M acetate and 0.1 to 0.7 M succinate - NF45 membrane - influence of the concentration ratio. Single solution: succinate (◇), acetate (○). Binary solution: succinate (◆), acetate (●). The lines were fitted by Eq. A.1 (See Appendix A).

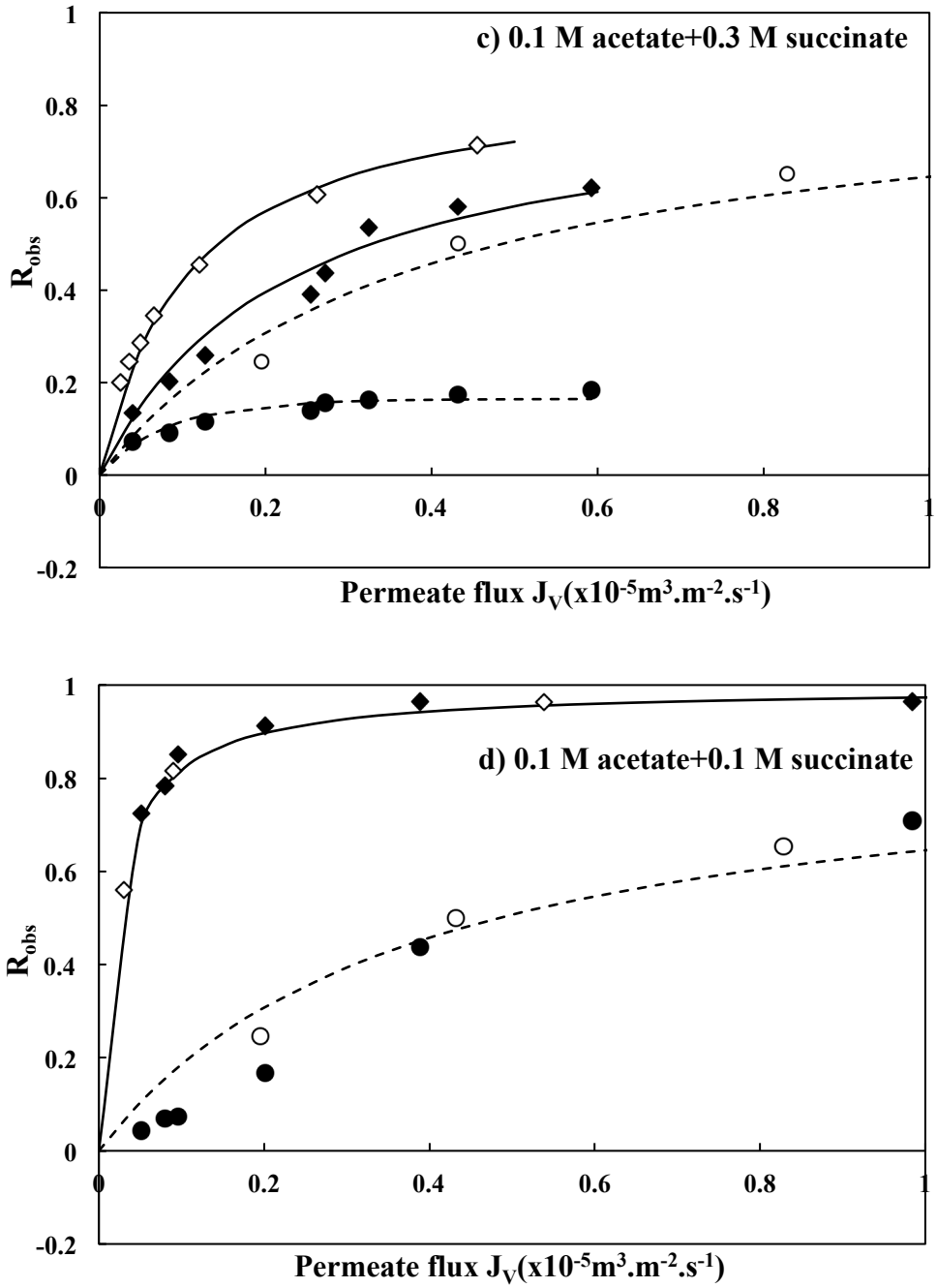


Figure 4.16 (continued).

Discussion

The results show that the retention of succinate increases for decreasing succinate concentrations. This increase is due to the strong electrostatic interaction between the organic acid salts and the membrane. Indeed, the transfer of succinate is governed by its charge at low salts concentration thus high succinate retention is obtained. On the other hand, at high salts concentration, the succinate retention is low since the transfer of succinate is mainly governed by its size (Section 4.4.2.1.1). Then, decreasing of succinate concentration in binary-solute solutions results in a higher retention because of the electrostatic repulsions become stronger. As shown in the Fig. 4.16, the succinate retentions in binary-solute solutions are almost similar to that observed in single-solute solutions due to the succinate retention is mainly fixed by its concentration and to the low acetate concentration. However, in binary mixture that contains 0.3 M succinate and 0.1 M acetate, the succinate retention was slightly lower than that observed in single-solute solution because the effect due to the additional concentration of acetate is no more negligible.

The variations of the acetate retention versus the permeate flux for various concentrations of succinate are depicted in Fig.4.17. The retention of acetate decreases for increasing succinate concentrations. As previously mentioned, at high succinate concentration, the electrostatic repulsion become weaker and the transfer of solute is fixed by their size. Then, the acetate retentions were lower than those observed in single-solute solutions.

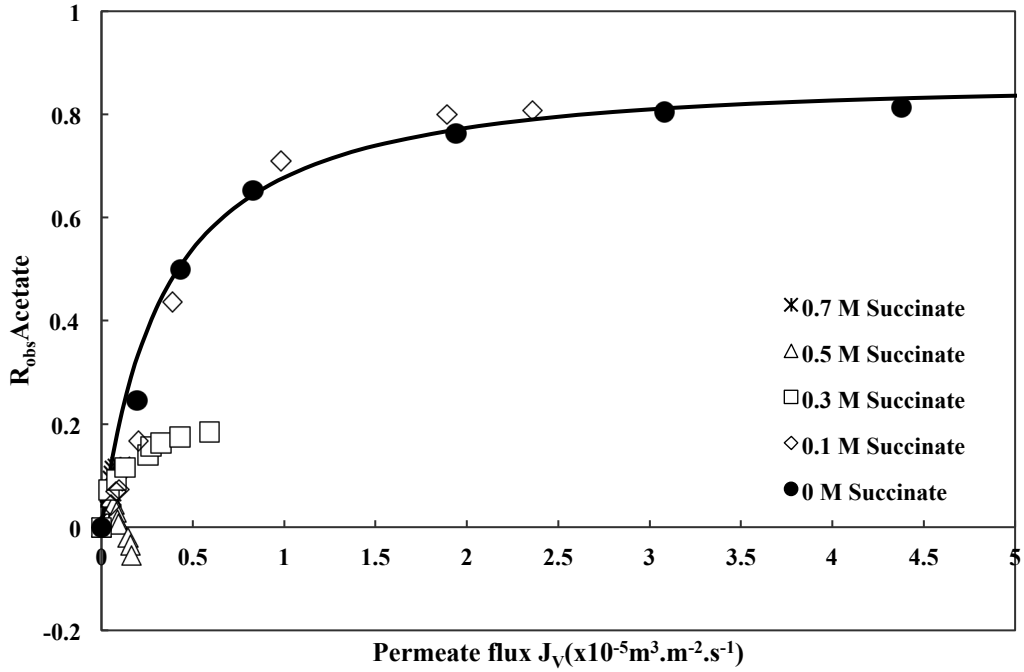


Figure 4.17 Observed retention of acetate as a function of the permeate flux in binary-solute solutions at pH 7, 0.1 M acetate and 0 M to 0.7 M succinate. The lines were fitted by Eq. A.1 (See Appendix A).

As mentioned above, lower salt retention is obtained at high salt concentration because of the screening effect. Indeed, the electrostatic repulsion depends on the ionic strength, *I*.

$$I = \frac{1}{2}(2^2[Suc^{2-}] + 1[Ace^-] + 3[K^+]) \qquad (4.7)$$

To demonstrate the impact of the ionic strength on the retention, we report the variation of the succinate and acetate retentions versus the ionic strength of the feed for various permeate fluxes in Fig.4.18 and 4.19, respectively. Fig. 4.18 shows that the succinate retention is strongly affected by increasing salt concentration due to the screening effect. It is observed that the succinate retention continuously decreases for increasing succinate concentration compared with the succinate retention at the same permeate flux, the succinate retention in binary-solute solutions

is slightly lower due to the presence of acetate. One can also observe that the acetate retention strongly decreases for increasing ionic strength (Fig. 4.19). Again, this decrease is due to the screening effect. Indeed, at low ionic strength, the transfer of both acetate and succinate are governed by their charge. On the contrary, the transfer of both solutes are fixed by their size at high ionic strength. Then, increasing ionic strength results in lower succinate and acetate retentions. In these conditions, one can observe that the low retention is obtained for an ionic strength higher than 1.6 M.

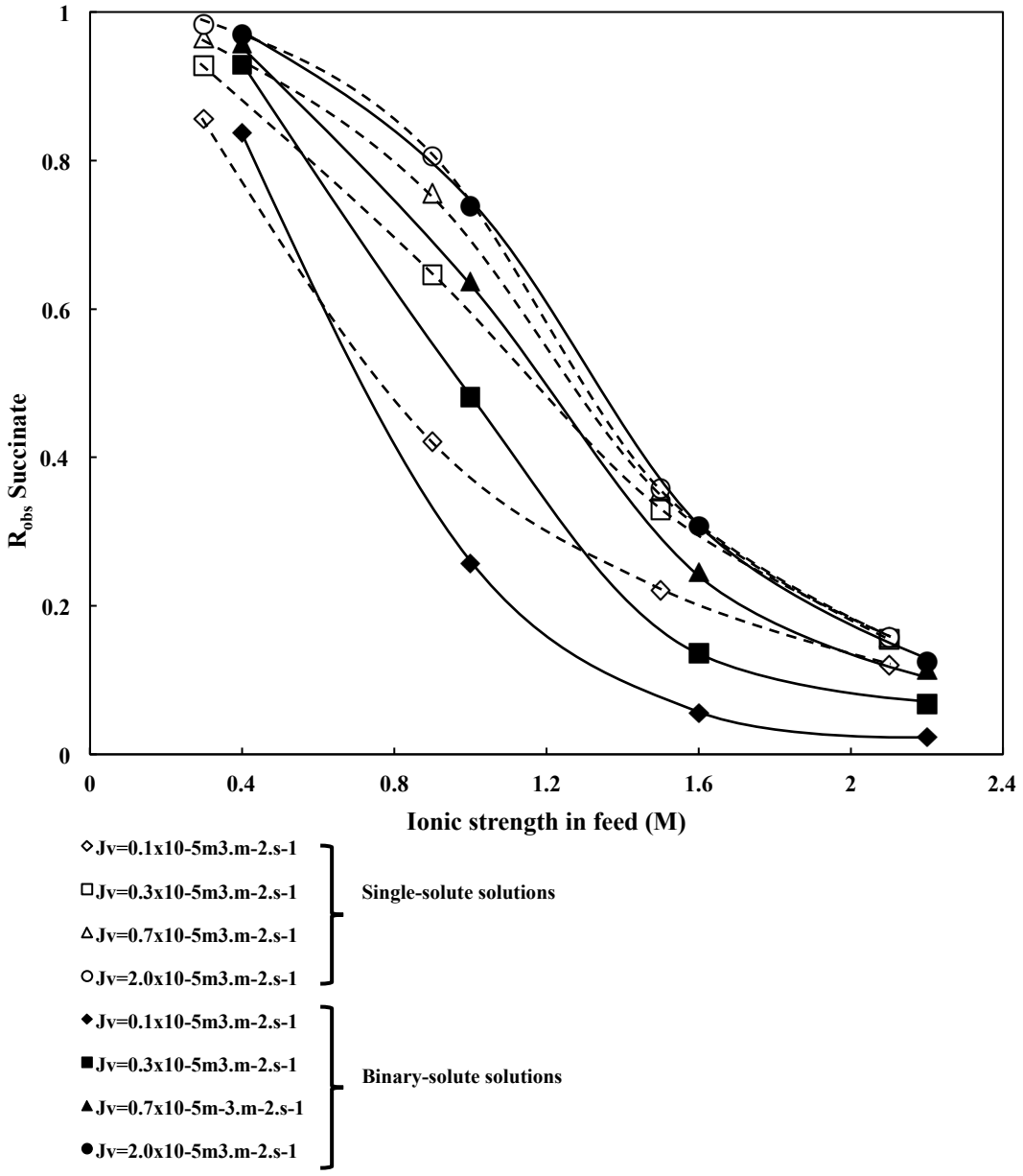


Figure 4.18 Observed retention of acetate as a function of the ionic strength. Single - solute solutions (Empty symbols) and binary-solute solutions (Filled symbols) at pH 7.0, 0.1 M acetate and 0.1 M to 0.7 M succinate.

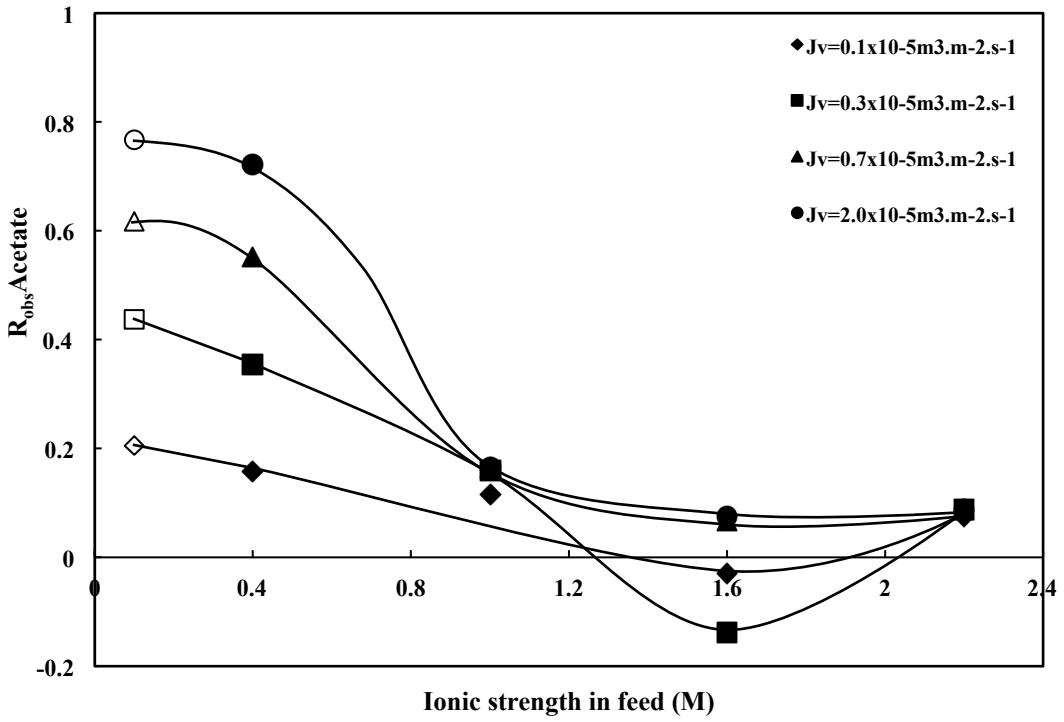


Figure 4.19 Observed retention of acetate as a function of the ionic strength. Single - solute solutions (Empty symbols) and binary-solute solutions (Filled symbols) at pH 7.0, 0.1 M acetate and 0 M to 0.7 M succinate.

Negative values of the retentions of ions in nanofiltration were already reported during nanofiltration of mixed solutions containing mono- and divalent ions (Hagmeyer *et al.*, 1998; Straatsma *et al.*, 2002). It is due to the competition for permeation between the membrane co-ions (showing the same sign of charge as the membrane), which have different size and/or charge. Divalent anions are more retained than monovalent ones through a negatively charged membrane. Then, in a solution containing succinate and acetate, succinate is more retained than acetate. Moreover, in order to maintain electroneutrality on both sides of the membrane, the acetate permeation increases. When the concentration of acetate in the permeate exceeds that in the retentate, negative value of the retention are obtained. The

permeation of acetate, which is the less retained co-ion, is facilitated by increasing the concentration of succinate ions, which is the more retained co-ion.

In addition, the negative retention of acetate in the presence of succinate can be explained by the concentration ratio of succinate and acetate in the feed. Through a simulation study using equation 2.8 (See chapter II), it is found that the factor influencing the retention of acetate is the concentration ratio of succinate to acetate. At a fixed retention of cation (K^+), the retention of acetate decreases with increasing concentration ratio between succinate and acetate in the feed (Fig. 4.20). Then, the retention of acetate becomes negative at high concentration ratio. For instance, at the retention ratio of succinate and potassium equal to 1.1, the negative retention of acetate is observed when the concentration ratio is greater than 5. However, the negative retention of acetate could also be observed at low concentration ratio when the retention of succinate increased (i.e. for increasing retention ratio of succinate to potassium (R_{Suc2-}/R_{K+})). These results are in an agreement with Kang and Chang (2005). They also proposed that the negative retention is affected by the concentration ratio between divalent and monovalent, and the charge of the membrane. With high concentration ratio and/or low membrane surface charge, the negative retention is likely occurred. The negative retention in this work is obtained at a concentration ratio about 5 at low permeate flux (Fig. 4.16b). Kang and Chang (2005) also reported that the negative retention of acetate is observed, at a concentration ratio about 3 for their conditions.

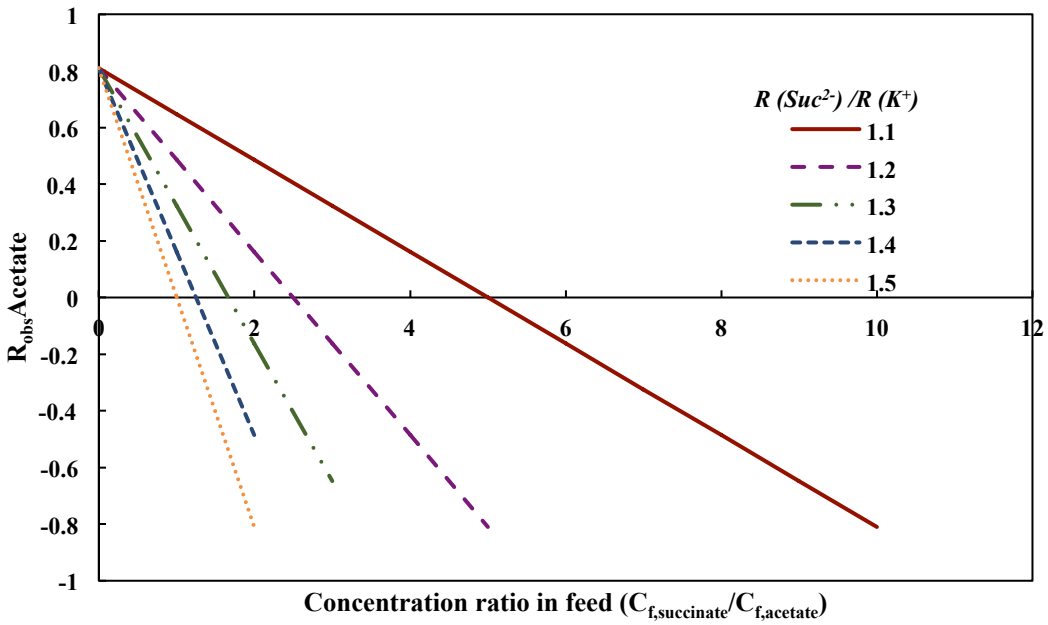


Figure 4.20 Acetate retention as a function of concentration ratio of succinate to acetate in the feed ($[Suc^{2-}]/[Ace^-]$) - influence of the retention ratio of succinate to potassium ion.

As previously mentioned, different succinate and acetate retentions were achieved by decreasing the salt concentration. In order to determine the succinate/acetate separation efficiency, the separation factor is used to express the ratio of the concentration of acetate and succinate in the permeate relative to the concentration ratio of these solutes in the retentate. Then, the variation of separation factor versus permeate flux are presented in Fig. 4.21. As expected, the separation factor was close to 1 for the concentration ratio about 5 and 7 since the transfer of succinate was governed by size effect. However, the separation factor was higher than 1 for the concentration ratio about 1 and 3 due to the fact that the transfer of succinate was fixed by its charge. This means that the retentate solution was enriched in succinate compared with the feed. Moreover, as expected, the separation factor increased by decreasing the succinate concentration. The highest separation factor was obtained at a concentration ratio about 1.

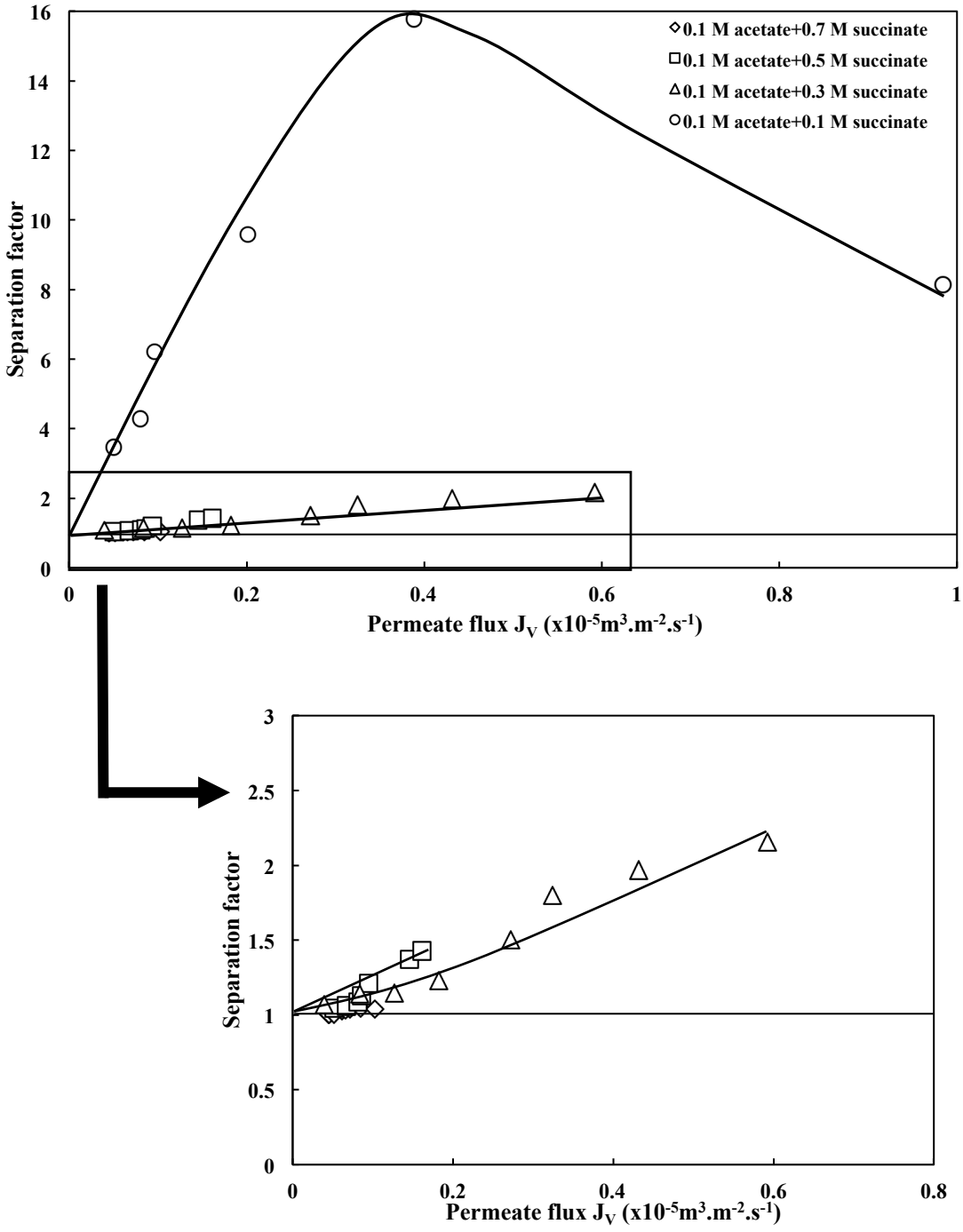


Figure 4.21 Separation factor of succinate as function of the permeate flux at feed pH 7.0 and feed solutions containing 0.1 M acetate and 0.1 to 0.7 M succinate

Conclusion

In this section, the influence of the concentration ratio of succinate to acetate was studied in binary-solute solutions. The results showed that the retention of both solutes depends on the concentration ratio. For a concentration ratio equal to 7 (0.7 M succinate/0.1 M acetate), the succinate and acetate retentions are low and too close. However, for decreasing concentration ratio to 1 (0.1 M succinate/0.1 M acetate), one can observe that the increase of succinate retention is higher than that of acetate. Then, the separation between succinate and acetate can be expected.

4.4.3.3 Influence of the dilution factor

From the previous experiments, it was concluded that the separation of succinate and acetate from a fermentation broth containing 0.7 M of succinate and 0.1 M of acetate is not achievable. Indeed, succinate and acetate retentions are too close and low (less than 20%) (Figs. 4.15b and 4.16a). However, the separation might be effective only at lower salt concentration and pH at 7 since succinate is then completely retained contrary to acetate (Fig. 4.16d). In order to evaluate the influence of the broth concentration on the transfer of both solutes and the separation efficiency, the nanofiltration of the binary-solute solution (0.7 M succinate/0.1M acetate) has been performed at different dilution factors (1 - 2 - 4 - 6 - 8 - 10) at pH 7.

The variations of succinate and acetate retentions versus permeate flux were in agreement with those previously observed at various concentrations (Fig. 4.22). For a dilution factor equal to 1, the retention of succinate and acetate are low and similar. Then, no separation is expected in this condition. But,

for increasing dilution factors, i.e. decreasing feed concentrations, it is observed that the increase of the succinate retention is higher than that of acetate. Then the succinate/acetate separation can be achieved for diluted solutions. Moreover, at a dilution factor 4, negative values are obtained for the retention of acetate.

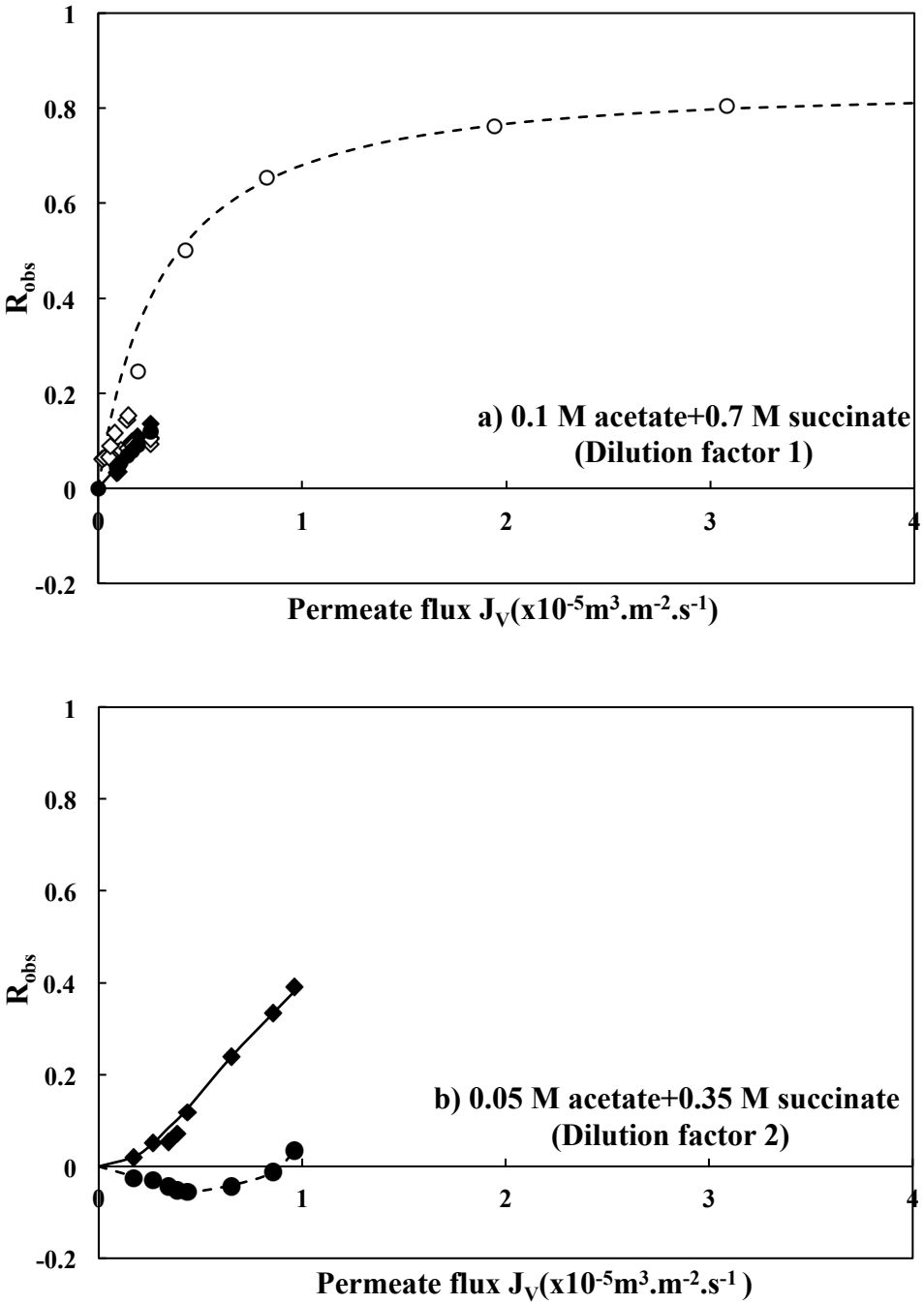
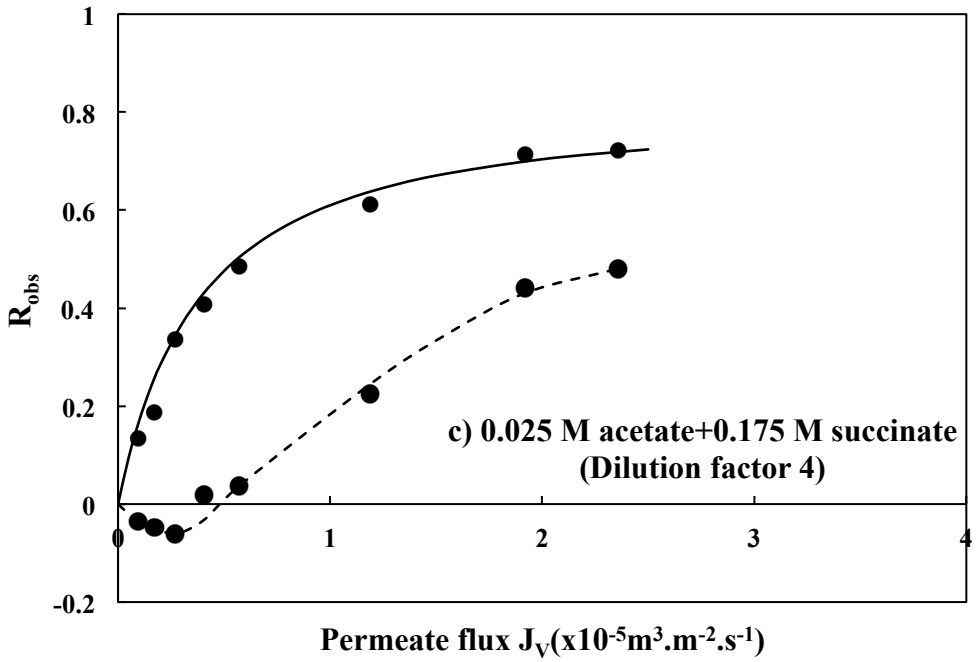


Figure 4.22 Observed retention of succinate and acetate in binary solute solutions (0.1 M acetate + 0.7 M succinate) at pH 7- NF45 membrane - influence of the dilution factor. Single solution: succinate (◇), acetate (○) and Binary solution: succinate (◆), acetate (●). The lines were fitted by Eq. A.1 (See Appendix A).



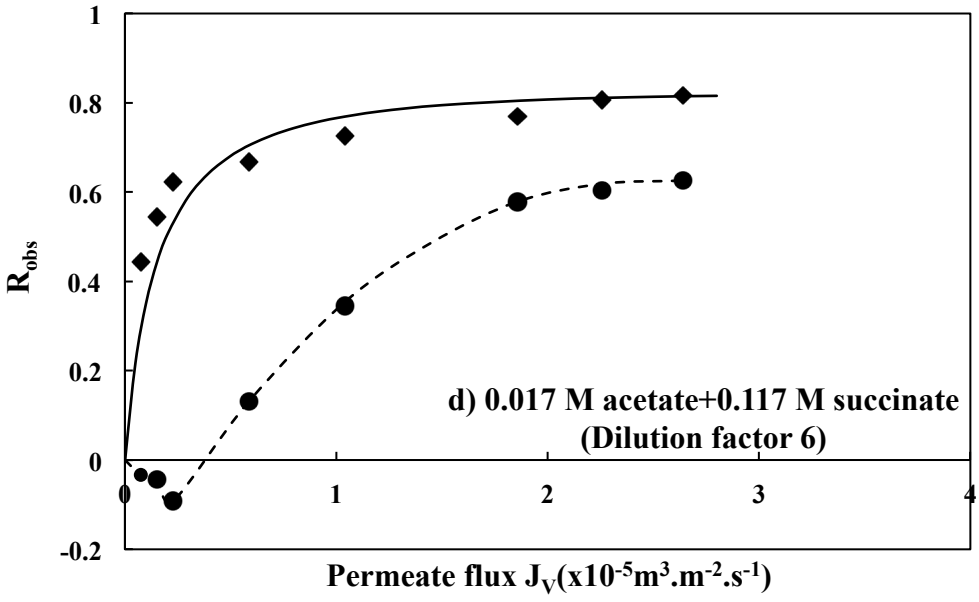


Figure 4.22 (continued).

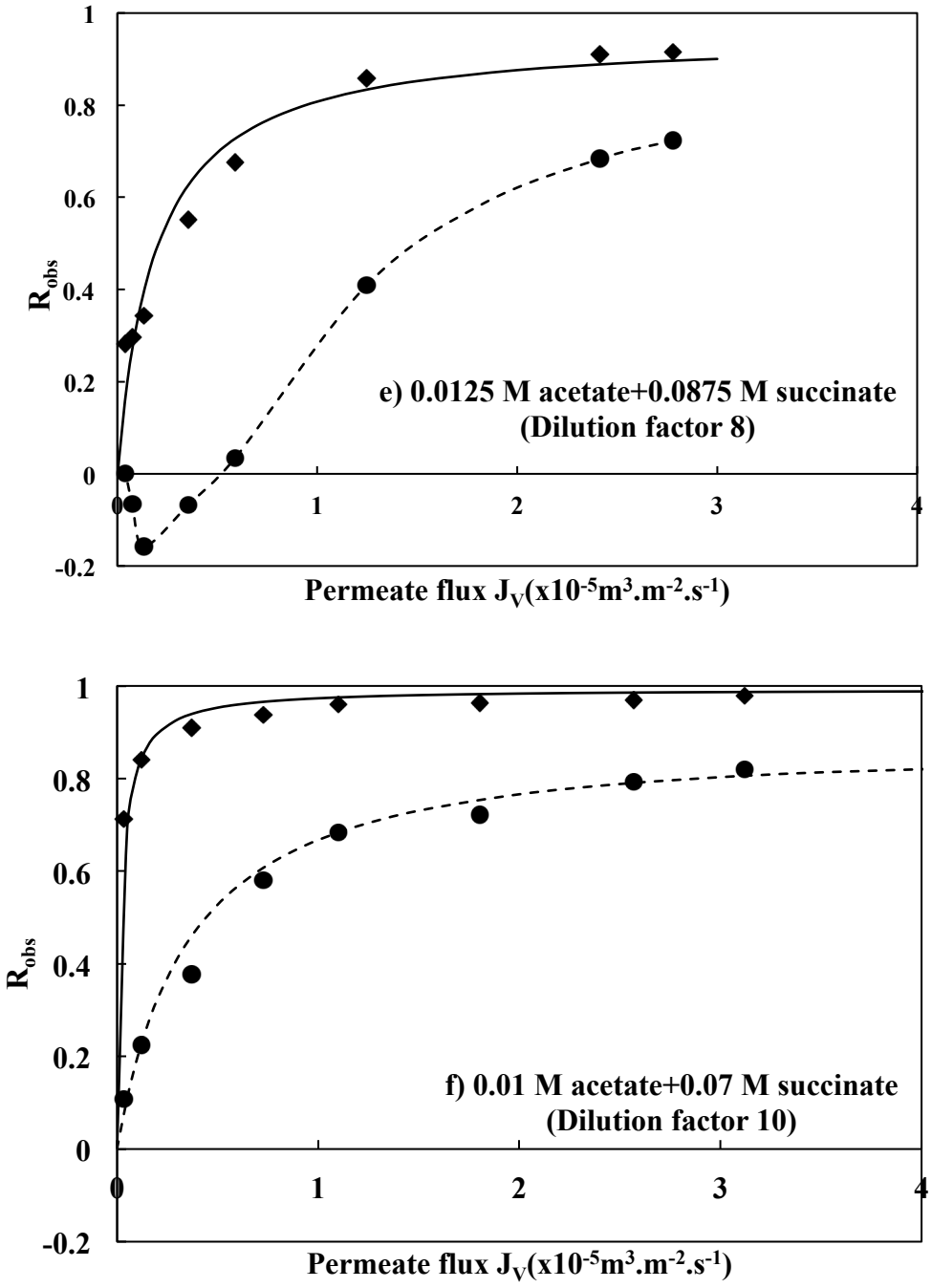


Figure 4.22 (continued).

Discussion

Based on the above results, the retention of succinate and acetate increases when increasing dilution factors, i.e. decreasing feed concentrations. Then, increasing dilution factor results in a higher retention because of increasing electrostatic replusions. It is also observed that the increase of the succinate retention is greater than that of acetate because the charge and size of succinate are higher than those of acetate. Consequently, the separation between succinate and acetate could be achieved for diluted solutions. Similar results have been previously reported (Li *et al*., 2003). It was also found that the retention of L-glutamine increased with increasing dilution factor due to increasing electrostatic repulsion. Moreover, the acetate retention had negative value for a dilution factor increasing from 2-8. As already explained, the negative retention of monovalent anions can be obtained in presence of divalent anions. However, the negative retention of acetate could not be observed for dilution factor equal to 10. As already mentioned, the electrostatic repulsion are dominant at low concentration. Consequently, acetate retention becomes higher and reached positive values.

For increasing dilution factor, the electrostatic repulsion become stronger and high salt retentions are obtained. As previously discussed, the electrostatic repulsion increases for decreasing the ionic strength. In order to demonstate the influence of ionic strength on the retention, the variation of corresponding retention of succinate and acetate versus ionic strength are reported in Fig. 4.23 and Fig. 4.24, respectively. At dilution factor equal to 1, i.e. high salt concentration and high ionic strength, the retentions of succinate and acetate are low and similar due to the screening effect. On the other hand, high retentions of both

solutes were observed at high dilution factors (low salt concentration and low ionic strength) due to the strong electrostatic repulsions. The negative retention of acetate could be observed at low permeate flux when increasing the ionic strength from 0.2 to 1.6 M.

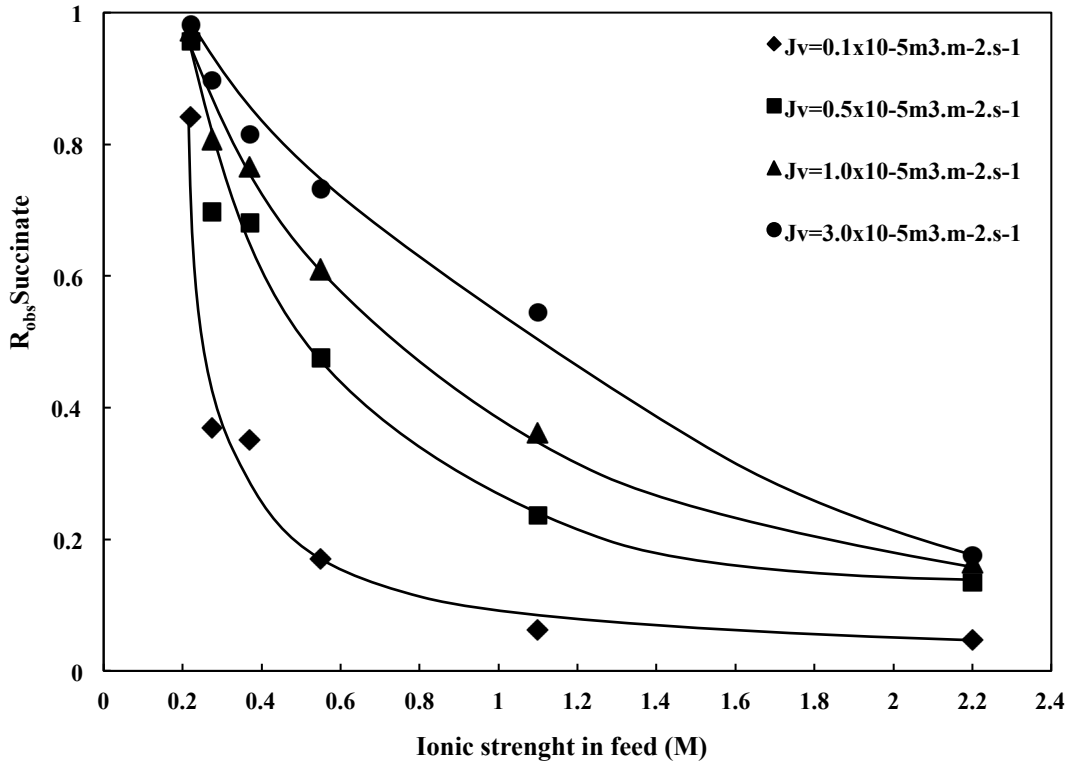


Figure 4.23 Observed retention of succinate as a function of the ionic strength in binary-solute solutions (0.1 M acetate + 0.7 M succinate) at pH 7.0 - NF45 - influence of the dilution factor.

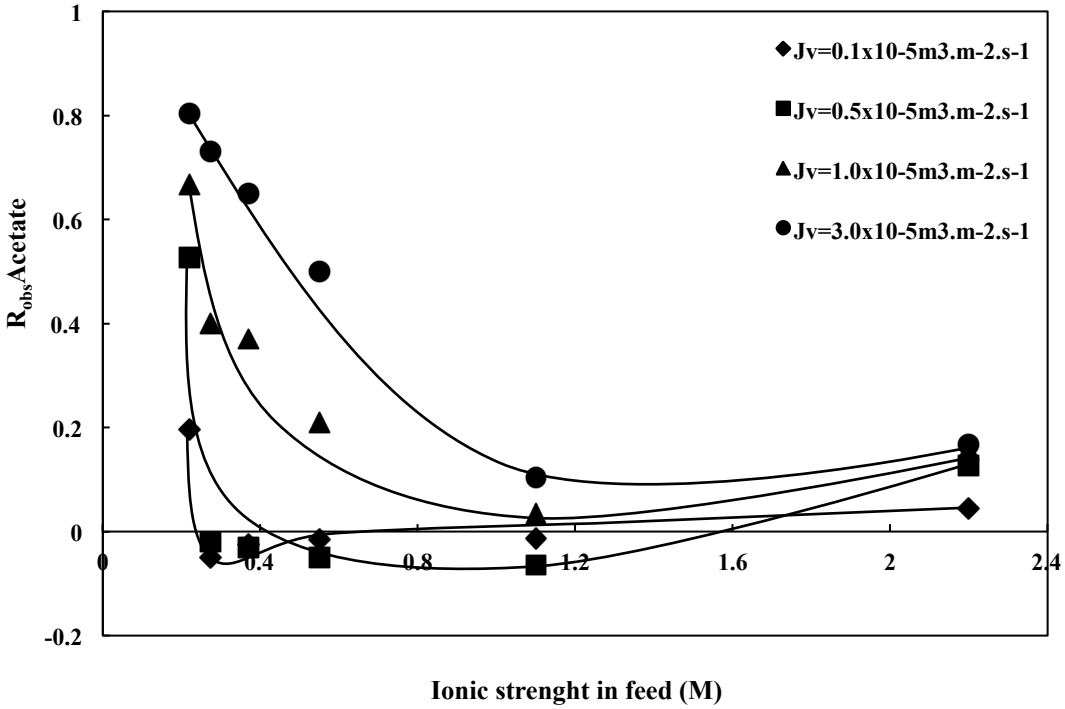


Figure 4.24 Observed retention of acetate as a function of the ionic strength in binary-solute solutions (0.1 M acetate + 0.7 M succinate) at pH 7.0 - NF45 - influence of the dilution factor.

As previously suggested, the retention of succinate became higher than that of acetate by increasing the dilution factor. In order to evaluate the influence of the dilution factor on the separation efficiency, the variations of the corresponding separation factor versus permeate flux are reported in Fig. 4.27. As expected, a better separation is obtained by increasing the dilution factor. It is found that the separation factor is close to 1.0 for non-diluted solution (dilution factor 1) while values greater than 1.0 are obtained for more diluted feed solutions. The maximum value increases from 2 to 6.5 for dilution factors increasing from 2 to 10. The fluxes corresponding to the maximum value of separation factor increased from J_v = 0.5 to 2.5 x 10^{-5} m^3 m^{-2} s^{-1} when dilution factors vary from 2 to 10. These results

indicated that the separation performances (separation factor as well as permeate flux) were improved for increasing dilution factor, i.e. lower succinate concentration.

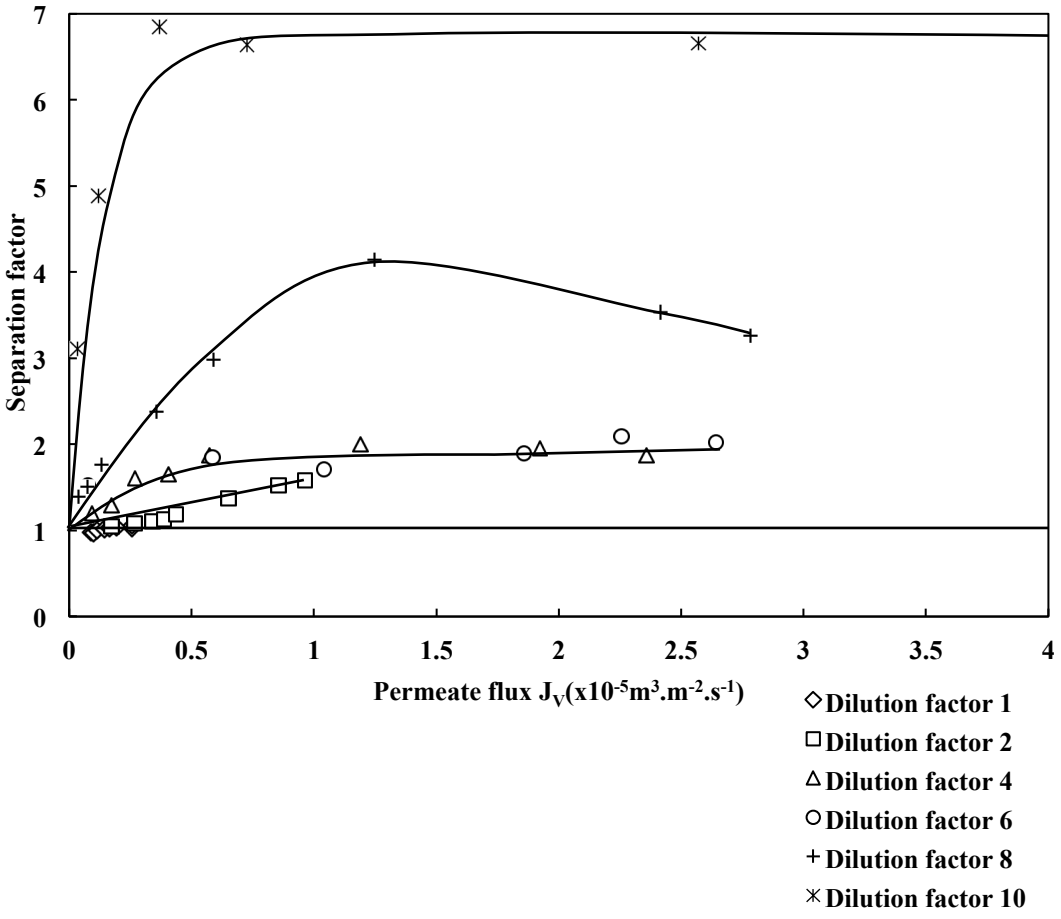


Figure 4.25 Separation factor of succinate as function of the permeate flux at feed pH 7.0 and feed solutions containing 0.1 M acetate and 0.7 M succinate - influence of the dilution factor.

Conclusion

Regarding the influence of the dilution factor on succinate and acetate retentions in binary-solute solutions, results showed that the retention of succinate and acetate increased when the dilution factor was increased from 1 to 10

since the electrostatic repulsion are dominant. Then, the separation between succinate and acetate could be achieved by increasing dilution factors.

4.4.4 Purification of fermentation broth by nanofiltration

In this section, the objective is to propose a methodology for the purification of succinate from the fermentation broth. This methodology is based on the knowledge of the previously investigated transfer mechanisms in order to determine the optimum condition to be used regarding the purity of succinate.

4.4.4.1 Composition of the fermentation broth

In this study, succinate fermentation broth was produced from glucose fermentation by *E.coli* KJ122 under anaerobic condition. *E.coli* KJ122 was grown in AM1 medium supplemented with 50 g/L glucose and 10 g/L $KHCO_3$. Fermentations were performed at 37 ^oC, 100 rpm with 0.35 L working volume in 0.5 L small anaerobic vessel and incubated 72 h. The pH was controlled at 7 by adding mixture solution of 6 M KOH and 3 M K_2CO_3. After fermentation, succinate was produced as major product and acetate was produced as the main impurity. Also, glucose and some ions such as Cl^- and PO_4^{3-} were still remaining in the fermentation broth. The composition of fermention broth is shown in the Table 4.14. To evaluate the influence of the broth composition on the separation performances, nanofiltration experiments are carrired out with synthetic fermentation broths of increasing complexity containing succinate and impurities.

Table 4.14 Composition of the fermentation broth.

	Fermentation broth
Organism	*E.coli* KJ122
Medium/Condition	AM1 containing 50 g/L glucose with 10 g/L $KHCO_3$, 500 ml small anaerobic vessel, 72 h incubation, pH maintained with 1:1 mixture of 6 M KOH + 3 M K_2CO_3
pH	6.98
Conductivity ($ms.cm^{-1}$)	55.85
Glucose	0.027 M (4.86 g/l)
Succinate	0.350 M (41.33 g/l)
Acetate	0.065 M (3.9 g/l)
PO_4^{3-}	0.017 M (1.62g/l)
Cl^-	0.0045 M (0.16 g/l)
K^+	0.80 M (31.28 g/l)
Na^+	0.01 M (0.23 g/l)
Total anion	0.82
$((2[Suc^{2-}]*95\%)+ ([Suc^{2-}]*5\%)+[Ace^-]+3[PO_4^{3-}]+[Cl^-])$	
Total cation ($[K^+]+[Na^+]$)	0.81

4.4.4.2 Preliminary experimental investigation

The composition of the fermentation broth considered in this study is shown in Table 4.14 at pH 7. The objective of this experiment was to investigate the transfer of succinate and acetate in the presence of glucose, chloride and phosphate. Then, experiments were performed at different composition of feed solutions.

Acetate is the major impurity in succinate fermentation broth. Then, a binary solution containing 0.35 M succinate and 0.065 M acetate was first investigated. The variations of the retention of succinate and acetate versus the permeate flux were plotted in Fig. 4.26a. It was observed that the retention of succinate is 60 % at $J_V=0.1x10^{-5}m^3.m^{-2}.s^{-1}$. The retention of acetate is negative in the ranges of flux tested. Furthermore, the transfer of succinate and acetate were investigated at feed solution of increasing complexity containing succinate, acetate and other impurities such as glucose, chloride and phosphate. With the feed solution that contains succinate, acetate and glucose, the succinate retention is similar to that observed with binary solute solution without glucose. It was also observed that the retention of glucose is lower than that of succinate and the negative retentions of acetate obtained in this condition (Fig. 4.26b). Further, Fig 4.26c shows the variation of the retention of succinate, acetate and chloride with the permeate flux. The retention of succinate is slightly lower than that obseverd in binary-solute solution containing succinate and acetate. The retention of acetate is less negative than that of chloride. For feed solution containing succinate, acetate and phosphate, the retention of succinate is much lower than that observed in binary-solute solution containing succinate and acetate. The retention of succinate and phosphate are similar and less than 60 % at $J_V=0.1x10^{-5}$ $m^3.m^{-2}.s^{-1}$ (Fig. 4.26d). Also, a negative retention of acetate is observed in the ranges of flux tested.

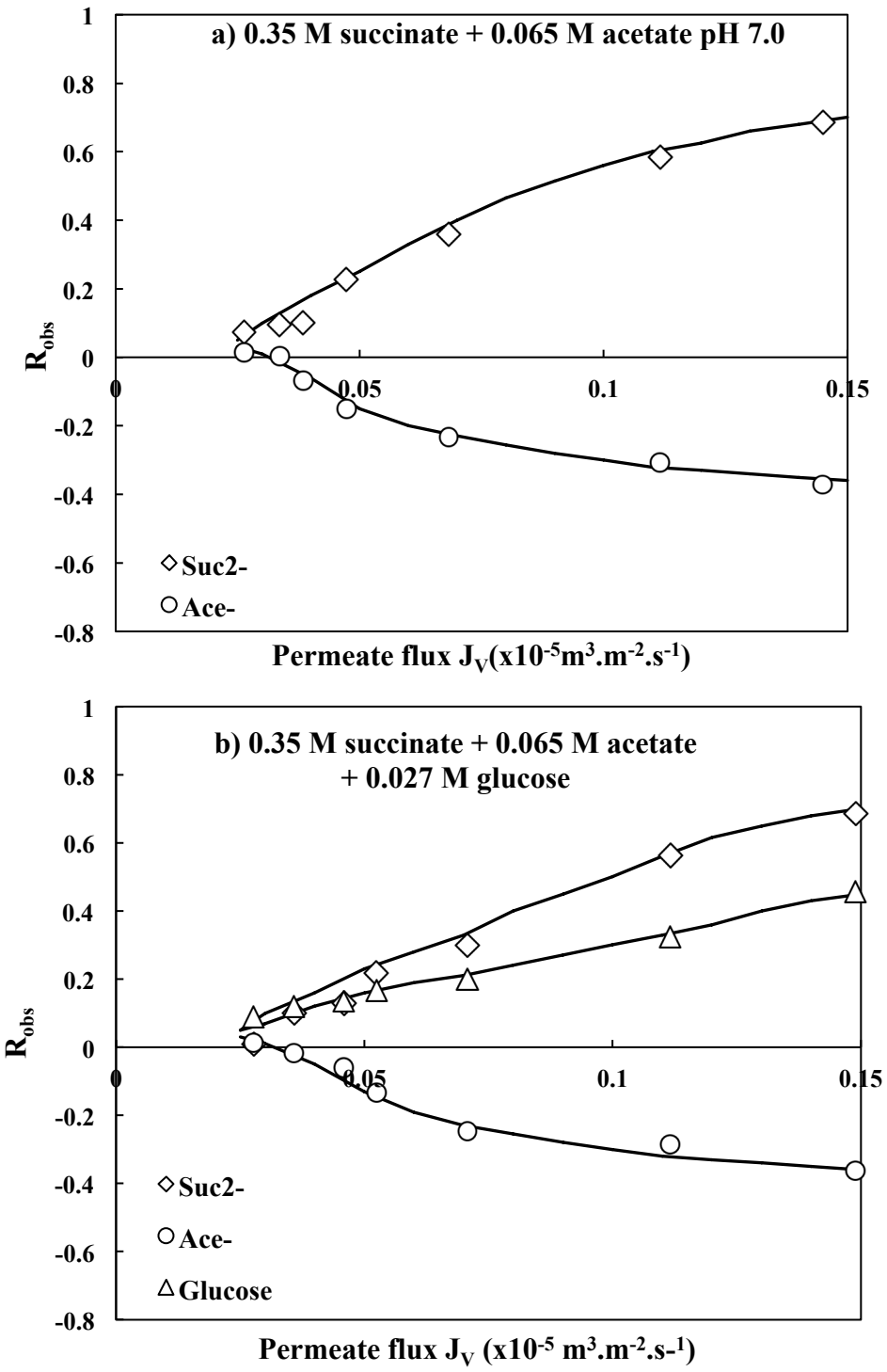
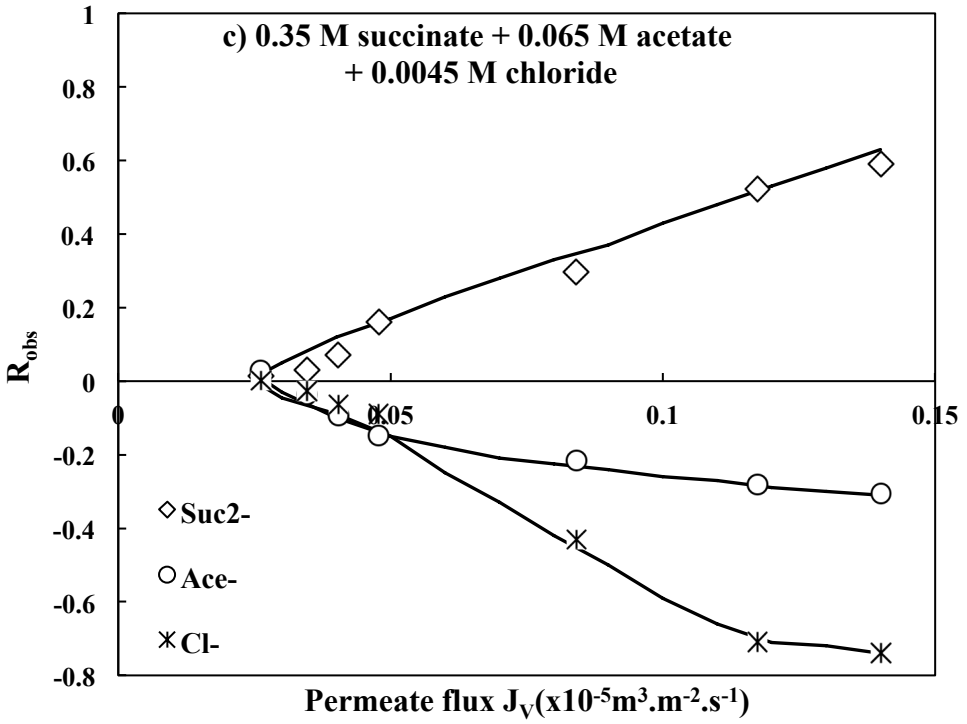


Figure 4.26 Observed retention of succinate and impurities as function of the permeate flux with increasing complexity of feed solutions.



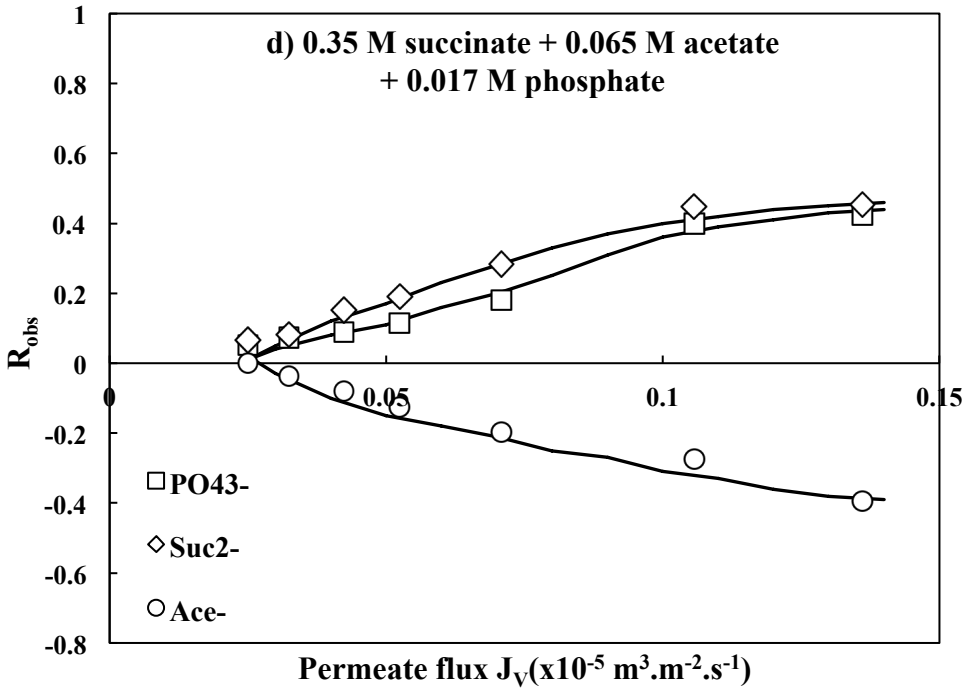


Figure 4.26 (continued).

As previously reported, the retention of succinate is low at concentration higher than 0.2 M (Fig. 4.9) due to the screening effect. Then, in binary-solute solution containing 0.35 M succinate and 0.065 M acetate, succinate is not completely retained since the electrostatic repulsion is less effective. Furthermore, in presence of inorganic salts, like chloride and phosphate in the feed solution, the ionic strength is higher so that electrostatic repulsion become weaker. Then, decreasing succinate retention is observed. As observed in other investigations, the addition of neutral solute like glucose have no impact on the transfer of charged solutes (Bargeman *et al*., 2005; Umpuch *et al*., 2010). Moreover, the retention of acetate was always negative and decreased with increasing permeate flux. Such results were in accordance with previous ones obtained with different ions of the same sign of charge thus showing a negative retention of the less retained one. However, the acetate retention became less negative in the presence of chloride since acetate has a bigger charge and size than chloride.

4.4.4.3 Purification of succinate using a two stages process

It was previously pointed out that the separation of succinate and acetate was not possible for a succinate concentration higher than 0.2 M. On the contrary, it was shown that the succinate was strongly retained by the membrane at succinate concentrations lower than 0.2 M at pH higher than 7, whereas the acetate retention is low (Fig. 4.14). Consequently, the fermentation broth had to be diluted before the nanofiltration step.

In order to evaluate the impact of the dilution factor on the separation of succinate and acetate, experiments were carried out with non-diluted and diluted synthetic fermentation broth, results are plotted in Fig. 4.27. The retention of

succinate was less than 60% and the acetate retention was negative for non-diluted synthetic fermentation broth (Fig. 4.27a). The separation of succinate and acetate was not expected under this condition since succinate was not completely retained by the membrane at the succinate concentration higher than 0.2 M. However, for decreasing feed concentrations by dilution factor 2, it was observed that the increase of the succinate retention was higher than that of acetate (Fig. 4.27b). Therefore, the separation of succinate and acetate could be expected for diluted synthetic fermentation broth.

Based on the above results, it was thus decided to carry out the nanofiltration of the diluted synthetic fermentation broth (dilution factor 2) in a diafiltration mode using NF membrane (NF45) in order to improve the removal of acetate and thus the purity of succinate. Finally, the retentate solution was concentrated using a RO membrane (XLE), which is known to ensure a complete retention of organic salts. The two stages process for succinate purification is illustrated in Fig. 4.28.

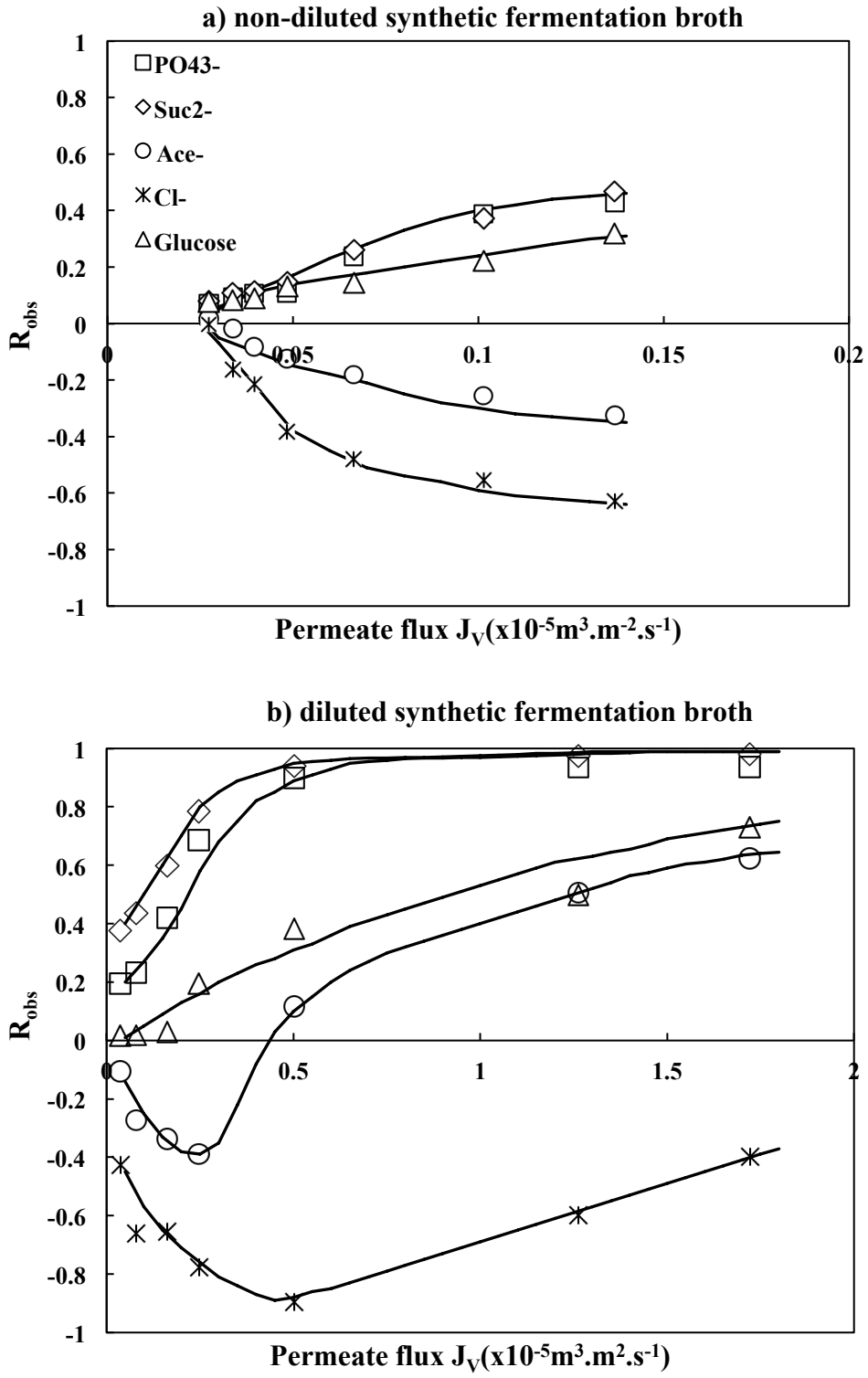


Figure 4.27 Observed retention of succinate and impurities as function of the permeate flux: (a) non-diluted synthetic fermentation broth ; (b) diluted synthetic fermentation broth (Dilution factor 2).

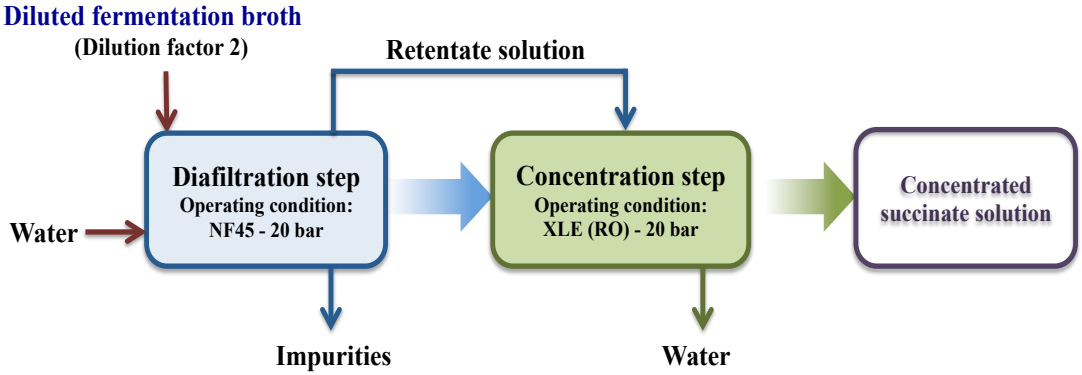


Figure 4.28 The two stages process operations for succinate purification.

4.4.4.3.1 Stage 1: Purification of succinate - Diafiltration

In this step, the diafiltration of the succinate fermentation broth diluted by a factor 2 has been carried out at 20 bar during 26 h. The results are firstly presented in terms of the variation of the permeate flux and retention of solutes versus the number of diavolumes (Fig. 4.29). A diavolume is defined as the total ultra-pure water volume (V_p) added during diafiltration divided by the initial retentate feed volume (V_0). The permeate flux increased during the first eight diavolumes because of the lower concentration and becomes constant for increasing diavolumes over eight (Fig. 4.29a). Furthermore, the variation of solute retention versus the number of diavolumes is also reported in Fig. 4.29b. One can observe that the retention tend towards a constant value after 3 diavolume. As expected, the succinate retention is about 99% and the retention of acetate is less than 70% over the whole range of the diavolumes. It was also observed that the retention of phosphate and glucose is ranged between 92% to 99% and 75% to 95% when the number of diavolumes increases from 0.2 to 14. The negative retention of chloride is obtained at low diavolume because of the presence of succinate and phosphate in feed solution

which is more retained. However, the chloride retention increases with increasing the number of diavolumes and reaches positive values due to the increasing permeate flux.

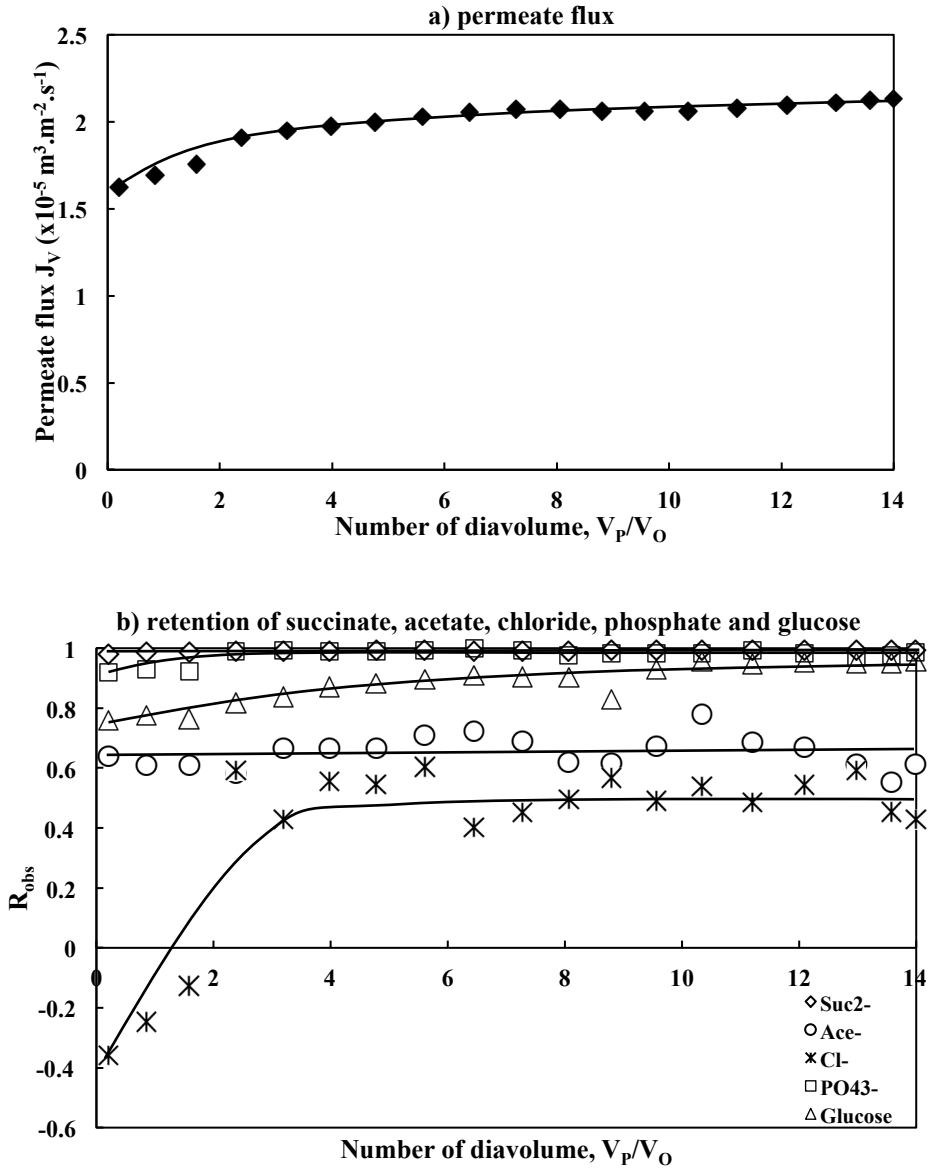


Figure 4.29 Permate flux and retention of solutes as function of numbers of diavolumes in a diafiltration of diluted synthetic fermentation broth at pH 7 - ΔP =20 bar - feed composition: 0.175 M succinate + 0.0325 M acetate + 0.0085 M phosphate + 0.0023 M chloride + 0.0135 M glucose (Dilution factor 2). (a) permeate flux; (b) retention of succinate, acetate, chloride, phosphate and glucose in retentate.

Knowing the values of the solutes retentions at the given composition of the fermentation broth, the solute concentration in retentate during the diafiltration could be predicted and simulated by the mass balance equation (Eq. 4.3) assuming a constant retention (the retention values equal to the constant/mean value obtained in Fig. 4.29b). The calculated solutes concentrations in the retentate versus the number of diavolumes are plotted in Fig. 4.30a. One can observe that the concentration of solutes slightly decreases with increasing the number of diavolumes. From the process point of view, it is also interesting to calculate the purity and the yield of succinate to evaluate the separation efficiency during diafiltration (Fig. 4.30b). The purity of succinate is expected to increase from 85% to 99.4% while the yield is expected to decrease from 100% to 91% when increasing the number of diavolumes to 14. Then, it is confirmed that NF in a diafiltration mode can be used to achieve the purification of succinate.

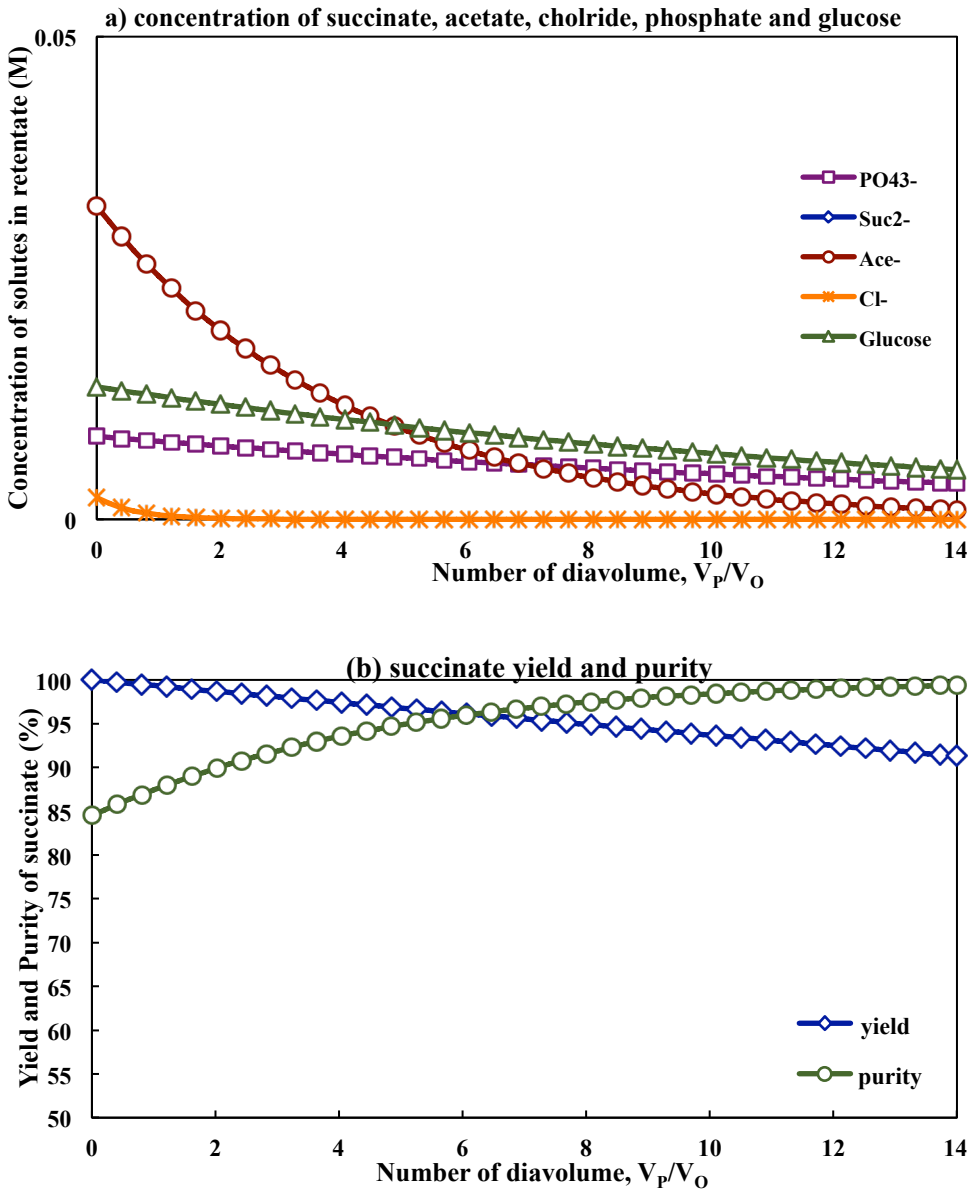


Figure 4.30 Concentration of solutes in retentate, succinate yield and purity as function of numbers of diavolumes in a diafiltration of diluted synthetic fermentation broth from simulation - at pH 7 - ΔP =20 bar – feed composition: 0.175 M succinate + 0.0325 M acetate + 0.0085 M phosphate + 0.0023 M chloride + 0.0135 M glucose (Dilution factor 2). (a) concentration of succinate, acetate, chloride, phosphate and glucose in retentate; (b) succinate yield and purity.

Further, the variation of the solute concentrations in the retentate versus the number of diavolumes from the experiment is illustrated in Figs. 4.31 and 4.32. The predicted values are also plotted for comparison. It was observed that the concentration of succinate ($R_{Suc} \approx 99$ %) slightly decreased during the diafiltration operation while the acetate ($R_{Ace} \approx 65$ %) concentration decreased much greater (Fig. 4.31). Moreover, the concentration of phosphate and glucose continuously decreased with increasing the number of diavolumes. The concentration of chloride is closed to zero after 2 diavolumes (Fig. 4.32). According to these results, one can observe that, the experimental values are in agreement with the predicted ones.

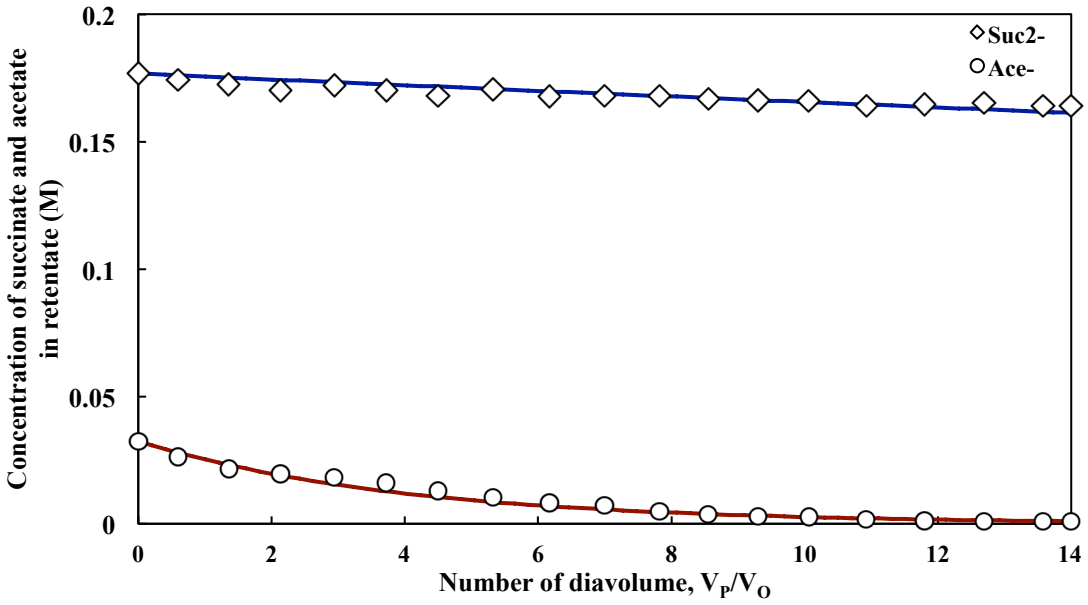


Figure 4.31 Concentration of succinate and acetate in retentate as function of numbers of diavolumes in a diafiltration of diluted synthetic fermentation broth at pH 7 - ΔP =20 bar - feed composition: 0.175 M succinate + 0.0325 M acetate + 0.0085 M phosphate + 0.0023 M chloride + 0.0135 M glucose (Dilution factor 2). The lines are predicted values.

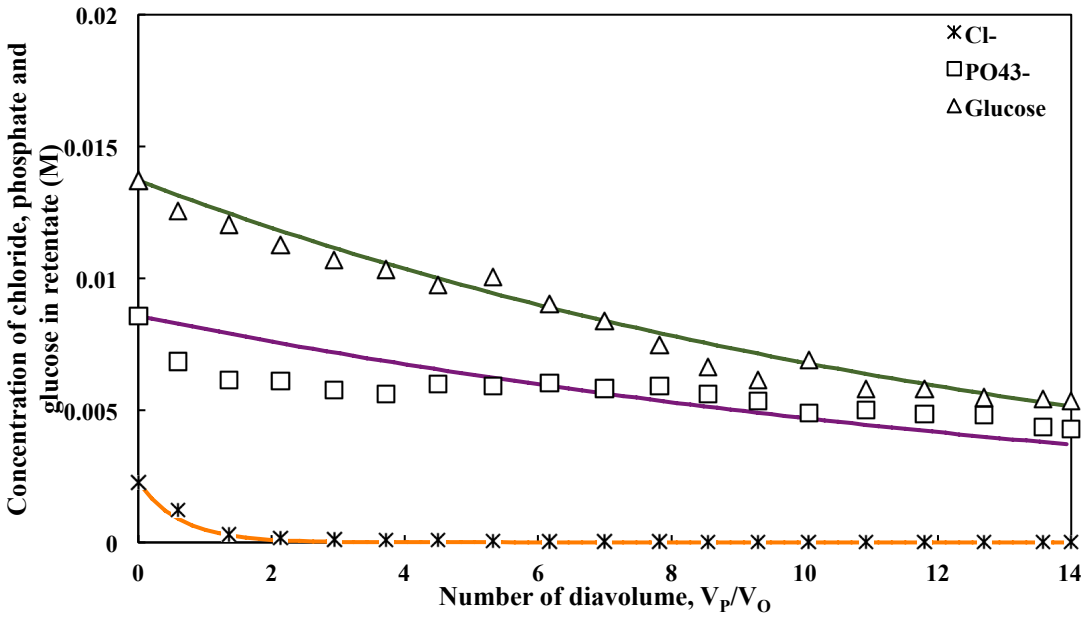


Figure 4.32 Concentration of chloride, phosphate and glucose in retentate as function of numbers of diavolumes in a diafiltration of diluted synthetic fermentation broth at pH 7 - ΔP =20 bar - feed composition: 0.175 M succinate + 0.0325 M acetate + 0.0085 M phosphate + 0.0023 M chloride + 0.0135 M glucose (Dilution factor 2). The lines are predicted values.

As previously reported, Fig. 4.31 shows that the concentration of acetate strongly decreased during operation. Then, the concentrations of solute in permeate versus the number of diavolumes is reported in Fig. 4.33 and Fig. 4.34. As expected, the concentration of acetate is higher than that of succinate over the whole range of the diavolume (Fig. 4.33). The concentration of glucose decreases with increasing the number of diavolumes (Fig. 4.34). For a diavolume over 2, the concentration of phosphate and chloride are close to zero.

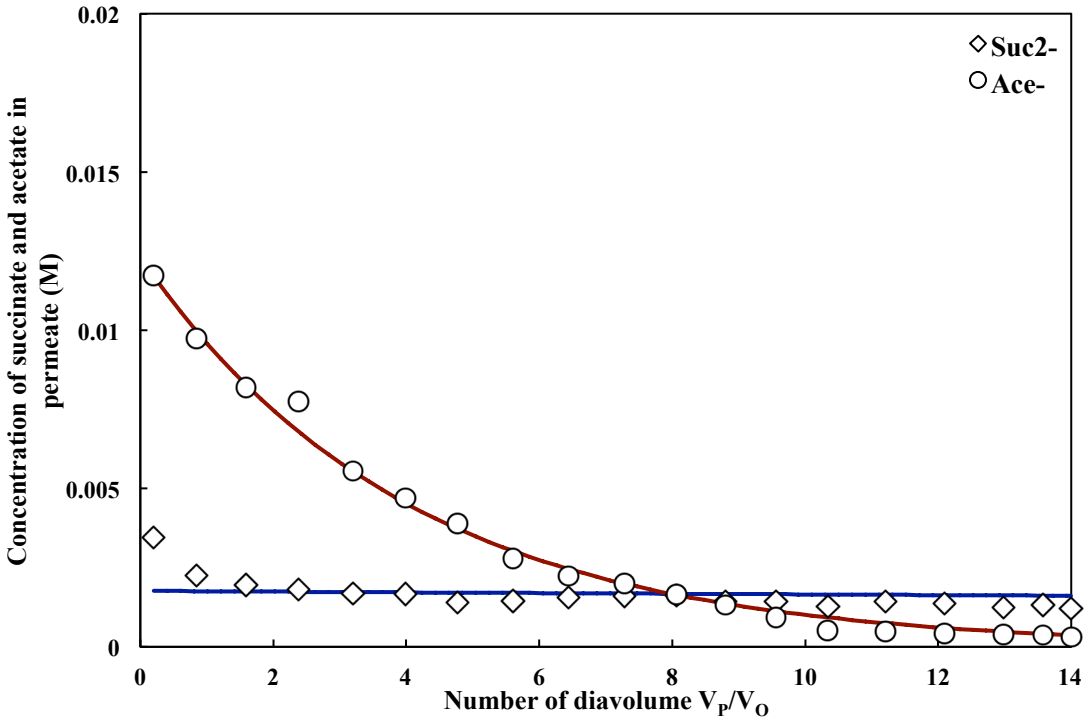


Figure 4.33 Concentration of succinate and acetate in permeate as function of numbers of diavolumes in a diafiltration of diluted synthetic fermentation broth at pH 7 - ΔP =20 bar - feed composition: 0.175 M succinate + 0.0325 M acetate + 0.0085 M phosphate + 0.0023 M chloride + 0.0135 M glucose (Dilution factor 2). The lines are predicted values.

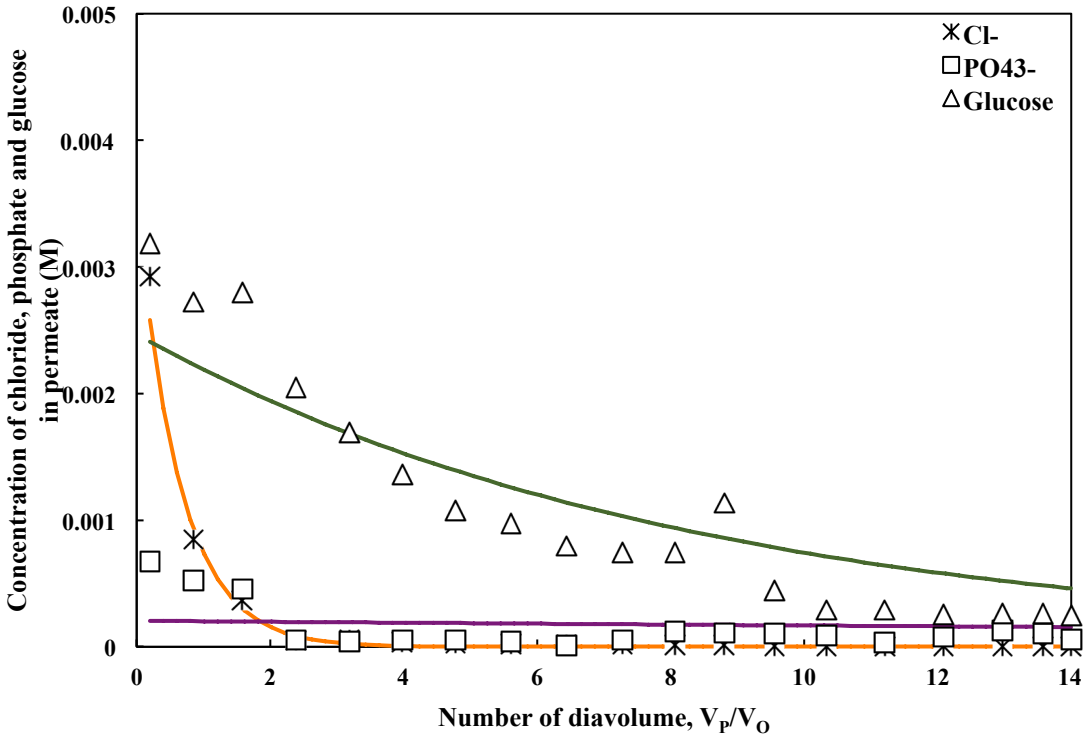


Figure 4.34 Concentration of chloride, phosphate and glucose in permeate as function of numbers of diavolumes in a diafiltration of diluted synthetic fermentation broth at pH 7 - ΔP =20 bar - feed composition: 0.175 M succinate + 0.0325 M acetate + 0.0085 M phosphate + 0.0023 M chloride + 0.0135 M glucose (Dilution factor 2). The lines are predicted values.

According to Fig. 4.31, the decrease in rate of acetate concentration is much higher than that of succinate. Consequently, the purity of succinate increased, from 85% (initial value in the feed) to 99.5% for 14 diavolumes (Fig. 4.35). Meanwhile the succinate yield remained higher than 93% after 14 diavolumes.

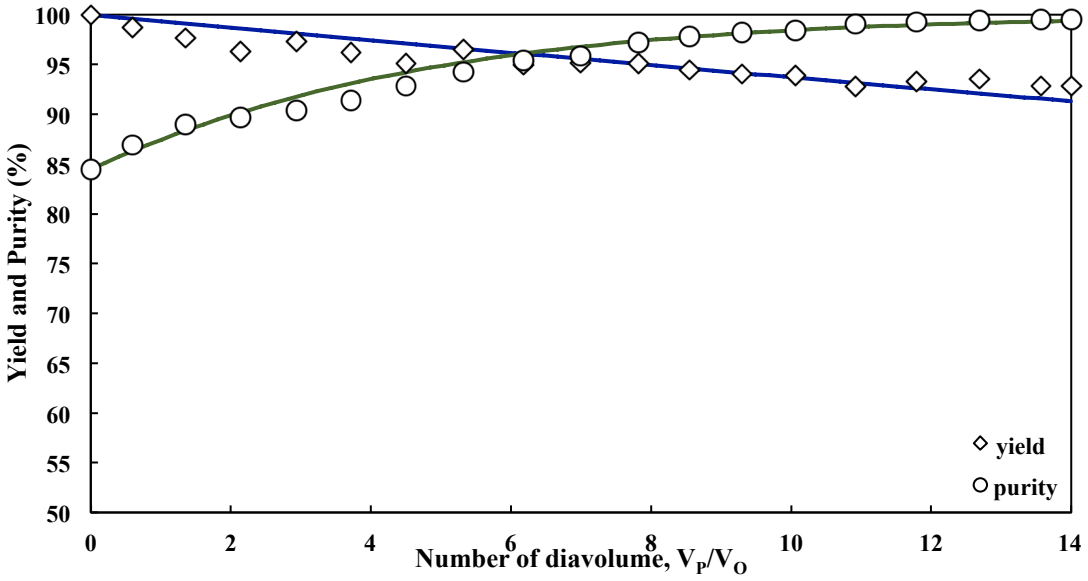


Figure 4.35 Yield and purity of succinate as function of numbers of diavolumes in a diafiltration of diluted fermentation broth at pH 7 - ΔP =20 bar - feed composition: 0.175 M succinate + 0.0325 M acetate + 0.0085 M phosphate + 0.0023 M chloride + 0.0135 M glucose (Dilution factor 2). The lines are the predicted values.

4.4.4.3.2 Stage 2: Concentration of the purified succinate - Reverse osmosis

Finally, the diafiltrated fermentation broth (diavolume = 14, [Suc] = 0.16 M) was concentrated using the XLE reverse osmosis membrane. This operation was carried out to recover the initial succinate concentration in the fermentation broth, i.e. 0.34 M (concentration factor ≈ 2). First, the results are presented in terms of the variation of solute retention and permeate flux versus the volume reduction factor (Fig. 4.36). A volume reduction factor is defined as the initial feed volume (V_0) divided by the retentate volume (V_R). One can observe that the retentions of succinate and phosphate slightly decreases, from 99.7% to 99.2% with

increasing the volume reduction factor. The retention of glucose is closed to 100 % for the whole range of the volume reduction factor. Moreover, the retention of acetate decreases from 95 % to 90 % when the volume reduction factor increases from 1 to 2.1. As previously mentioned, the higher salt concentration in retentate results in a decrease of the solute retention. One can also observe that the permeate flux increases during operation in concentration mode due to the high osmotic preesure.

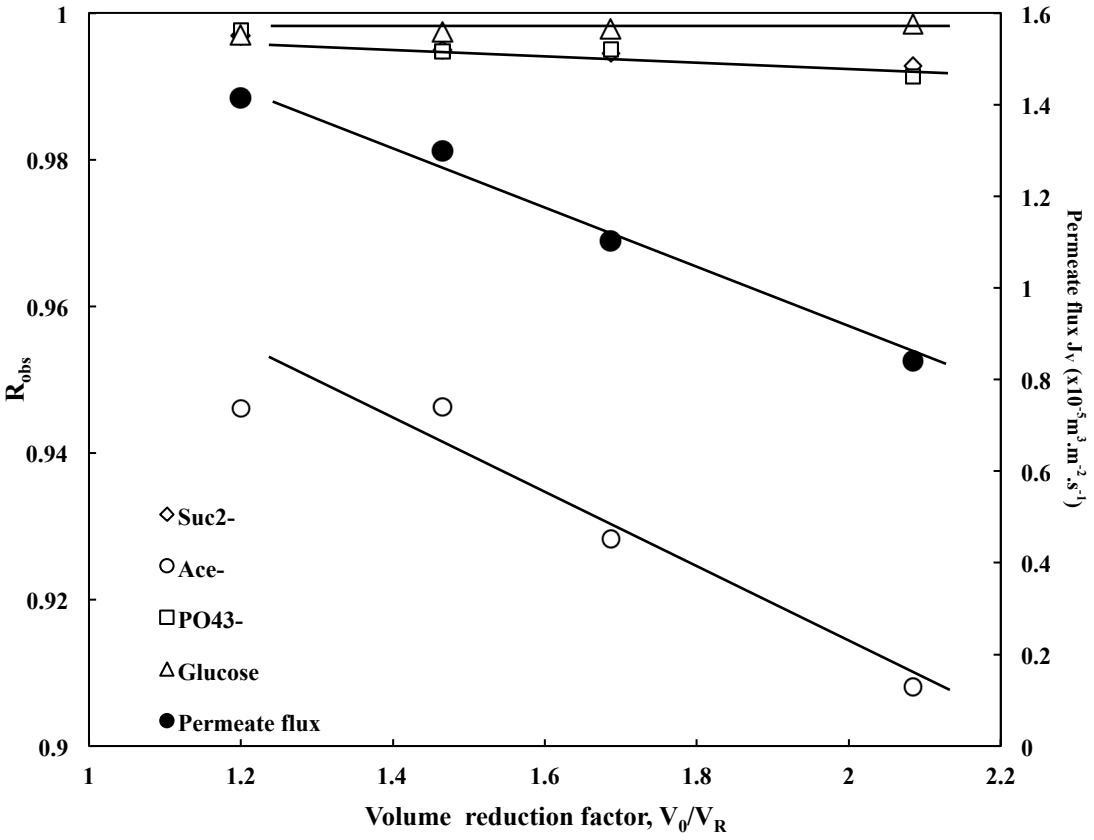


Figure 4.36 Retention of succinate, acetate, phosphate, glucose and permeate flux as function of volume reduction factor in a concentration mode - at pH 7 - ΔP =20 bar.

From the mass balance equation in concentration mode (Eq. 4.5), the volume reduction factor depends on the concentration factor (C_r/C_0) and succinate retention. Then, the solute concentration in retentate can be calculated by Eq. 4.5 as constant retention (the retention values equal to the constant/mean value obtained in Fig. 4.36). Fig 4.37 shows the predicted values of solute concentration in retentate versus the volume reduction factor. As expected according to succinate retention ($R_{Suc} \approx 99$ %), the succinate concentration is about 0.34 M for the volume reduction is higher than 2.

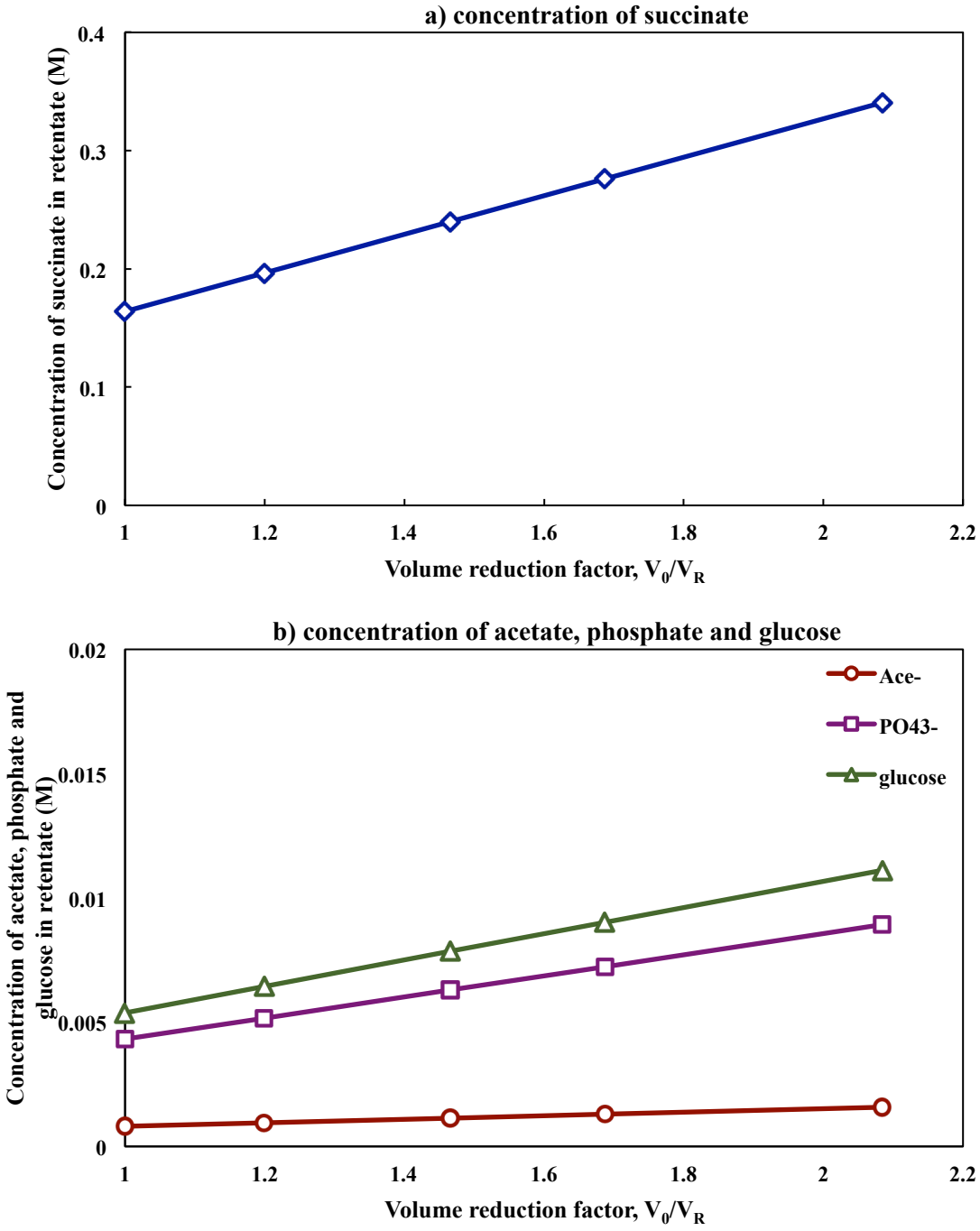


Figure 4.37 Concentration of succinate, acetate, phosphate and glucose as function of the volume reduction factor in a concentration mode - at pH 7 - ΔP =20 bar. (a) concentration of succinate; (b) concentration of acetate, phosphate and glucose.

The concentration of purified succinate solution has been carried out at 20 bar. As expected, the concentration of solute increases with increasing the volume reduction factor since retention of solute is close to 100%. The variation of solute concentration in retentate versus the volume reduction factor is plotted in Fig. 4.38. It can observe that the concentration of succinate ($R_{Suc} \approx 99$ %) strongly increases from 0.16 M to 0.34 M when the volume reduction factor increases from 1 to 2.1, while the concentration of acetate ($R_{ace} \approx 93$ %) is slightly increases. The concentration of phosphate ($R_{PO43-} \approx 99$ %) and glucose ($R_{glucose} \approx 99$ %) increased during concentration step. Also, these results are in agreement with the predicted values. Furthermore, the concentration of solute versus the volume reduction factor is also reported in Fig. 4.39. One can observe that the concentration of succinate, phosphate and glucose continuously increases with the volume reduction factor.

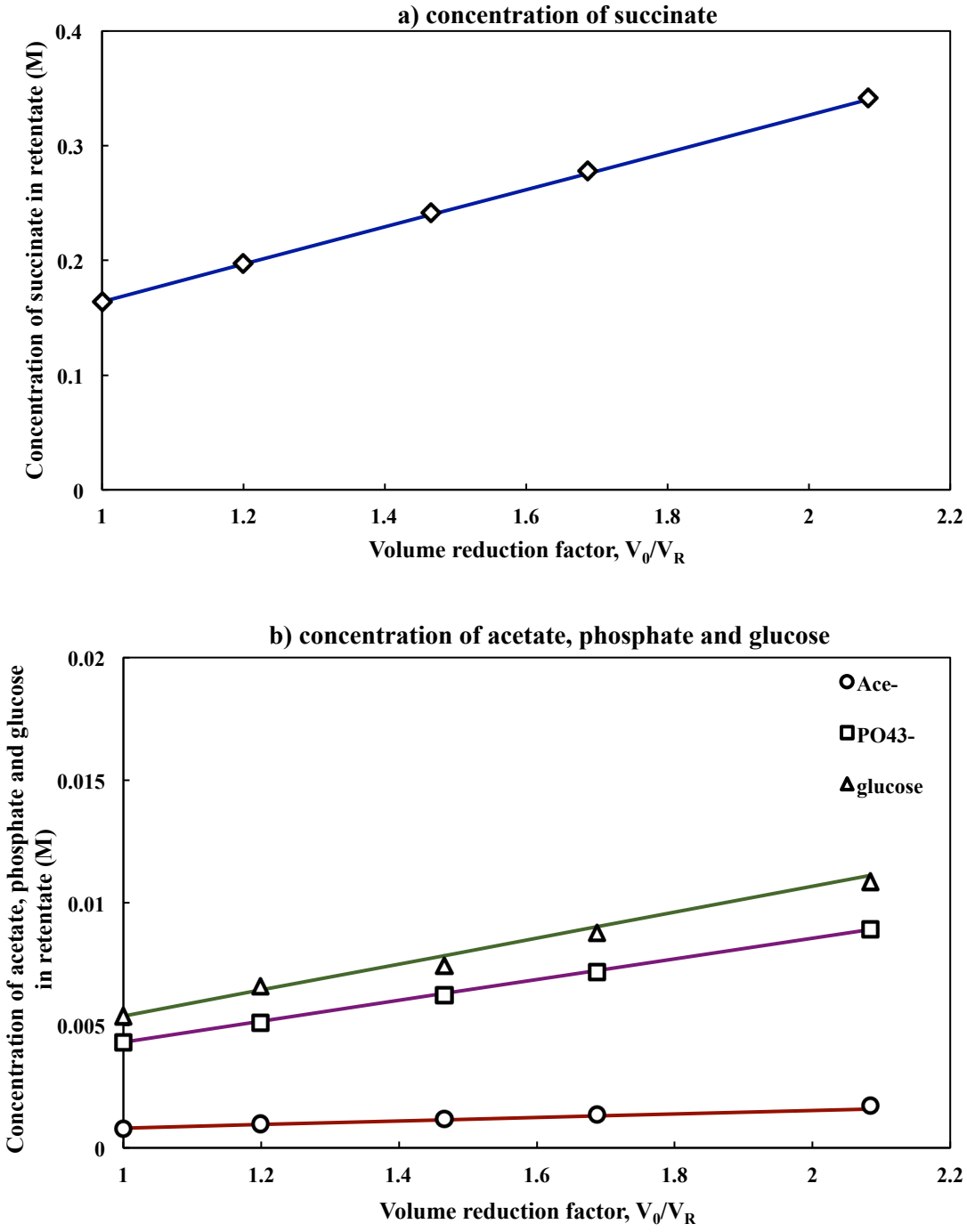


Figure 4.38 Concentration of succinate, acetate, phosphate and glucose in retentate as function of the volume reduction factor in a concentration mode - at pH 7- ΔP =20 bar. (a) concentration of succinate; (b) concentration of acetate, phosphate and glucose. The lines are the predicted values.

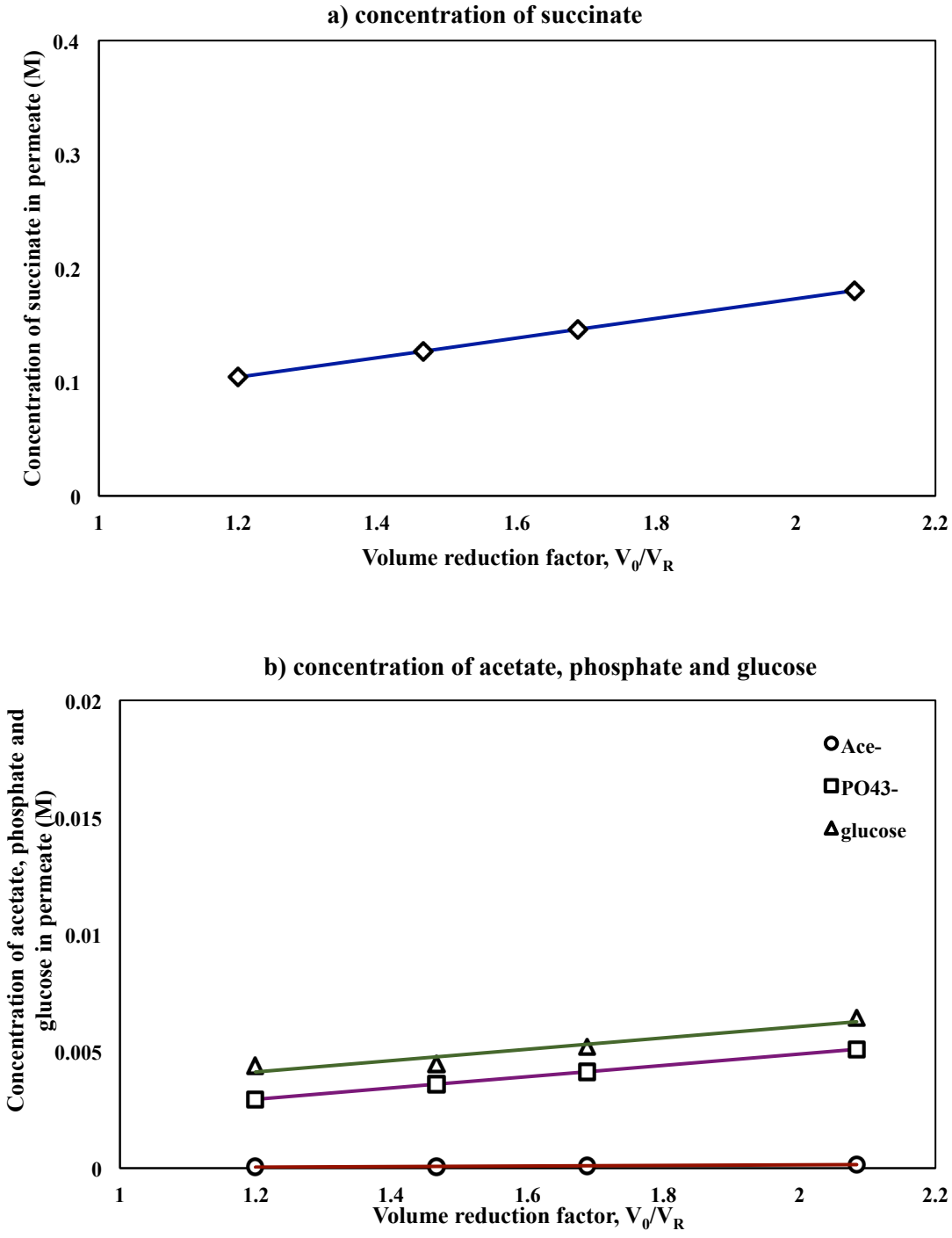


Figure 4.39 Concentration of succinate, acetate, phosphate and glucose in permeate as function of the volume reduction factor in a concentration mode - at pH 7- ΔP =20 bar. (a) concentration of succinate; (b) concentration of acetate, phosphate and glucose. The lines are the predicted values.

In addition, the composition of initial fermentation broth, before/after diafiltration and after concentration step shown in Table 4.15. As expected, the concentration of solutes decresases during diafiltration. One can observe that chloride ions are completely removed from fermentation broth. After concentration step, the solute concentrations increases by 2 times its initial value for the volume reduction factor equal to 2.1 since the solute retentions (≈ 99 %). Then, it was possible to recover the initial succinate concentration by using concentration step.

Table 4.15 The composition of initial fermentation broth, before/after diafiltration and after concentration step.

Feed composition	**Initial fermentation broth**	**Before diafiltration (in the retentate)**	**After diafiltration (in the retentate)**	**After concentration step (in the retentate)**
succinate	0.35 M	0.175 M	0.16 M	0.34 M
acetate	0.065 M	0.0325 M	0.001 M	0.002 M
chloride	0.0045 M	0.0023 M	-	-
phosphate	0.017 M	0.0085 M	0.004 M	0.009 M
glucose	0.027 M	0.0135 M	0.005 M	0.01 M

As already reported, the succinate concentration increases during concentration step. Then, the succinate yield slightly decreased with increasing the volume reduction factor while the succinate purity was constant (Fig. 4.40). The succinate purity and yield obtained with this operation were 99.5% and 99.3%, respectively.

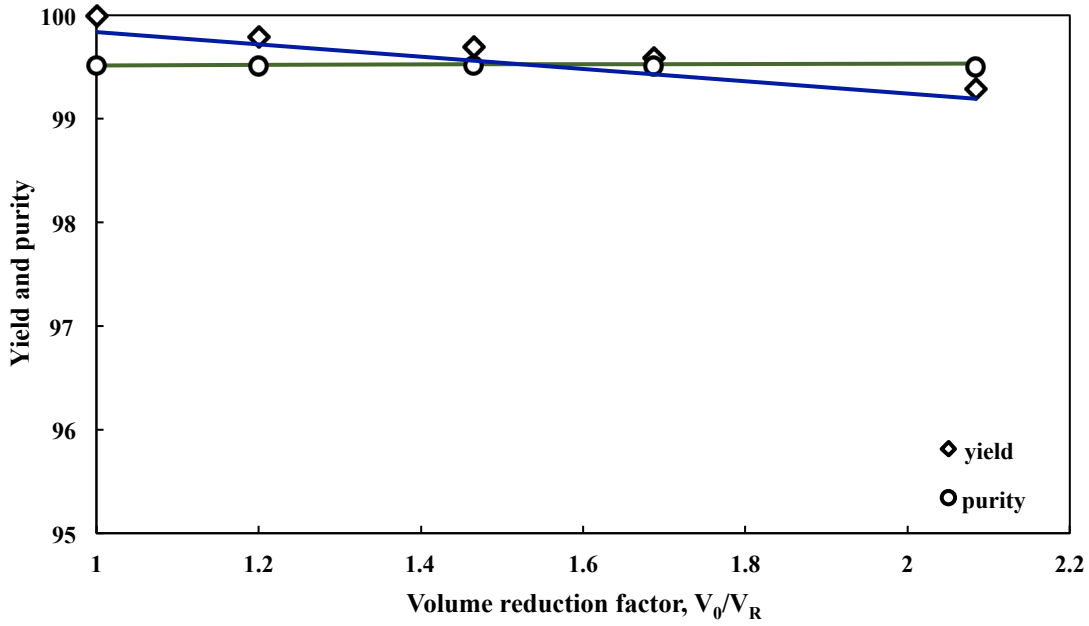


Figure 4.40 Yield and purity of succinate as function of volume reduction factor in a concentration mode.

Discussion

As observed in previous results, the concentration of succinate in retentate decreased slower than that of acetate during diafiltration. This result is in agreement with the work of Kang and chang (2005). They reported that the succinate concentration slightly decreased, while the concentration of other organic acid salts (lactate, acetate and formate) strongly decreased with time, i.e. for increasing the number of diavolumes. However, the retention of succinate in present work was higher than that observed in Kang's work. Since the initial succinate concentration in this work was lower than 0.2 M, a high retention was obtained.

The combination mode of the two steps process is proposed in this work, i.e. dilution/diafiltration/concentration operations. It was possible to achieve the purification of the fermentation broth, i.e. to increase the

succinate purity in the fermentation broth from 85% to 99.5% while minimizing the succinate loss, keeping the total yield higher than 92%.

Conclusion

The separation between succinate and acetate in the fermentation broth containing high succinate concentration (0.3 - 0.7 M) is achievable by using the combination of dilution/diafiltration/concentration operations. The synthetic fermentation broth was first diluted to a given concentration to make the succinate/acetate separation feasible. NF was then used in a diafiltration mode in order to achieve the purification of succinate, i.e. the removal of acetate. Finally, a concentration step by RO was used to recover the succinate concentration. In this manner, the succinate purity is remained higher than 92% and the succinate purity is about 99.5%.

4.5 Conclusion

The aim of this work was to investigate nanofiltration as a purification step in the production of succinic acid from fermentation i.e removal of impurities such as acetate. Experiments were carried out with synthetic solutions of increasing complexity were used to investigate the influence of the operating conditions as well as of the broth composition on the transfer mechanisms. It was observed that both succinate and acetate transfer are strongly affected by the organic salt concentration due to charge effects. More precisely, a good correlation has been observed between succinate retention and its divalent ionic fraction. Considering the succinate /acetate separation it was shown that the nanofiltration performances are improved for decreasing salt concentration. Then, based on these knowledge of the transfer

mechanisms, a methodology has been proposed to achieve the purification of a succinate fermentation broth. The succinate/acetate separation has been carried out using the combination of dilution/diafiltration/concentration operations. As expected, the succinate purity in the fermentation broth increases from 85 % to 99.5 % while minimizing the succinate loss, keeping the total yield higher than 92 %. Then, the purification of a succinate fermentation broth can be achieved.

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CHAPTER V

CONCLUSION AND PERSPECTIVES

5.1 Conclusion

This research works reported the enhancement of succinate production from xylose in *E. coli* KJ122 and its purification by nanofiltration. There are two parts comprised in novelty of this study including improvement of xylose fermentation in *E. coli* KJ122 by using metabolic engineering and metabolic evolution and purification of succinate from fermentation broth by nanofiltration.

First, *E. coli* KJ122 was developed for the fermentation of xylose to succinate by deleting *xylFGH* genes and using metabolic evolution. The resulting strain, named KJ12201 showed an increase in xylose consumption and succinate production compared with *E. coli* KJ122. After metabolic evolution of *E. coli* KJ12201, the 14^{th} transfer-isolated strain, named KJ12201-14T exhibited a high ability to consume xylose with high accumulation of succinate (84 g/L) in mineral salts medium (AM1) under batch fermentation. Under fed-batch fermentation, KJ12201-14T could produce succinate at 84 g/L with the yield and productivity of 0.85 g/g and 1.01 g/L.h, respectively within 84 h. Also, acetate was detected at 14 g/L as a by-product. Considering these results, KJ12201-14T could be a potential strain for the economic succinate production using abundant and feasible renewable substrates such as xylose, which are readily available in Thailand.

Second, nanofiltration was investigated as a purification step in the production of succinate from fermentation. The experimental investigation was performed with solutions of increasing complexity, i.e. single-solute solutions of succinate and acetate, binary-solute solutions containing succinate and acetate. A synthetic fermentation broth containing succinate and impurities was also performed. The investigation of the mass transfer of solute has been carried out with single and binary-solute solutions in constant concentration mode. In single-solute solutions, the retention of succinate depends on its concentration because its mass transfer was governed by charge effects. Succinate retention increases with increasing pH from 2.2 to 7.6 due to the increase of electrostatic interaction between the charged solute and the fixed charge on the membrane surface. More precisely, a good correlation has been observed between succinate retention and its divalent ionic fraction. The counter-ions such as Na^+ and K^+ have no effect on succinate retention because the retention is fixed by charge and size of succinate. In addition, it was observed that the retention of succinate is higher than that of acetate as expected from its size and charge. Further, experiments were carried out with binary-solute solutions containing succinate and acetate. At high salts concentration containing 0.7 M succinate and 0.1 M acetate, pH has no influence on the separation between succinate and acetate due to the screening effect. According to the results, the separation was expected to be feasible at low salts concentration. Then, the separation between succinate and acetate was evaluated at various succinate concentrations (0.1 - 0.7 M succinate). As expected, the separation was found to be unachievable in binary mixture containing 0.7 M succinate and 0.1 M acetate. For decreasing in succinate concentration from 0.7 to 0.1 M, the succinate is more retained than acetate. Then, the succinate/acetate

separation can be achieved. In addition, it was shown that both succinate and acetate transfer are strongly affected by the organic salt concentration due to charge effects. Furthermore, the experiments were performed with binary solutions containing 0.7 M succinate and 0.1 M acetate at different dilution factor. Indeed, it was confirmed that the separation factor is higher when decreasing salts concentrations. Then, significant improvement of the succinate/acetate separation can be achieved by diluting a feed solution. Considering the succinate/acetate separation it was shown that the NF performances are improved for decreasing salt concentration

In order to evaluate the influence of the fermentation broth composition on the separation, the experiment was carried out with synthetic fermentation broths containing succinate and impurities. The composition of the synthetic fermentation broth considered in this study which was given in table 4.14 at pH 7. Based on the knowledge of the transfer mechanisms, the separation of succinate and acetate is not achievable for succinate concentration higher than 0.4 M. Then, the synthetic fermentation broth was first diluted down to a given concentration to make the succinate/acetate separation feasible. NF was then used in a diafiltration mode in order to achieve the purification of succinate, i.e. the removal of acetate. Finally, the concentration step by RO was used to recover the initial succinate concentration. In this manner, the succinate purity was increased from 85 % to 99.5 % while the total yield remained higher than 92 %.

The outcome of the studies can contribute to the current biotechnological production of succinate include the following 4 main point (Fig. 5.1):

1. The combination of metabolic engineering and metabolic evolution can be useful for the improvement of xylose metabolism and succinate yield in *E. coli* KJ122;

2. Introducing the fed-batch fermentation to improve productivity in succinate production from lignocellulosic biomass like xylose;

3. The knowledge of the transfer mechanisms of solutes through NF membrane can use to design the process for succinate purification from fermentation broth;

4. A diafiltration combined with concentration operations can be used as final purification step in the downstream processing.

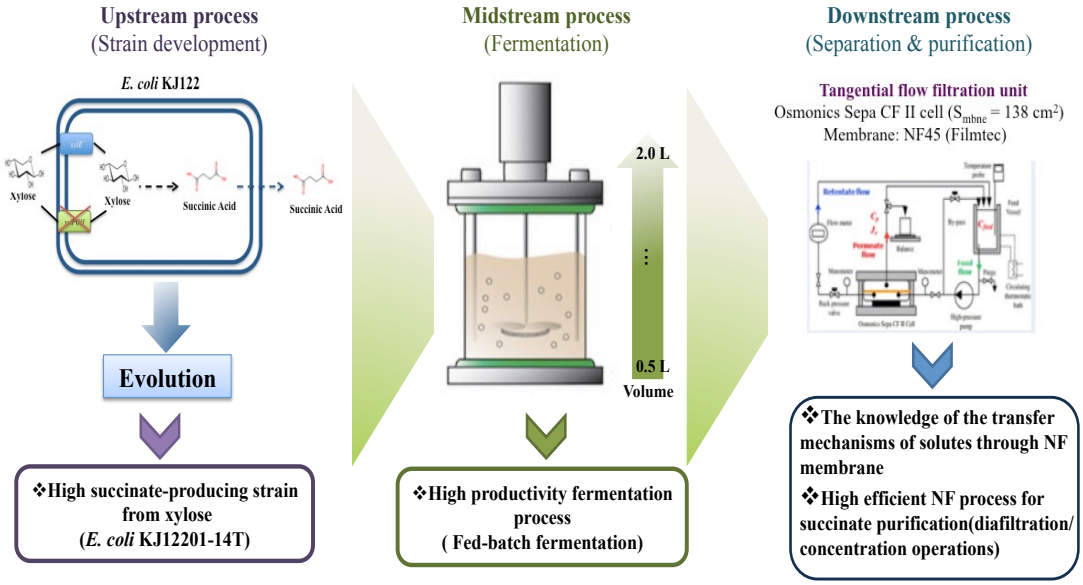

Figure 5.1 The diagram for an overview outcome implemented in this work.

5.2 Perspectives

In this work, *E. coli* KJ122 was developed to ferment pure xylose into succinate using *xylE* for xylose uptake. Lignocellulosic hydrolysates is comprised of glucose, xylose and other sugars . Therefore, in order to achieve maximum product yield and productivity, a complete utilization of mixed sugars derived from lignocellulosic hydrolysates is essential (Saha 2003). Further development, strain KJ122-14T will be optimized with sugar mixtures, and in lignocellulosic hydrolysates using a simple mineral salts medium. Indeed, bioreactor design and scale-up are important in the

development of bioprocess. Hence, the optimization process of succinate production in commercial scale by derivative of KJ122 (strain KJ12201-14T) using lignocellulosic feedstock will be proposed to find out the most suitable condition for succinate production.

In order to produce succinate by fermentation, the purification process for succinate should be required due to the many impurities in the fermentation broth, such as by-produced acids, carbon sources and salts. Traditional downstream processes are designed around one or several precipitation stages which produce large amounts of effluents with a high salt content (Bailey and Ollis, 1986). In order to reduce the environmental impact, membrane technologies (nanofiltration or electrodialysis) were proposed to replace precipitation. According to these results, nanofiltration could achieve the purification of succinate by removing acetate; however, the succinate and acetate separation was found to be hardly achievable for high salts concentration (Narebska and Kurantowicz, 1998; Li and Shahbazi, 2006). Electrodialysis (ED) is also a novel membrane process that is very attractive since the generation of effluents or by products can be significantly reduced when compared with the chemical separation process. Bailly *et al.* (2001) reported ED can be used as organic acid recovery process. Therefore, ED will be proposed on succinate purification from fermentation broth to compare the selectivity of succinate.

5.3 References

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APPENDICES

APPENDIX A

IRREVERSIBLE THERMODYNAMICS

For NF membrane, the transport of solute through a membrane can be described by irreversible thermodynamics. The solute flux through the membrane can be described as the sum of convective and a diffusive flux. Solute transport by convection takes place because of an applied pressure gradient across the membrane. A concentration difference on both sides of the membrane causes diffusive transport. The retention of solute is defined as:

$$R = \frac{(1-exp(-J_v A))\sigma}{1-\sigma \exp(-J_v A)} \quad \text{(A.1)}$$

and

$$A = \frac{1-\sigma}{P_{SK}} \quad \text{(A.2)}$$

where σ is a reflection coefficient and the solute permeability (P_{SK}) can be estimated by

$$P_{SK} = \frac{\bar{P}}{\Delta x} \quad \text{(A.3)}$$

where $\bar{P}$ is the solute permeability coefficient and Δx is the effective membrane thickness.

Moreover, σ and P_{SK} can be estimated directly from the R_{obs}, since R depends on values of the two coefficients, σ and P_{SK}.

By plotting the data of solutes retention as a function of the permeate flux, σ is the maximum value of the solutes retention at high permeate flux. Also, P_{SK} can be estimated from the slope of the plot.

$$\lim_{J_v \to \infty} R = \sigma \tag{A.4}$$

$$\lim_{J_v \to 0} \frac{dR}{dJ_v} = \frac{\sigma}{P_{SK}} \tag{A.5}$$

APPENDIX B

DISSOCIATION EQUILIBRIUM OF SUCCINIC ACID

An organic acid like succinic acid is the carboxylic acid. The succinic acid form depends on pH. Succinic acid act as two carboxyl groups then the two forms of succinate were generated. At pH 4.2 (pK_{A1}), one of carboxyl group is dissociated then the monovalent from of succinate was fond. When increasing the pH to 5.6 (pK_{A2}), the divalent from of succinate was presented since the carboxyl group is deprotonated. The dissociation equilibrium equation of succinic acid can be follows:

$$H_2Suc \rightleftharpoons HSuc^- + H^+ \tag{B.1}$$

and

$$HSuc^- \rightleftharpoons Suc^{2-} + H^+ \tag{B.2}$$

The dissociation of succinic acid described by the acid dissociation constant, K_A. As succinic acid has two carboxyl groups, K_A is defined by the following equation.

$$K_{A1} = \frac{[HSuc^-]+[H^+]}{[H_2Suc]} = 6.2 \times 10^{-5} \tag{B.3}$$

and

$$K_{A2} = \frac{[Suc^{2-}]+[H^+]}{[HSuc^-]} = 2.3 \times 10^{-6} \tag{B.4}$$

As K_{A1} and K_{A1} of succinic acid are 6.2 x 10^{-5} and 2.3 x10^{-6}, respectively. Then, pK_{A1} and pK_{A2} can be estimated by

$$pK_{A1} = -\log K_{A1} = -\log(6.2 \times 10^{-5}) = 4.2 \quad \text{(B.5)}$$

and

$$pK_{A1} = -\log K_{A2} = -\log(2.3 \times 10^{-6}) = 5.6 \quad \text{(B.6)}$$

Another important point is the relationship between pH and the pK_A of succinic acid. This relationship is described by the following equation.

$$\frac{[HSuc^-]}{[H_2Suc]} = 10^{[pH-pK_{A1}]} \quad \text{(B.7)}$$

and

$$\frac{[Suc^{2-}]}{[HSuc^-]} = 10^{[pH-pK_{A2}]} \quad \text{(B.8)}$$

At pH=pK_{A1}= 4.2, the ratio of $[HSuc^-]/[H_2Suc]$ is equal to 1. This mean that the half of the succinate has dissociated to monovalent ions (50 % H_2Suc and 50 % $HSuc^-$). In addition, pH=pK_{A1}= 5.6, the ratio of $[Suc^{2-}]$ / $[HSuc^-]$ is one. Succinic acid is completely dissociated. Then, two forms of succinate ions (monovalent ions and divalent ions) are observed (50% of $HSuc^-$ and 50 % of Suc^{2-}). Moreover, at pH =1/2(pK_{A1} + pK_{A2}), the concentration of H_2Suc is equal to Suc^{2-}. The variation of the different forms of succinic acid versus the pH is plotted in Fig. B.1.

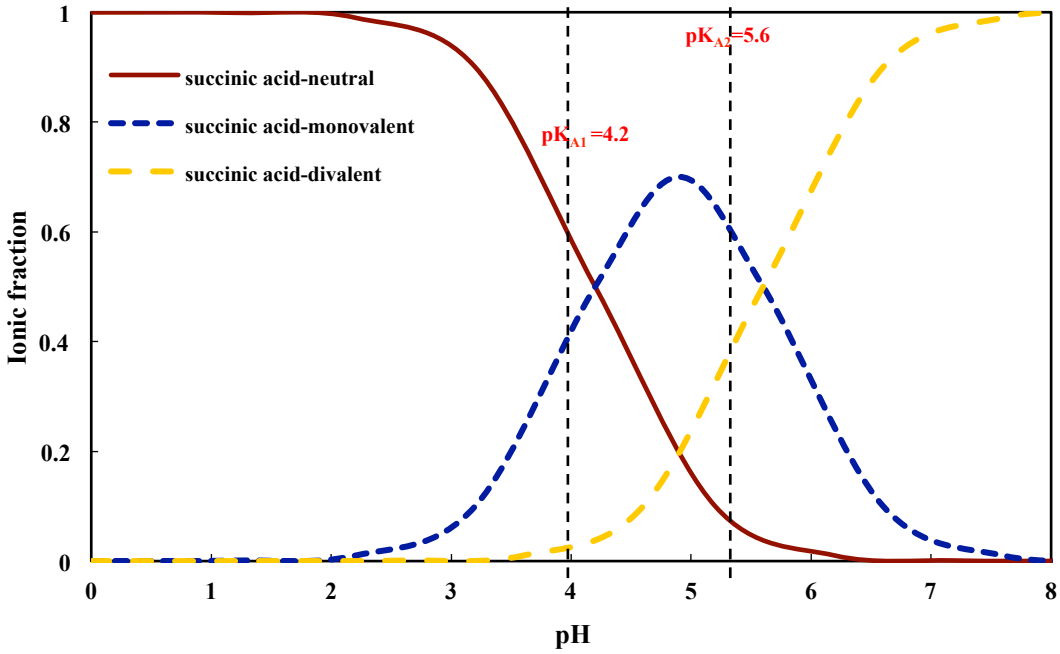

Figure B.1 Ionic fraction of succinic acid as function of pH.

APPENDIX C

THE NEGATIVE RETENTION OF ACETATE

In a mixed salts solution containing a potassium succinate and potassium acetate, the negative retention of acetate in the presence of succinate can be explained by the electroneutrality equation.

In general, the retention of solute can be defined by:

$$R = 1 - \frac{C_P}{C_R} \tag{C1.1}$$

Therefore,

$$\frac{C_P}{C_R} = 1 - \mathrm{R} \tag{C1.2}$$

and

$$C_P = C_R(1 - R) \tag{C1.3}$$

For the constant feed concentration mode, the electroneutrality in the permeate and in the retentate can be defined as:

$$2[Suc^{2-}] + [Ace^{-}] = [K^{+}] \tag{C1.4}$$

Then, the ratio of the ion concentrations in the permeate to the retentate:

$$\frac{2[Suc^{2-}]_P + [Ac^{-}]_P}{2[Suc^{2-}]_R + [Ac^{-}]_R} = \frac{[K^{+}]_P}{[K^{+}]_R} \tag{C1.5}$$

and the retention of K+ can be expressed as:

$$R_{K+} = 1 - \frac{[K^{+}]_P}{[K^{+}]_R} \tag{C1.6}$$

Then,

$$\frac{[K^+]_P}{[K^+]_R} = 1 - R_{K+} \quad \text{(C1.7)}$$

Combining Eqs. A7 and A5, we obtain:

$$\frac{2[Suc^{2-}]_P + [Ace^-]_P}{2[Suc^{2-}]_R + [Ace^-]_R} = 1 - R_{K+} \quad \text{(C1.8)}$$

As the expression of the succinate concentration in the permeate is presented by:

$$[Suc^{2-}]_P = [Suc^{2-}]_R\,(1 - R_{Suc2-}) \quad \text{(C1.9)}$$

Then,

$$\frac{2([Suc^{2-}]_R\,(1-R_{Suc2-})) + [Ace^-]_P}{2[Suc^{2-}]_R + [Ace^-]_R} = 1 - R_{K+} \quad \text{(C1.10)}$$

Eqs. A1.10 can be divided by the acetate concentration in retentate $[Ac^-]_R$:

$$\frac{2\,{[Suc^{2-}]_R}/{[Ace^-]_R}\,(1-R_{Suc2-}) + {[Ace^-]_P}/{[Ace^-]_R}}{2\,{[Suc^{2-}]_R}/{[Ace^-]_R} + 1} = 1 - R_{K+} \quad \text{(C1.11)}$$

From Eqs. A1.11, α can be used instead of the ratio of succinate to acetate in retentate:

$$\alpha = {[Suc^{2-}]_R}/{[Ace^-]_R} \quad \text{(C1.12)}$$

and

$$\frac{[Ace^-]_P}{[Ace^-]_R} = 1 - R_{Ace-} \quad \text{(C1.13)}$$

Then,

$$\frac{2\alpha\,(1-R_{Suc2-}) + (1-R_{Ac-})}{2\alpha + 1} = 1 - R_{K+} \quad \text{D1.14}$$

and

$$R_{Ace-} = 2\alpha(R_{K+} - R_{Suc2-}) + R_{K+} \quad \text{(C1.15)}$$

Eqs. A1.15 can be divided by the retetntion of potassium (R_{K+});

$$\frac{R_{Ace-}}{R_{K+}} = \frac{2\alpha(R_{K+}-R_{Suc2-})}{R_{K+}} + \frac{R_{K+}}{R_{K+}} \quad \text{(C1.16)}$$

Then,

$$R_{Ace-} = R_{K+}\left[1 + 2\alpha\left(1 - \frac{R_{Suc2-}}{R_{K+}}\right)\right] \quad \text{(C1.17)}$$

Using β as the ratio of succinate retention to potassium retention:

$$\beta = {R_{Suc2-}}/{R_{K+}} \quad \text{(C1.18)}$$

Therefore, the acetate retention in the mixing solution can be defined by:

$$\boldsymbol{R_{Ac-} = R_{K+}[1 + 2\alpha(1 - \beta)]} \quad \text{(C1.19)}$$

APPENDIX D

LIST OF PRESENTATIONS

2016 **Khunnonkwao P.**, Jantama K., Kanchanatawee S., Galier S., and **Roux-de Balmann H.** Integration of nanofiltration in the production of succinic acid from fermentation of lignocellulosic material, 5th International Congress on Green Process Engineering (GPE 2016), June 19-24, 2016, Mont Tremblant, Quebec, Canada (Oral presentation).

2015 **Khunnonkwao P.**, Jantama K., Kanchanatawee S., Galier S., and **Roux-de Balmann H**. Purification of succinic acid from fermentation broth using nanofiltration, Euromembrane, September 7-10, 2015, Aachen, Germany (Poster Presentation).

APPENDIX E

LIST OF PUBLICATION

2016 **Khunnonkwao P.**, Jantama K., Kanchanatawee S., Galier S., and Roux-de Balmann H. Integration of nanofiltration in the production of succinic acid from fermentation of lignocellulosic material. 5th International Congress on Green Process Engineering (GPE 2016), June 19 - 24, 2016, Mont Tremblant, Quebec, Canada (article in press).

BIOGRAPHY

Miss Panwana Khunnonkwao was born on January 19th, 1985 in Chaiyaphum province, Thailand. She attended and finished Bachelor Degree of Science (1st Class HONS.) in Food Technology, Ubon Ratchathani University, Thailand in 2008. After graduation, she obtained a scholarship awarding to graduate students whose lecturers are awarded research funding by outside sources (OROG) to further participate a graduate study at the Suranaree University of Technology (SUT). In 2010, she finished her master degree in school of Biotechnology, Institute of Agriculture Technology. Her research topic was entitled of "Purification of L-(+)-lactic acid from fermentation broth by pervaporation-assisted esterification technique" in 2012 her work had been published in Process Biochemistry Journal in the title of "Purification of L-(+)-lactic acid from pretreated fermentation broth using vapor permeation-assisted esterification". After accomplished master degree, she continued her PhD program in the same year. In 2013, she had awarded an international joint PhD program between SUT and Université de Toulouse III-Paul Sabatier (UPS), Toulouse, France. Her research work emphasized on improved xylose uptake and utilization in metabolically engineered *Escherichia coli KJ122* for succinate production taken a place in school of Biotechnology, SUT, Thailand. She performed a part of her research on purification of succinate by nanofiltration in Laboratoire de Génie Chimique, UPS, France. Thailand. During her attendance in France, she was fully financial funded under the 2013 Franco-Thai Joint Research Project (PCH- SIAM) and Franco-Thai Scholarship 2014/2015.