DEVELOPMENT OF A THERMOCHEMICAL PROCESS FOR HYDROLYSIS OF POLYLACTIC ACID POLYMERS TO L-LACTIC ACID AND ITS PURIFICATION USING AN ENGINEERED MICROBE

By

DIANE SYLVIE CHAULIAC

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To my aunt “Lolo”, who taught me how precious life is, I’ll live it fully, stronger and happier.

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$(NH_4)_2SO_4$	Ammonium sulfate
[D-LA]	D-Lactic acid concentration
[L-LA]	L-Lactic acid concentration
[LA]	Lactic acid concentration
[Lac]	Lactic acid mass fraction at time t
$[Lac]_{max}$	Maximum lactic acid concentration
[Na-L-LA]	Sodium L-lactic acid concentration
[NaOH]	Sodium hydroxide concentration
°C	Celsius degree
BCA	Bicinchoninic acid assay
BL	(R)-β-Butyrolactone
CAGR	Compound annual growth rate
CAPSO	*N*-Cyclohexyl-2-hydroxyl-3-aminopropanesulfonic acid
cm	Centimeter
Compatibilizer	Also referred to as coupling agents, are additives, that when added to a blend of immiscible materials during extrusion, modify their interfacial properties and stabilize the melt blend
$CuSO_4$	Cupric sulfate
D-LA	D-Lactic acid
D-LDH	D-Lactate dehydrogenase
DCPIP	2,6-Dichlorophenolindophenol
DNA	Deoxyribonucleic acid
DNase	Desoxyribonuclease
DXO	1,5-Dioxepan-2-one
E. coli	*Escherichia coli*

e.g.	*Exempli gratia*
E_a	Activation energy
EDTA	Ethylenediaminetetraacetic acid
etc.	*Et cetera*
FAD	Flavin adenine dinucleotide
FDA	Food and drug administration
$FeSO_4$	Ferrous sulfate
Flexural modulus	Pressure used as a gauge to compare relative bending stiffness of various plastics. Measure obtained through a 3 points bending test
Flexural strength	Pressure (load per surface area) to apply to break the material during a 3 points bending test
FMN	Flavin mononucleotide
FRT	FLP Recombination Target
g	Gram
g/L	Gram per liter
GA	Glycolic acid
$g_{D\text{-}LA}$	Gram of D-LA
g_{DW}	Gram cell dry weight
gras	Generally regarded as safe
h	Hour
H_20	Water
H_2SO_4	Sulfuric acid
HEPES	4-(2-HydroxyEthyl)-1-piperazineethanesulfonic acid
HPLC	High-performance liquid chromatography
i.e,	*Id est* (That is)
K	Kelvin

k’	Apparent maximum hydrolysis rate
kan	Kanamycin resistance gene
kDa	Kilo daltons
KH_2PO_4	Monopotassiun phosphate
*K*m	Michaelis constant
ktpa	kilo tons per annum
L	Liter
L-LA	L-Lactic acid
L-LDH	L-Lactate dehydrogenase
LA	Lactic acid
LB	Luria Bertani broth
LDH	Lactate dehydrogenase
M	Molar
MES	2-(*N*-Morpholino)ethanesulfonic acid
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4.7\ H_20$	Magnesium sulfate
min	Minute
mL	Milliliter
MM	Minimal medium
mM	millimolar
mm	millimeter
Modulus	(Young’s Modulus) Measurement of the stiffness of an elastic material. It is the flexural modulus obtained in the initial linear stress to strain curve
MS	Microsoft
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt

MW	Molecular weight
N. meningitidis	*Neisseiria meningitidis*
Na	Sodium
Na_2HPO_4	Sodium phosphate dibasic
NaCl	Sodium chloride
$NaMoO_4$	Sodium molybdate
NaOH	Sodium hydroxyde
nm	Nanometer
nt	Nucleotide
O_2	Oxygen
OD_{420}	Optical density (420 nm)
PAGE	Polyacrylamide gel electrophoresis
PCL	Polycaprolactone
PCR	Polymerase chain reaction
PDLA	PLA made of D-Lactide
PDLLA	PLA made of D- and L-lactide
PEG	Poly(ethylene glycol)
PEO	Poly(ethylene oxide)
PET	Polyethylene terephtalate
PHB	Polyhydroxybutyrate
PLA	Polylactic acid
PLLA	PLA made of L-Lactides
R	Universal gas constant
RNase	Ribonuclease
ROP	Ring Opening Polymerization

rpm	Rotation per minute
RT	Room temperature
s	Second
Sb-PLA	Stereoblock-PLA
Sc-PLA	Stereocomplex PLA
SOC	Super Optimal Broth
SSP	Solid-State Polycondensation
Strain (Mecanics)	Normalized measure of deformation, dimensionless
t	Time
T	Absolute temperature in Kelvin
t_0	Time at the start of the experiment
Td	Doubling time (h)
Tensile strength	Also called ultimate strength, is the maximum stress that a material can withstand while being stretched or pulled before necking
Tg	Glass transition temperature
Tm	Melting temperature
TMC	Trimethylene carbonate
UV	Ultraviolet
Vm	Maximum specific activity
w/v	Weight / Volume
λ	Lag phase duration
µg	Microgram
µL	Microliter
µm	Micrometer
µmole	Micromole

Abstract of Dissertation Presented to the Graduate School
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DEVELOPMENT OF A THERMOCHEMICAL PROCESS FOR HYDROLYSIS OF POLYLACTIC ACID POLYMERS TO L-LACTIC ACID AND ITS PURIFICATION USING AN ENGINEERED MICROBE

By

Diane Sylvie Chauliac

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Polylactic acid polymer (PLA), produced from renewable resources is an alternative to petroleum-derived plastics. Thermochemical hydrolysis of the PLA-based plastics is an effective method of recycling them at the end of use to generate the constituent monomer, lactic acid (LA) that can be reused to produce PLA. Temperature-dependent release of LA from PLA beads in water follows apparent first order decay kinetics after a short lag. In the presence of limiting amount of NaOH, a concentration-dependent immediate release of LA, apparently from the amorphous regions of the beads, was detected. The rate of hydrolysis of PLA was higher in the presence of NaOH compared to water alone and this was dependent on particle size. Racemization of released LA was not detected during hydrolysis in water or with limiting amount of NaOH. D-LA removal from the resulting syrup was achieved using an *Escherichia coli* lacking all three L-lactate dehydrogenases identified. This bacterial biocatalyst was able to metabolically remove all the D-LA present in 1.5 M PLA-derived syrup in about 25 h, at 37°C. This represents an average productivity of 0.173 $g_{D\text{-}LA}/(g_{DW}.h)$. These results show that PLA-based plastics can be rapidly converted to optically pure L-LA for reuse using a combination of thermochemical and bio-based processes.

CHAPTER 1
REVIEW OF THE SYNTHESIS AND DEGRADATION OF POLY LACTIC ACID POLYMERS

Biodegradable Polymers

Drivers and Rationale for the Use of Biodegradable Polymers

In 2011, 280 million tonnes of petroleum derived plastics were produced world-wide and current average per capita plastics consumption of about 26 kg is estimated to increase by about 4% every year through 2016 (124). Manufacture of these non-renewable plastics utilizes toxic non-renewable chemicals. In addition, these non-biodegradable polymers accumulate in the environment creating environmental hazard both in the land, rivers and ocean (1, 59, 154, 155). Accumulating information points to potential health hazards and diseases that can be directly related to the use of certain plastics in consumer products (132, 155). Food stored in some plastic containers is being recognized as carrying toxic leachates from the containers making it unfit for human consumption (94, 95). Owing to concerns over the effects of such leachates, government agencies in Australia, Canada, the European Union, and the United States have restricted or prohibited the use of phthalates in consumer products (189). These concerns led researchers to create new forms of plastics, which are renewable, biodegradable and biocompatible (26, 106, 144, 174). Bio-based polymers, also called biopolymers when the polymer naturally exists in nature, reduce oil consumption and alleviate some of the environmental burden petroleum-based plastics cause. Various biodegradable polymers are available on the market and classified as follows:

- Agro-polymers from agricultural such as the agro-polymers from agro-resources, e.g., starch, cellulose,
- Biopolymers produced by microbial activity, e.g., the Polyhydroxyalkanoates,

- Bio-based polymers conventionally and chemically synthesized and whose monomers are obtained from agricultural resources, e.g., polylactic acid (PLA),

- Polymers whose monomers and polymers are obtained conventionally by chemical synthesis, e.g., polycaprolactones.

Natural polymers and synthetic polymers based on renewable resources are the basis for a sustainable production of eco-efficient plastics that are expected to gradually replace the oil-based family of plastic (110). Compared to various biodegradable polyesters, PLA has one of the highest potential as a replacement for petroleum-based plastics due to its availability on the market and its low price (98, 141, 173). Rising prices of oil make PLA a cost-competitive alternative to petroleum-derived plastics. PLA polymers are aliphatic polyesters produced from lactic acid. PLA products are approved by the FDA for their use in therapy, and can be used for food contact packaging since they are generally regarded as safe (GRAS) (16).

Biodegradable Polymers Market

The global biodegradable plastics market has been witnessing the fastest growth among all plastic and polymer markets in recent times. This high growth is driven by several factors such as increasing awareness and the push for sustainable solutions. In 2005, the global biodegradable plastics market was 94.8 ktpa (kilo tons per annum) compared with 28 ktpa in 2000. Forecast made in 2005 predicted a bio-degradable plastic production of 214.4 ktpa in 2010 (17% CAGR) (125), while the actual amount turned out to be almost double this figure with 422 ktpa of biodegradable plastics consumed in 2011 (6). This latter report also predicted a 22.5% annual growth rate leading to a forecast of 1,200 ktpa of biodegradable plastic by 2016. Despite the rapid increase of the biodegradable plastics market, these values do not compare with the 31,000 ktpa of plastic waste generated in 2010 in the United States alone (30). Western Europe is

the leading consumer of biodegradable plastics and represents 60% of the global market. North America and Asia Pacific regions represent 22% and 18% of the market of biodegradable plastics, respectively. In terms of end use markets, packaging (including rigid and flexible packaging, paper coating and foodservice and loose-fill packaging) is the largest sector in 2005 with 63% of total market volume (125). The packaging segment accounted for 70% of total volume in 2010 and is expected to slightly decrease to about 65% by 2016 as other uses increase. This sector was worth 297 ktpa in 2011 and should increase at a 20.5% compound annual growth rate (CAGR) to reach nearly 770,1 ktpa by 2016. The fibers/fabrics segment is expected to show substantial growth over the forecast period, especially in the hygiene market. The use of biodegradable polymers in fibers and fabrics was valued at an estimated 61 ktpa in 2011 and is expected to reach 197 ktpa in 2016, reflecting a 26.6% compound annual growth rate (CAGR) (6).

PLA Polymers and Lactic Acid Market

Since Ethicon companies introduced a high strength polymer in 1972, PLA has been widely utilized as sutures, dental implants, bone screws and pins because of its biocompatibility with the human body. The PLA market was then primarily related to medical applications due to its high production price. Advances made in the 1980's in the fermentation of corn for production of lactic acid allowed the PLA technology to be accessible for applications other than medical (98). Polylactic acid has emerged as a key product in the biodegradable plastic group. It is not only a biodegradable polymer, but also a complete bio-based polymer. The physical and mechanical properties of PLA make it a good candidate for replacement of petroleum-based plastics in several application areas (102). In 2005, PLA market share represented 38% of the biodegradable plastics market volume, starch polymers and other synthetic polymers constituting 47% and 15% respectively. The PLA market was expected to grow faster than other

biodegradable polymer markets, raising the PLA market share to 43% of the sales of biodegradable plastic in 2011, overpowering the market for starch polymers (i.e., 41%) (125). .The global demand for PLA was 248.8 kilo tons in 2010 and is expected to reach 870.8 kilo tons in 2016, growing at a CAGR of 20.8% from 2011 to 2016. In terms of revenue, the demand was estimated to be worth $1.2 billion in 2010 and is expected to reach $3.8 billion in 2016, growing at a CAGR of 18.7% from 2011 to 2016 (102). European Union is the largest consumer of PLA, mainly owing to stringent packaging regulations. Demand for PLA in Europe is expected to reach $1.4 billion in 2016, growing at a CAGR of 18.3% from 2011 to 2016. Cargill-Dow was the first company to produce PLA on a large scale in 2002. Given the strong market demand and expected PLA market growth, many companies have embarked on polylactic acid production with increased production capacities and building new plants; PURAC biomaterials (The Netherlands), Futtero (Joint Venture between Total petrochemicals and Galactic, Belgium), Mitsui (Japan), LG Chem (Korea) and Hycail (Finland) are other companies worldwide focusing on industrial production of PLA plastics.

PLA Production

Synthesis

Different routes of PLA production are summarized in Figure 1-1. There are three possible processes for the production of PLA. The cheapest method is the condensation of lactate into a prepolymer of low molecular weight (top part of Figure 1-1). This prepolymer is brittle and glassy, which for the most part, is unsuitable for any application unless some external coupling agent is added to the mixture, for increasing the polymer molecular weight. However, the added adjuvants and solvents necessary to achieve a high molecular weight polymer increase the cost and complexity of this method (8, 11, 64, 139, 145). An alternate method uses a solvent base process in which a high molecular weight polymer is produced by direct condensation of

lactic acid molecules (middle section of the diagram Figure 1-1). Release of water molecules accompanies the condensation of each monomer of lactate to the polymer. Because water molecules can affect and hydrolyze the polymer as it is being produced, this process requires the use of solvents and continuous azeotropic distillation to remove water and is currently used by Mitsui Toatsu company in Japan (29). Another route for PLA production is a solvent free process in which a low molecular weight prepolymer is first produced by condensation of lactic acid (bottom part of Figure 1-1). This is followed by a controlled depolymerization to produce lactide, a cyclic dimer of lactic acid. Lactides are maintained in solution and purified by distillation. Catalytic ring opening of the purified lactide intermediates results in production of the desired molecular weight polymer in a continuous manner, without any additional purification step to remove the residual lactide molecules (55). The following sections will detail the molecular mechanism of this process; production of lactide followed by ring opening polymerization (ROP) to produce PLA in the USA.

Lactic acid production

Production of high molecular weight PLA at high yield relies on the availability of high quality lactic acid (41, 60). The attractive cost of lactic acid and its availability on the market are the reasons why PLA was the first bio-based polymer produced in mass. Currently, almost all the lactic acid (2-hydroxy propionic acid) available on the market is produced by fermentation of corn or potato starch. Briefly, homolactic *Lactobacilli* ferment carbohydrates to produce optically pure L-lactic acid as sole fermentation product. In these fermentations, over 1.8 moles of L-LA per mole of hexose is produced, representing 90% of the theoretical yield of 2 mol/mol (21). During the fermentation process lasting three to six days, calcium hydroxide (or calcium carbonate) is added to the medium to neutralize lactic acid in the medium as calcium lactate. Medium is then filtered to remove cells and insoluble fractions, dried and acidified with sulfuric

acid to yield crude lactic acid that still contains residual carbohydrates and proteins. Further purification could utilize distillation of the lactic acid as methyl or ethyl ester, followed by hydrolysis of the purified ester to the acid form. D-lactic acid is also produced by fermentation (48, 130, 176).

In 1990, the worldwide production volume of lactic acid was approximately 40 ktpa with two primary manufacturers, CCA Biochem in The Netherlands and Sterling Chemicals in Texas City, TX, USA (21). Since the 90's, the global demand for lactic acid increased ten times to 482.7 kilo tons in 2010 and is expected to reach 1,077 kilo tons in 2016, growing at a CAGR of 14.2% from 2011 to 2016 (102).

Production of lactide molecules

Production of lactide, a cyclic dimer of lactic acid, was first described by Pelouze in 1845. He studied self-esterification of lactic acid under heat and reported the formation of a prepolymer no longer miscible with water (121). There are three main steps in the current production process of lactide: prepolymerization (dehydration), lactide synthesis from the prepolymer usually in the presence of a catalyst such as tin octoate (thermal cracking), and purification of lactide. The prepolymerization step is typically conducted in a 6 h batch process, where a vacuum of 70-250 mbar and temperature up to 190°C are applied to dehydrate the lactic acid, causing it to self- esterify into a prepolymer of 8 to 25 residues long linear chain (116). The prepolymer is then fed into a cracking zone operated at a temperature no higher than 240°C and pressure sufficient to cause vaporization of lactide after its formation. During this step, the hydroxyl group located at one end of the polymer attacks the carbonyl carbon of the following lactate unit and cyclicyze into a molecule whose formula is $C_6H_8O_4$ (Figure 1-2) This mechanism is referred to as "back-biting" mechanism (8, 64, 145). Presence of a catalyst enhances lactide formation by facilitating the backbiting mechanism. Tin octoate (stannous 2-ethylhexanoate) is

the best among the catalysts tested and exhibited the lowest level of racemization (114). Besides, this catalyst is widely available at food grade. In this process, lactide formation is in equilibrium with lactic acid and therefore, lactide must be removed as it is produced to ensure completion of the reaction (114, 142). This is achieved by vaporization of neo-formed lactide to quickly remove it from the reaction chamber. After condensation and subsequent purification to remove any residual water, lactic acid and oligomers, concentrated pure lactide is maintained as a liquid (116) or turned into a powder. Purification involves distillation and crystallization steps to remove impurities but also to remove most of the meso-lactide produced as the rate of hydrolysis of meso-lactide (back into lactic acid) is much higher than that of D or L-lactide. Different percentages of the lactide isomers formed depend on the purity of the lactic acid feedstock, temperature of the reaction and the catalyst used (60). D-lactide contains only D-LA, L-lactide contains only L-LA, and optically inactive *meso*-lactide contains one molecule of each of the isomers (Figure 1-2).

Lactides used for PLA production must be at least 99% optically pure, must not contain more than 0.2% of meso-lactide because meso-lactides are highly hygroscopic and undergo rapid hydrolysis in water (182). Because racemization can be controlled throughout lactide production, it is possible to project the optical purity of the resulting product based on the starting material (54). Therefore, lactic acid used as a raw material must have an optical purity of higher than 99% (184).

Ring-opening polymerization (ROP)

The ring-opening polymerization of lactide (ROP) was first demonstrated by Carothers in 1932 (13). This process of ROP has been shown to be an equilibrium reaction between the cyclic dimer and polymer form (13). The ROP can be cationic or anionic (41), depending on the type of initiator used to break the cyclic lactide and introduce it into the polymer. The molecular

mechanism of cationic ring opening and anionic ring opening is presented in Figures 1-3 and 1-4, respectively. Polymerization starts with the opening of positively charged lactide at the alkyl-oxygen bond by an S_N2 nucleophile attack by the triflate anions, one to activate the first lactide, and a second anion to break down the dimer of lactate. Then, the carbonyl of another lactide attacks the carbon that was activated by the triflate anion. This reaction leads to binding of the lactide molecule to polymer and the release of one molecule of catalyst triflate anion. The cycle can then start over with the nucleophile attack from trifle to the backbone of the newly linked lactide (Figure 1-3), opening of the ring and subsequent attack from a new lactide molecule.

Many different chemicals have been tested for their ability to initiate polymerization of PLA, but potassium or calcium ions are the chemical of choice in the production of polymers that are considered biocompatible (60). Among other initiators tested, tin (II) and zinc have been shown to yield the purest PLA polymers (19). Coupling agents are also added to the mixture to allow ramification of the polymer, which greatly increase the final molecular weight of the polymer. These chemicals react the same way as catalyst, but attack preferentially the hydroxyl or carboxyl groups present in the polymer. Some examples of coupling agents are isocyanates, acid chlorides, anhydrides, epoxides, thiirane and oxazoline (60). By controlling the residence time, temperature and the type of catalyst employed, it is possible to control the ratio and sequence of D- and L-LA units in the final polymer. This mixture dictates the mechanical properties of the polymer such as tensile strength, the elongation at break, the modulus and the melting point, among others. The architecture of the backbone defines the degree of crystallinity and the processing temperature of the polymers. It should be noted that racemization could occur at any step in the polymer production (50, 86).

Chemistry of PLA Polymers

There are three types of polylactide polymers that can be produced; homopolymers, copolymers, and stereocomplexes. Homopolymers are typically made of 100% L-lactide and called PLLA. PDLA, made out of D-lactides exclusively, has the same characteristics as PLLA but is more expensive due to the higher price of the D-LA monomer (77). The common commercial copolymer of PLA is PDLLA (Poly (D-L-)Lactic Acid), composed of predominantly L-lactide, with small amounts of D- and *meso*-lactide. Properties of PLA are highly related to the ratio between the two isoforms D and L. Stereocomplex PLA (sc-PLA) consists of both PLLA and PDLA fibers. PLLA and PDLA chains are packed side by side to form a super structure with improved thermal and mechanical properties over PLLA. Their properties will be briefly detailed here as the present study principally focuses on PDLLA copolymers and not on sc-PLA.

Aside of the polymers mentioned above, numerous molecules have been copolymerized with PLLA. Glycolic acid (GA), poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO), (R)-β-butyrolactone (BL), 1,5-dioxepan-2-one (DXO) and trimethylene carbonate (TMC) are examples among many other possible comonomers. Plasticizing PLA with other molecules can help tailor the properties of the created polymer to fit the needs of a specific application. For instance, PLA-PEO polymers are more hydrophilic, flexible and biodegradable than PLA homopolymer (146), which makes them a more suitable candidate for controlled drug delivery systems. Starch has also been investigated as a blend with PLLA to reduce its cost while maintaining its biodegradability (69, 79, 80). Blending PLLA with starch requires the addition of compatibilizers that act as glue between the hydrophobic PLLA and the hydrophilic starch (63, 81, 177, 186, 187). PLLA has also been blended with non-biodegradable polymers such as polyefins, vinyl polymers, elastomers, and rubbers and with other biodegradable polymers such as polyanhydrides, polyvinyl alcohols, poly(ε-caprolactone) and polyhydroxyalkanoates. There is

a plethora of copolymers that have been engineered and commercialized but for the most part, these degradable materials are of interest for the pharmaceutical industry and are not further discussed here.

Properties of PLLA and PDLLA

PLA homopolymers available on the market are typically made of a 100% L-lactide (PLLA) with high crystallinity and good mechanical properties (122). Research and development allowed researchers to understand that the chiral purity of the polymer controls the ability of the polymer to crystallize (33, 56). These polymers are transparent and their properties approach some of the petroleum derived plastics (111). PLA presents a medium water and oxygen permeability level (180) comparable to polystyrene (90). For instance, noteworthy PLA properties include a flexural modulus higher than that of polystyrene, a resistance to fatty foods and dairy products equivalent to PET, an excellent flavor and aroma barrier, and good heat stability (98). Other advantages of controlled polymer crystallinity relate to the storage, transfer and processing of polylactic acid resins into fibers, non-woven fabrics, films, and other end products (33). PLLA is a semicrystalline material with a modulus of 2.7 GPa, a tensile strength of 50 to 70 MPa, an elongation at break of 4%, a flexural modulus of 5 GPa, and a flexural strength of 100 MPa (28, 34, 49, 70). Its melting point is about 180°C and the glass transition Tg is around 60 to 65°C (4), which when annealed properly, has a crystallinity of 35% to 70%. From these values, PLLA can be characterized as being brittle with a rather low elongation at break, which limits its applications. To broaden the applications of PLA polymers, chemists developed copolymers with tailored valuable properties. The common commercial copolymer of PLA is PDLLA (Poly (D-L-)Lactic Acid), composed of predominantly L-lactide, with small amount of D- and *meso*-lactide. Typically, the feed of D-LA ranges from 1 to 8% of the total LA used during production of the polymer. PLA resins containing more than 93% of L-lactic acid

are semicrystalline whereas PLA composed of 50 to 93% L-lactic acid are fully amorphous (3). For identical L-isomer contents, copolymers of L- and D-lactides have higher crystallinities than those made with L- and meso-lactides, and therefore exhibit lower melting temperatures (100). These different properties associated with PDLLA favor its actual development in different packaging applications (trays, cups, bottles, films, etc.) (141, 146). Amorphous and biaxial films made of PDLLA exhibit clarity and gloss that exceeds PET. Besides, PDLLA is easily printed on, which means that stickers and labels are not necessary (98). Kolstad investigated the crystallization behavior of copolymers of L-lactides and meso-lactide (86). He observed a loss of 3°C in the melting temperature with every 1% of meso-lactide (or D-isomer) added to the feed. 3% meso-lactide was enough to double the crystallization time of the polymer over the PLLA crystallization kinetics, 6% meso-lactide incorporated into PLA slowed the crystallization kinetics of the corresponding polymer by an order of magnitude (86). Lower crystalline melting points achieved by introducing meso-lactide into the polymerisation also allow lower melt-processing temperatures of the final polymer (98). PDLLA has a melting temperature ranging from 130 to 180°C, again directly related to the amount of D-LA added to the feed (51, 57, 60, 86). The glass transition of PDLLA varies from 60°C for PLLA to 34°C when PLA is prepared with 50% of each isomer of lactic acid (118). Overall, copolymerizing PLLA with small amount of D-isomer offers materials with lower brittleness and better film properties (4). Annealing improves the thermo mechanical properties of PLA by letting the polymer crystalize. Therefore, extrusion or injection molding processes must be optimized. Proper crystallization of PLLA and PDLLA will improve the behavior of the plastic when applications require temperatures higher than 50°C. Biaxial orientation of PLA film is an important method to increase tensile strength

and elongation at break. Thus, PLA is a versatile polymer that can be converted into several materials having useful properties for many different applications.

Properties of sc-PLA

While PLLA and PDLA are relatively heat sensitive, stereocomplexes made from both polymers exhibit a melting point increase of 51°C as compared to their respective pure polymers (65, 164). On the other hand, blending PLLA with PDLA lowers its biodegradability as it increases its stability (23). Sc-PLA can act as high performance polymers because their melting temperature, i.e., 230°C, is 50°C higher than that of enantiomeric PLLA or PDLA (65). The glass transition of sc-PLA is 65 to 72°C, which is slightly higher than that of PLLA (71), probably due to the limited mobility of PLA fibers in the amorphous regions surrounded by crystalline states. Sc-PLA typically has a tensile strength of 880 MPa, a Young's modulus of 8.6 GPa, and a elongation at break of 30%, which are values considerably higher than the ones reported for PLLA (4). To promote sc crystallization of PLA, stereoblocks type PLA have been created (sb-PLA), which are block copolymers of enantiomeric PLLA and PDLA. Sb-PLA can be synthesized by solid-state polycondensation (SSP) of a mixture of PLLA and PDLA having medium molecular weight or by stepwise ROP of D- and L-lactides. It is believed that van der Waals interactions of the enantiomeric polymer chains are responsible for the stereocomplex formation (4). "Stereocomplexation" of sb-PLA remains a challenge and several research efforts are currently ongoing towards a process that is fast enough and yields high molecular weight sc-PLA (138, 165, 166, 168). Even though an optimum process for the production of sc-PLA is not in place yet, stepwise ROP seems to be a promising way (68, 87). In this method, a primary polymer is synthesized using L- or D-lactide as a feed. When the molecular weight of 50 kDa is reached, the polymer is precipitated to remove the residual monomer. The second lactide monomer and a catalyzer such as the commonly used tin octanoate is then added to the newly

formed polymer to start the ROP using the counterpart lactic unit exclusively. This way sb-PLA of 200 kDa can be obtained, with various PLLA/PDLA block ratio for producing fibers with high thermal stability for various electric appliances and automobile parts since sc-PLA is incombustible by nature (92, 169).

PLA Degradation

Recycling and Composting

The world's annual consumption of plastic materials has increased from around 5 million tons per year in the 1950's to nearly 100 million tons a year in 2010 (179). The widespread use of plastics demands proper end of life management. In 2011, plastics made up about 12 percent of the municipal solid waste, a dramatic increase from 1960, when plastics were less than one percent of the waste stream (30). The general assumption is that biopolymers quickly degrade via microbial activity. However, PLA should be composted at industrial facilities since the appropriate conditions required for degradation of biopolymers are not met in the typical "backyard" compost pile or in landfills (53, 175). PLA and other organic matter, when composted, can be used as fertilizers and soil amendment. Biodegradation of PLA containers, although eco-friendly, is effective only under appropriate conditions, i.e.,60°C and 80% relative humidity (9). Biodegradation of PLA polymer occurs in two steps: the first phase consists of fragmentation of the polymer due to absorption of water. Fragmentation of PLA is faster at higher temperatures compared to room temperature (98). In an industrial composting facility operating at a temperature of about 70°C, fragmentation starts in less than 2 days and biodegradation starts at about day 4. When the average molecular weight of the released polymer reaches around 10, 000, microorganisms present in the soil begin to digest these short polymers releasing carbon dioxide and water, which constitutes the second step of the biodegradation process. Several A*ctinomycetes* and thermophilic bacteria have been reported to possess low-

molecular weight PLA-degrading ability and these include *Brevibacillus, Bacillus smithii, Geobacillus* spp. and *Bacillus licheniformis* (2, 83, 134, 158, 160).

Despite the compostability of PLA, landfill is currently the dominant waste management method in the United States (24, 52, 137). When disposed in landfills, PLA products do not biodegrade and may even contribute to the release of greenhouse gases into the atmosphere, cancelling the beneficial effects of utilizing such plastics over petroleum-derived polymers (174). In 2005, NatureWorks implemented a large-volume "buy back" program in North America for post-consumer Ingeo bottles sorted from mixed-plastic recycling streams. (112). Galactic (Belgium), another major PLA producer, recently developed a process to recycle 1 ktpa of PLA as a start towards an effort to recycle the plastic. One of their methods is mechanical recycling that moulds the PLLA polymer back into PLA beads. Their chemical process involves melting the plastic and separating the two isomers chemically in order to reintroduce pure lactic acids into the polymer production stream. An optical purity of over 99% is required for both D-Lactic acid and L-Lactic acid entering the PLA production process (62). Chemical separation of the two enantiomers is expensive, usually using liquid or solid enantioselective membranes (58) or High Performance Liquid Chromatography (HPLC).

Enzymatic Degradation

Microbial and enzymatic degradation of PLA are sustainable ways of recycling the polymer. At present, available information on PLA-degrading enzymes is less than that available for other biodegradable plastics such as PCL or PHB (148). Studies on enzymatic degradation of PLA mainly focus on the changes occurring on the polymeric material itself by following the weight loss or the surface morphology changes, among other criteria. Enzymatic degradation of low molecular weight PLA films by enzymes such as *Rhizopus delemer* lipase, hog pancreatic

lipase and carboxylic esterase have been studied (37). A recent study evaluated the use of two commercial enzyme preparations from Novozyme (Japan); i.e.,lipase CA (produced by *Candida antarctica*) immobilized on acrylic resin, and lipase RM (produced from *Rhizomucor miehei*) immobilized on a macroporous anion exchange resin to break down PLA into cyclic oligomers of low molecular weight that can be purified and redirected into the production of the plastic. Lipase RM was reported to degrade PDLLA at 60°C and lipase CA degraded PLLA at 100°C (151). Besides the higher temperature, the enzymatic reaction was conducted in a mixture of chloroform and hexane or *o*-xylene. In 2006, Jarrerat et al. demonstrated biological recycling of PLA at 40°C without the use of organic solvents (75). The extracellular PLA-degrading enzyme produced by an A*ctinomycete*, *Amycolatopsis orientalis* showed high activity; 2.0 g/L of PLLA powder was completely degraded within 8 h at 40°C by 20 mg/L of purified enzyme (12.5 mg PLA hydrolyzed per mg of enzyme per hour). An optically active L-LA was obtained as degradation product without undesirable racemization. However, the enzyme produced was stereospecific and did not cleave D-isomer based PLA (73). At present, there are about 30 microbes that have been isolated for their PLA-degrading ability and are listed in Table 1-1 (148). So far, only a few PLA depolymerases have been isolated and purified from these organisms. Their biochemical characteristics are presented in Table 1-2 (148). For enzyme-based recycling of PLA to become a reality, large-scale cost-effective production of PLA depolymerases need to be achieved. Since the PLA polymers sold on the market are PDLLA containing L-lactide with small amount of D-lactide or meso-lactide, enzymes that can degrade both types of ester bonds that are present in the polymer, i.e., L-L, D-D, L-D and D-L are required for effective depolymerization of PDLLA. As of today, only one organism, *Bacillus stearothermophilus*, was isolated for its ability to degrade PDLA (161). The enzyme degrading

PDLA has not been characterized yet, and it is not known if such enzyme may be able to cut L-D and D-L ester bonds. This gap in knowledge needs to be addressed before efficient enzyme-based recycling of PLA becomes a reality.

Thermohydrolysis

An alternative to biodegradation of PLA is hydrolysis of the polymer at high temperature, also called chemical recycling (163). Optical purity of the starting material is also a critical factor for PLA synthesis from hydrolysis-derived LA (172).

The mechanism of hydrolysis of PLA is covered in numerous reports (3, 4, 22, 171, 183) (Figure 1-5). While extensive literature is available on degradation characteristics of PLA polymers used in the medical field, information on chemical recycling of PLA to monomer is minimal (10, 163, 170, 183). Tsuji *et al.* reported on thermohydrolysis of pellet-shaped PLA in water in the temperature range of 120°C to 350°C while Yagihashi and Funazukuri (2009) evaluated depolymerization of polymer in an alkaline solution. Use of large excess of NaOH in comparison to the amount of polymer in this study did not permit critical evaluation of the effect of NaOH on the hydrolysis of the polymer, including potential racemization of the released monomer (96). The present study compares the kinetics of PLA hydrolysis in water and NaOH towards understanding the role of NaOH in this process. Hydrolysis kinetics were evaluated using a modified version of the Gompertz equation (47, 190) to describe the thermochemical hydrolysis of PLA via a single equation relating the LA concentration as a function of time, rate of hydrolysis and lag phase duration.

Table 1-1. PLA-degrading organisms and detection method (148)

Strain	Detection method of PLA degradation (Ref.)
Amycolatopsis sp. HT 32	Film-weight loss; monomer production (127)
Amycolatopsis sp. 3118	Film-weight loss; monomer production (66)
Amycolatopsis sp. KT-s-9	Clear zone method (157)
Amycolatopsis sp. 41	Film-weight loss; monomer production (128)
Amycolatopsis sp. K104-1	Clear zone method (109)
Lentzea waywayandensis	Film-weight loss; monomer production (74)
Kibdelosporangium aridum	Film-weight loss; monomer production (74)
Tritirachium album ATCC 22563	Film-weight loss; monomer production (72)
Brevibacillus	Change in molecular production and viscosity (159)
Bacillus stearothermophilus	Change in molecular production and viscosity (161)
Bacillus smithii PL 21	Change in molecular production and viscosity (160)
Bacillus licheniformis PLLA-2	Biodegradation test (83)
Paenibacillus amylolyticus TB-13	Molecular technique (140)
Bacillus clausii strain pLA-M4	Molecular technique (105)
Bacillus cereus pLA-M7	Molecular technique (105)
Treponema denticola pLA-M9	Molecular technique (105)
Paecilomyces	Molecular technique (136)
Thermomonospora	Molecular technique (136)
Thermopolyspora	Molecular technique (136)
Actinomadura keratinilytica T16-1	Clear zone and turbidity method (149)
Micromonospora echinospora B12-1	Clear zone and turbidity method (149)
Micromonospora viridifaciens B7-3	Clear zone and turbidity method (149)
Nonomuraea terrinata L44-1	Clear zone and turbidity method (149)
Nonomuraea fastidiosa T9-1	Clear zone and turbidity method (149)
Bacillus licheniformis T6-1	Clear zone and turbidity method (149)
Laceyella Sacchari T11-7	Clear zone and turbidity method (149)
Thermoactinomyces vulgalis T7-1	Clear zone and turbidity method (149)

Table 1-2. Characteristics of purified PLA-degrading enzyme from various strains (148)

Strain	MW (kDa)	Opt. pH	Opt. temp (°C)	Enzyme type (Ref.)
Amycolatopsis sp. 41	40	6	34-45	Protease (128)
Amycolatopsis sp. K104-1	24	9.5	55-60	Serine protease (109)
B. smithii	63	5.5	60	Acyltransferase (133)
Cryptococcus sp. S-2	21	-	-	Lipase (103)
Amycolatopsis orientalis ssp. *orientalis*	24 19 18	9.5 10.5 9.5	50-60	Serine protease (91)
Actinomadura keratinilytica T16-1	30	10	70	Serine protease (149)

Adapted from (148) with permission from Interchopen

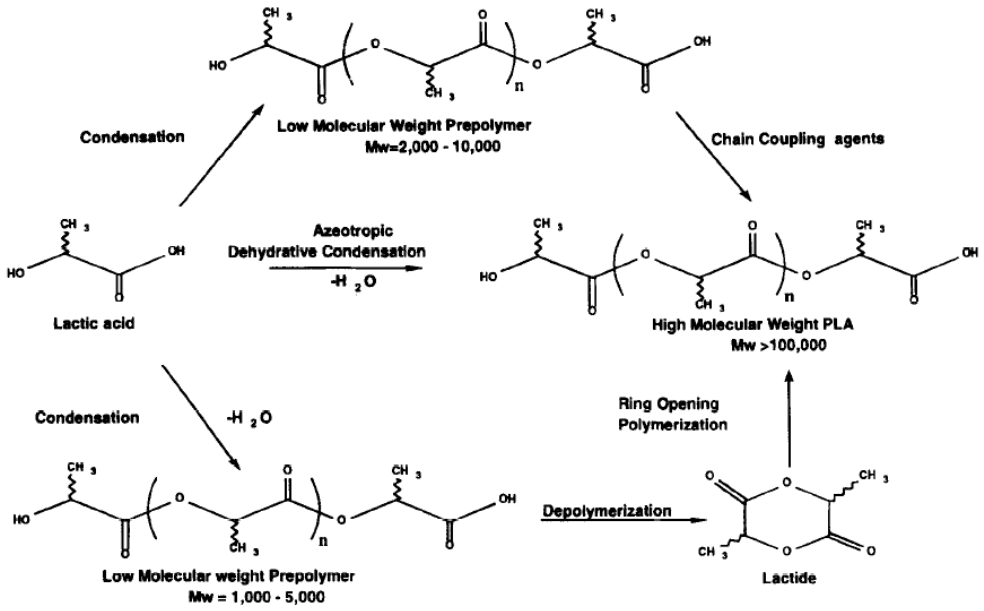

Figure 1-1. Possible routes towards the industrial production of PLA (98). Reproduced with permission from Elsevier.

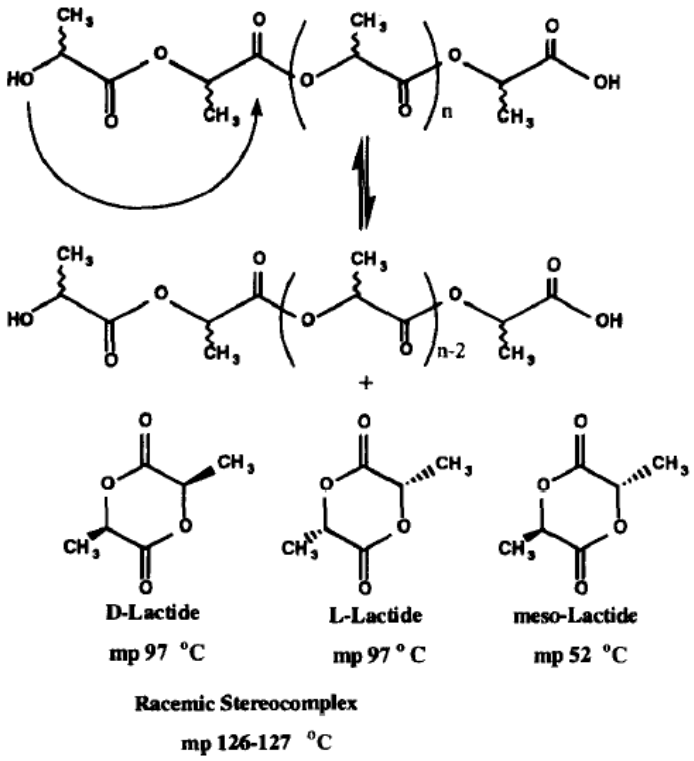

Figure 1-2. Molecular mechanism describing the production of Lactide (98). Reproduced with permission from Elsevier.

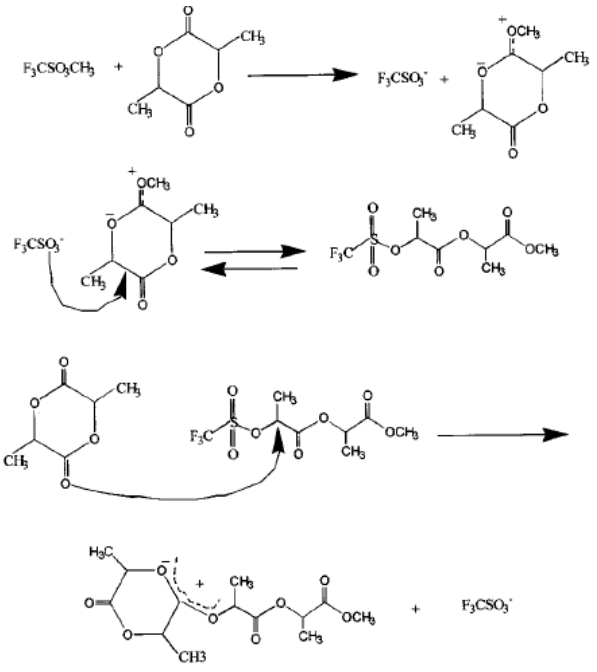

Figure 1-3. Molecular mechanism of cationic ROP (41). Reproduced with permission from Elsevier.

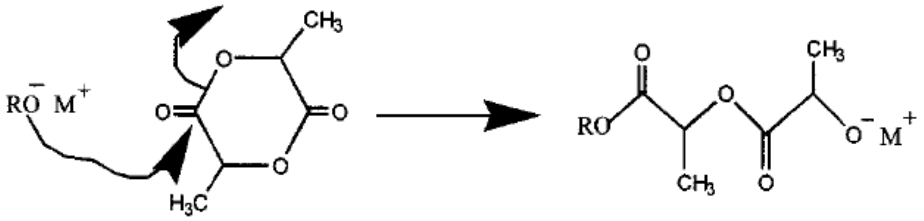

Figure 1-4. Molecular mechanism of anionic ROP (41). Reproduced with permission from Elsevier.

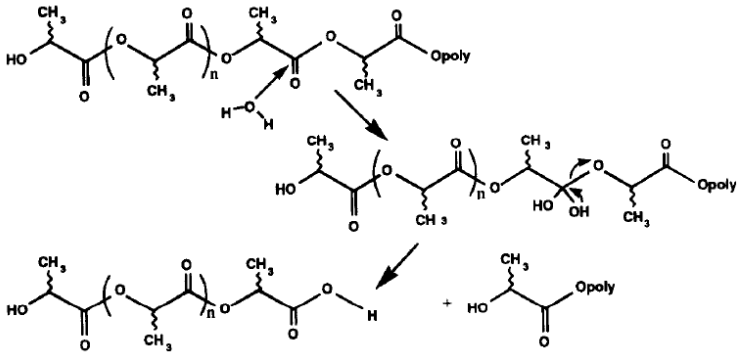

Figure 1-5. Hydrolysis of PLA in the presence of water (98). Reproduced with permission from Elsevier.

CHAPTER 2
ESCHERICHIA COLI AS A BIOCATALYST FOR THE D-LA REMOVAL OF THE HYDROLYZED PLA MATERIAL

Cradle-to-Cradle Life Cycle of PLA

Recovering the lactic acid contained in the polymer for reuse seems the logical application to develop. Viscous PLA syrup obtained after hydrolysis is composed of a mixture of D- and L-lactic acid. An optical purity of over 99% is required for both D-Lactic acid and L-Lactic acid entering the PLA production process (62). Chemical separation of the two enantiomers is expensive, usually using liquid or solid enantioselective membranes (58) or High Performance Liquid Chromatography (HPLC). A bio-based enantiomer separation is expected to be cost-effective compared to several other abiological processes. Therefore, a cost-effective biological system needs to be developed that purifies the L-lactic acid from the contaminating D-lactic acid. Using a microbe to offer new ways of separating two isomers has never been described before. However, this concept might be of interest only when the presence of one substance contaminates and decrease the purity of its isomer, present at a much larger scale. The present study proposes to fill this gap of knowledge by engineering a bacterium such as *Escherichia coli* to selectively metabolize D-LA contained in PLA syrup to result a pure L-LA substance. This way, pure L-LA could be redirected into the PLA production stream.

Escherichia coli: a biocatalyst of choice

Many bacteria have the ability to utilize D- and L-lactic acid for growth. However, no commonly used strain in laboratory or industrial setting has the ability to use D-lactic acid isomer exclusively. Among the commonly used laboratory strains, *Escherichia coli* seems of choice due to the vast amount of information available on its genetics and physiological characteristics. Qualified as “user-friendly” biocatalyst, *E. coli* has minimal growth

requirements; a high specific growth rate and a high cell yield on various substrates. Additionally, countless molecular biology tools are readily accessible to investigators and multiple genome sequences are published. Information is available for *E. coli* in terms of aerobic lactic acid metabolism. Wild type *E. coli* possesses two distinct pathways, each enabling use of L- and D-lactic acid as a carbon source under aerobic condition, respectively, and reviewed in the next paragraph.

Lactic Acid Metabolism of *Escherichia coli*

Known LDHs in Wild Type *E. coli*

E. coli grows well on L- or D-lactic acid as sole source of carbon aerobically and also produces D-LA during anaerobic growth and fermentation of sugars. As of today, three lactic acid dehydrogenases have been identified in *E. coli*.

Fermentative LDH

E. coli gene *ldhA* codes for the fermentative lactic acid dehydrogenase and is responsible for D-lactic acid formation from pyruvate (104). This enzyme is a cytoplasmic tetramer that is NADH dependent and is produced only under oxygen-limited conditions (152, 153). Under physiological conditions, the fermentative LdhA protein is not known to oxidize L- or D-lactic acid and therefore does not contribute to the aerobic lactic acid catabolism.

Respiratory LDH

Several lactate-oxidizing enzymes have been characterized in bacteria and these are membrane associated flavoproteins that are components of the electron transport system coupling to O_2 or to other electron acceptors such as nitrate, fumarate or trimethylamine-N-oxide (42, 43). These LDHs oxidize lactate to pyruvate and the electrons are transferred to ubiquinone. Quinones deliver the electrons to terminal oxidoreductases and ultimately to O_2 (43). Under anaerobic conditions and in the presence of nitrate or fumarate, lactate oxidation is coupled to

reduction of these alternate electron acceptors utilizing appropriate enzyme complexes. Pyruvate is used as the source of cellular carbon for biosynthesis. Both D-LA and L-LA induce their cognate LDH activities in the cell as long as the terminal acceptor is also present in the medium (85, 113, 119).

The *dld* gene located at 47 min on the *E. coli* chromosome encodes a protein with FAD as a cofactor (E.C.1.1.2.5) that is responsible for the oxidation of D-LA into pyruvate (129, 131). D-lactate oxidation is coupled to reduction of ubiquinone in *E. coli* (45, 61). D-LDH activity is detectable in membrane vesicles prepared from cells grown in glucose, lactate, glycerol and succinate, but virtually no activity was observed under anaerobic condition (40, 113). The enzyme consists of a single subunit of 64 kDa (131). D-LDH remains firmly attached to the membrane and requires detergents or strong chaotropic agents to release the enzyme from the cytoplasmic membrane (162). D-LDH from *E. coli* ML308-225 was purified 400-fold and the pure product showed that D-LDH was able to oxidize L-LA at a relative rate of 14% of the D-LA oxidation rate (38). The measured *K*m for L-LA was about 30 times higher than the *K*m for D-LA, i.e., 18 mM and 0.6 mM, respectively for this purified enzyme (38). Also in *N. meningitis*, Vm and *K*m measured with L-LA as a substrate using purified D-LDH were 50 μmole MTT reduced /(min.mg protein) and 32.2 mM respectively; Dld activity towards L-LA is 3 times lower than the activity recorded for D-LA as a substrate, and the *K*m of D-LDH towards L-LA is 55 times higher than the *K*m measured for D-LA (32).

The *E. coli lldD* gene (80 min) is part of an operon that codes for a lactate permease, a DNA-binding regulatory protein and the L-LDH (*lldPRD*) (25). The L-LDH encoded by *lldD* contains FMN as cofactor (E.C.1.1.2.3) and is anchored in the inner surface of the cytoplasmic membrane (25, 40, 120). Originally designated as *lct*, the L-LA utilization locus has been

renamed *lld* (43). The purified L-LDH appears to be a protein with a single subunit of 43 kDa (40) with a K*m* for L-LA of 120 μM (40). Production of L-LDH in the cell is dependent on the presence of either L-LA or D-LA and oxygen, but no D-LA dependent reduction of the electron acceptor could be measured with the L-LDH (40, 85, 113, 119). The activity measured on cells grown in LA was 20 times higher than in cells grown in other carbon sources (40). ArcA protein has been shown to repress transcription of *lldD* under anaerobic condition (25, 99). Interestingly, a mutation in the *lldD* did not abolish L-LA dependent reduction of DCPIP at 600 nm, suggesting that one or more genes encoding proteins with L-LDH activity exists in the cell (120).

Evidence of Other L-LDHs

As with *E. coli*, *Neisseria meningitides* and *Neisseria gonorrhoeae* seem to possess at least two distinct membrane bound L-LDHs based on growth of an *lldA* deletion mutant (homolog of *lldD* in *E. coli*) on L-LA as sole carbon source (31). In *Bacillus subtilis*, deletion of the *lutABC* operon, shown to code for an L-LDH in the cell, did not completely abolish growth of the mutant on L-LA (14), another evidence that bacteria may possess more than one L-LDH in the cell. Interestingly, the proteins encoded by the *B. subtilis lut*ABC operon are not homologs of the proteins expressed from the *lldPRD* operon of *E. coli* but they exhibit 54%, 57% and 38% sequence identities with an uncharacterized *ykgEFG* operon (14). The proteins encoded by the *lutABC* of *B. subtilis* and *ykgEFG* in *E. coli* are also homologs of the proteins encoded by *lldEFG* (SO_1520 to SO_1518) of *Shewanella oneidensis* MR-1. The YkgEFG proteins of *E. coli* exhibit 30%, 38% and 32% identity and 49%, 57% and 48% similarities with *Shewanella* SO_1520, SO_1519, and SO_1518 respectively. In *S. oneidensis*, mutating any of the genes of the operon led to a mutant that did not grow on L-LA (123). Growth on L-LA was restored in *Shewanella* mutant by transforming with *E. coli ykgEFG* genes (123). Bioinformatics studies have predicted a dehydrogenase-type activity for the *YkgE* orthologs of several bacteria; *YkgE* is

predicted to be a putative dehydrogenase subunit in *Shigella sonnei* SS046 (99% identity and similarity with the *E. coli* gene sequence) (76), and HN41 as a putative L-lactate dehydrogenase Fe-S oxidoreductase subunit in *Shewanella* sp. (31% identity and 49% similarities) (82).

The database (117) also reports that numerous bacterial genomes include these two sets of genes (*lld* and *ykg*), underlying that various organisms may possess two distinctive L-LDHs. Among them are the *Neisseria* strains reported to have multiple L-LDHs as well as *E. coli* K-12 (31).

Only two published studies describe a mutant of *E. coli* unable to grow on L-LA aerobically. Both of these studies used random mutagenesis to isolate a mutant unable to grow on L-LA aerobically (25, 120). Interestingly in one of these studies, this mutation appears to also disrupt the permease activity (gene upstream of the dehydrogenase), and a plasmid harboring only the dehydrogenase gene failed to complement the mutation (25). Considering there are at least two distinct L-LDH activities encoded by the *lldD* and *ykgE* in *E. coli*, the genotype of the L-LA defective mutant described by Dong et al. is unclear. In the other study, a mutation in the *lldD* did not abolish L-LA dependent reduction of DCPIP at 600 nm, in agreement with the presence of other L-LDHs in the cell (120).

The present study proposes a novel process for the post consumer use of PLA polymers. In this process, PLA-based plastics are hydrolyzed followed by purification of the lactic acid syrup using a bacterial biocatalyst to optically pure L-lactic acid that can reenter the PLA production. This study also presents kinetics of PLA hydrolysis in water and in the presence of NaOH towards a better understanding of the potential of NaOH as a catalyst for rapid hydrolysis of the polymer. The genetics and physiology of the engineered *Escherichia coli* biocatalyst developed for purification of the L-LA syrup (D-LA removal) is presented.

CHAPTER 3
OBJECTIVES AND CHOSEN STRATEGIES

The specific objectives of the research are:

1) Construct a kinetic model for breaking down of PLA polymers into its simplest components: L- and D-lactic acid. Thermohydrolysis will be performed with water and catalyst such as NaOH towards a better understanding of the potential of NaOH as a catalyst for rapid hydrolysis of PLA. Racemization that may occur throughout hydrolysis will be evaluated, 2) Most of the PLA plastics present on the market as packaging material contain a small amount of D-LA. Therefore, small amount of D-LA is present in the hydrolyzed material. Isomer separation is possible, though expensive. To overcome this, *E. coli* will be engineered and evolved to specifically and efficiently metabolize PLA-derived D-LA, leaving the remaining and abundant L-LA in the medium to be further purified and reused. Ultimately, the recombinant strain will be used to perform D-LA removal experiments in which cells, in presence of hydrolyzed PLA material, will actively oxidize the pool of D-LA contained in the syrup. Following D-LA removal, L-LA remaining in the culture broth can be purified following the process already in place and following production of L-LA by fermentation, 3) Explore and supplement the knowledge on the aerobic L-lactic acid metabolism of *Escherichia coli*. Attempts will be made to uncover and identify the genes encoding L-LDHs in the cell besides *lldD*.

Overall, the present study proposes a novel process for the post-consumer use of PLA polymers. In this process, thermohydrolysis is the first step, followed by the D-LA removal from the hydrolyzed material to yield pure L-LA that could be redirected into the production of the polymer itself.

CHAPTER 4
MATERIAL AND METHODS

PLA Hydrolysis

Materials

PLA beads (~2 mm in diameter) obtained from NatureWorks® LLC (grade 3052D and grade 4032D) and were utilized without further treatment unless otherwise noted. These two polymer grades represent a large portion of the PLA used in food packaging in USA. PLA grade 3052D is designed for injection-moulding applications whereas grade 4032D is typically converted into biaxially oriented films. Properties of PLA pellet grade 4032D that contains an average of 1.2% D-LA are as follows: Mw 155,000, number-average molecular weight Mn 93,200, Tm, 160°C and Tg, 61°C (150). On the other hand, PLA pellet grade 3052D contains 4.15% (±0.45) D-LA (NatureWorks® LLC). Pure Na-L-LA and D-LA were purchased from Sigma Aldrich (St. Louis, MO, USA) and were of the highest purity available.

Thermohydrolysis Conditions

Fifteen g of PLA beads were mixed with 15 g of liquid in a 200 mL sealed canister (Werner Mathis AG, Switzerland). In the experiments, unless specified otherwise, the total weight of solid and liquid was set at 30 g. A total of 24 canisters were prepared identically and heated to the desired temperature in a Mathis® oven (Werner Mathis AG, Switzerland). At different time periods, canisters were removed from the oven and cooled in water at room temperature to stop further hydrolysis. The Mathis® oven harbors a temperature probe that measures the internal temperature within a canister in line. Canisters are locked onto a circular device rotating alternatively for 30 s at 60 rpm clockwise and counterclockwise, allowing mixing. The oven was set to increase the temperature of the canisters by 6°C per minute. The

oven was preheated to the desired temperature and the contents of the canisters reached 160°C in about 18 min. When noted in the text, PLA beads were ground using a laboratory mill (Thomas-Wiley Company model 4, Swedesboro, NJ, USA). The powder was then passed through a bouillon strainer (WINCO model CCB-8R, China) and used in hydrolysis experiments.

Analysis

After heating and cooling to room temperature, canisters were opened and liquid and solid fractions were separated and weighed and a mass balance was performed. Once the liquid fraction was removed, canisters were rinsed with distilled water and dried at 160°C. Water bound to the solids was included as part of the solid fraction. The pH of the liquid fraction was measured using an Orion 420A pH meter (Thermo Scientific). Density of the liquid fraction was measured by weighing 1 mL of PLA syrup. LA concentration in the syrup was determined by HPLC using an HP 1090 chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a Bio-Rad Aminex HPX-87H ion exclusion column (45°C; 4 mM H_2SO_4; flow rate, 0.4 mL/min) and dual detectors (UV detector at 210 nm and refractive index monitor, in series). Optical isomers, D- and L- lactic acid, were determined by chiral HPLC (Chirex 3126(D)-penicillamine column; 150 × 4.6 mm; Phenomenex) using 2 mM $CuSO_4$ as mobile phase (flow rate, 0.6 mL/min) with a diode array detector set at 210 nm.

Kinetics Parameters Measurements

Apparent rate of hydrolysis k' and lag phase duration

For each condition tested, lactic acid concentration as a function of time of incubation was generated and an equation (2-1) derived from the Gompertz model was used to fit the data set. The equation used was as follows:

$$[Lac] = [Lac]_{max}.exp\left(-exp\left[\frac{k'exp^1}{[Lac]_{max}}(\lambda - t) + 1\right]\right) \quad \text{(Equation 2-1)}$$

where [Lac] is the mass fraction of lactic acid at time t , $[Lac]_{max}$ is the maximum theoretical mass fraction of lactic acid, k' is the maximum rate of hydrolysis of PLA into LA (1/h), t is the heating time (h), and λ is the lag phase duration (h). The constants $[Lac]_{max}$, k' and λ, were determined using the non-linear regression approach with the aid of the solver function available from the MS Excel ToolPak. Solver converges the sum of square error between the experimental data and the estimation to a minimum value. This way, hydrolysis rate and lag phase duration could be compared throughout the study. This equation, established in 1825 by Gompertz was modified later to describe bacterial growth (47, 190, 191). Since, the modified version of the equation has been reused in many other areas of research to predict yield of biogas, growth patterns of cancerous cells, *etc.* (12, 89), it is also used here to predict the rate of hydrolysis of PLA to lactic acid.

Activation energy E_a

The natural logarithm form of the Arrhenius equation was used to evaluate the activation energy for the hydrolysis of PLA into LA (eq. 2-2) (190).

$$Ln(k') = Ln\,(A) - \frac{E_a}{RT} \qquad \text{(equation 2-2)}$$

where E_a is the activation energy (kcal/mole), k' is the rate of hydrolysis (1/h), T is the absolute temperature in Kelvin (K), R is the universal gas constant ($1.9858*10^{-3}$ kcal/mol).

Calculations

LA M_w is 90.06 g/mol and PLA repeat unit M_w is 72 g/mol. Consequently, 15 g of PLA represents a maximum theoretical amount of 18.7 g of LA or 0.2076 mole after complete hydrolysis. Density of the PLA beads was established as 1.26.

Optical Purity Study

To evaluate the effect of heat and alkaline condition on the racemization of LA, 7 g of water or 7 g of 1 M NaOH was mixed with 7 g of PLA syrup obtained from a previous experiment in a sealed canister and subjected to heat treatment at 160°C for 2 h. The PLA syrup used in this experiment (ran in triplicate) was obtained by mixing equal amounts of syrup obtained at 160°C after 150 min with water or 1M NaOH. In another set of canisters, 7 g of 1 M L-LA solution was mixed with either 7 g of 1 M NaOH or 7 g of water and subjected to the same heat treatment. After heat treatment and cooling to RT, a sample from each of the canister was subjected to chiral HPLC to assess the D-LA content after treatment, and compared with the D-LA content of the starting sample.

Engineering *E. coli* for the D-LA Removal from Hydrolyzed PLA Material

Bacterial Strains, Plasmids and Growth Conditions

The *E. coli* K-12 strains and plasmids used in this study are listed in Table 2-1. *E. coli* Top10 or *E. coli* EPI300 was used for plasmid library construction. During strain construction, cultures were grown at either 37°C in Luria-Bertani (LB) broth (per liter: 10 g of Difco tryptone, 5 g of Difco yeast extract, and 5 g of sodium chloride) or on this medium solidified with agar (1.5%). Antibiotics were added to the medium as needed at the following concentrations: kanamycin (50 $\mu g.ml^{-1}$), tetracycline (12.5 $\mu g.ml^{-1}$), Chloramphenicol (30 $\mu g.ml^{-1}$) and ampicillin (100 $\mu g.ml^{-1}$). Engineered strains were cultured in minimal medium (per liter: 6.25 g Na_2HPO_4, 0.75 g KH_2PO_4, 2 g NaCl, 27.3 mg $NaMoO_4.2H_2O$, 27.3 mg $FeSO_4.7H2O$, 2.7 g $(NH_4)_2SO_4$, 0.55 g $MgSO_4.7H_2O$). In some experiments, an additional 100 mM sodium phosphate buffer, pH 7.0 was also added to the medium to provide higher buffering capacity. Cultures were maintained in minimal medium containing 2% lactic acid purchased from Fisher scientific (Fairlawn, NJ, USA). When used, glucose concentration was 1% unless otherwise specified, L-

LA concentration was 0.5% and D-LA concentration was 0.3%. SOC medium was used as a recovery medium after transformation: 20 g/L Bacto-Tryptone, 5 g/L yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose.

Toxicity Induced by PLA Syrup on the Growth of Wild Type *E. coli*

E. coli K-12 strain W3110 was used in this study as the wild type (Table 2-1). Preculture was grown in mineral salts minimal medium containing 10 g/L of lactic acid (Fisher) and used to inoculate 20 mL of minimal medium (125 mL flask) containing either Na-L-LA (Sigma) or hydrolyzed PLA syrup at an initial OD_{420} of 0.05. 1 mM betaine was also added to each culture to help the strain resist high osmotic pressures induced by high [LA]. The syrup used in this experiment was obtained by hydrolyzing soft-drink PLA cups (obtained from Kangaroo™ gas station) at 160°C for 135 min. These cups, harboring the company logo directly printed on the plastic, were hydrolyzed after cutting them into pieces of about 2 cm^2 to fit the canister diameter. Before adding PLA syrup to the culture medium, pH was adjusted to 7 by direct addition of NaOH pellets. After inoculation, flasks were incubated at 37°C and agitated at 200 rpm. A 500 µL sample was removed from the flasks at different time periods and the OD_{420} was measured using a spectrophotometer (Beckman spectrophotometer model DU 640) in order to determine the specific growth rate of the cultures. [Na-L-LA] tested were: 4, 8, 16, 26, 31, 37, 41, 56, and 79 g/L. Concentrations of LA derived from hydrolyzed PLA used in this study were: 4, 10, 25, 32, 37, 42, 49, 54, 65 and 111 g/L, as determined by HPLC.

Mutagenesis

Mutations constructed by transduction

Strains DC825, DC8255, DC82551, DC8261X, DC826170X, DC*lldR*, DC*ykgD* and DC*bglX* were created using the same protocol. Bacteriophage P1 cm clr100 was used in transduction experiments (107). P1 phage was prepared using appropriate *E. coli* "Keio mutant"

(5) as the donor and transduced into the appropriate recipient strain. After transduction, colonies were picked and streaked onto LB-kanamycin agar medium and incubated at 42°C overnight to eliminate the lysogens. Isolated colonies were picked, resuspended in sterile water, and the suspension was used as a DNA template to perform a set of PCR reactions using specific primers (Table 2-2), to ensure that the correct mutation is present in the transductant. A thermo-sensitive plasmid pCP20 expressing FRT recombinase, allowed curing the *kan* gene from the cassette (15). Strains with the letter "c" at the end of their names denote that their kanamycin cassettes were cured.

Deletion of *ykgEFG* operon

Deletion of *ykgEFG* operon was performed using methods reported elsewhere (20). Primers F*ykgG-kan* and R*ykgE-kan* (Table 2-2) were used to amplify the kanamycin gene using pKD4 as the DNA template. PCR amplified DNA of the correct size was gel-purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Pittsburgh, PA, USA) and electroporated into electrocompetent DC8255c [pKD46] cells. Following recovery at 30°C in 2 mL of SOC medium, cells were centrifuged and spread onto LB agar medium with kanamycin and incubated at 30°C for 24 to 48 h. Colonies were then streaked onto fresh LB agar with kanamycin and incubated at 37°C to confirm kanamycin resistance as well as to cure the thermo-sensitive plasmid pKD46 from the transformants. Colony-PCR reactions were performed to ensure that the double cross over deleted *ykgEFG* in the cell. The strain generated was named DC8255*ykgEFG*.

Tn5 random insertion into the genome of DC82551

Tn5 random insertion into the genome of DC82551 (Δ*lldD* Δ*ykgE::kan*) was performed using EZ-Tn5 insertion kit (Epicentre, Madison, WI). Cells were made competent by washing cells harvested at mid-exponential phase of growth four times with 10% glycerol sterile solution.

A total of 5 μL of complex (Tn5 transposon complexed with transposase enzyme) was electroporated into 250 μL of competent cells (OD_{420} above 100). A total of 500 tetracycline resistant transpositions were obtained. An alternative protocol consisted in the construction of a Tn5 P1 phage library, prepared as followed. The random insertions were generated by electroporating 1 μL of complex transposase:Tn5 with 35 μL of NEB electrocompetent cells (NEB, Ipswich, MA) using EZ-Tn5 insertion kit (Epicentre, Madison, WI). A large number of tetracycline-resistant transpositions (>10,000) were obtained and P1 phage was grown on the pool of tetracycline-resistant mutants. This phage preparation was used to transduce DC82551 ($L\text{-}LA^+$) and transductants were selected on LB-tetracycline agar medium. The tetracycline resistant colonies were transferred to minimal medium with L-LA as the carbon source and tetracycline and LB-tetracycline by replica plating technique and incubated at 37°C. The colonies that did not grow on lactate minimal medium but grew on LB-tetracycline medium were selected and resuspended in 50 μL minimal medium salts without carbon source. These cells were used to inoculate 4 culture tubes containing each 1.5 mL of minimal medium with different carbon sources; 5 g/L L-LA, 3 g/L D-LA, 5 g/L glucose, or 5 g/L succinate. Culture tubes were incubated at 37°C on a rotator operating at 75 rpm for aeration. Mutants that failed to grow on L-LA as sole source of carbon in liquid medium also were selected and their genomic DNA was sequenced using Tn5-F primer to map the transposon location.

UV mutagenesis of strain DC82551

Strain DC82551 was grown in 10 mL of Luria-Bertani broth (LB) to an OD_{420} of about 1.0. Cells were collected by centrifugation, washed twice with 5 mL of minimal medium salts (free of carbon source) and resuspended in 1 mL of the same medium. One mL of the suspension was subjected to UV-light (254 nm) for different time periods; from a few seconds to 1 min. Subsequently, 2 mL of SOC medium were added to the culture tube and incubated at 37°C with

agitation for two hours before plating serial dilutions of the culture on LB agar to assess the cell survival rate. Cells from time of exposure that yielded about 1% survival were plated to yield approximately 100-200 colonies per plate. "Replica plating" of these colonies to glucose-MM and LB plates were performed to determine the proportion of auxotrophs. The UV-treated sample that exhibited about 1% of auxotrophs was retained for further screening. Subsequently, colonies were once again "replicated" from Glucose MM agar medium onto L-LA MM, glucose MM and LB-agar and the plates were incubated at 37°C. Colonies identified to grow on LB and glucose MM but not on L-LA MM were selected and retested on L-LA MM medium to confirm their inability to grow on L-LA as the carbon source. Upon confirmation, selected isolates were inoculated into 2 mL each of the following minimal medium with different carbon sources in 13 x 100 mm glass tubes: 1% L-LA MM, 1% D-LA MM, 1% succinate MM, 1% glucose MM and incubated in a rotator at 37°C. The desired phenotype was Glucose^{+}, Succinate^{+}, D-LA^{+} and L-LA^{-}. Testing for growth on succinate MM ensured that the mutant possesses the machinery to grow on a non-fermentative carbon source whereas aerobic growth on D-LA MM suggested that the transport of lactate was not altered. Mutant strains that exhibited the required phenotype were stored at -80°C for future use. All the L-LA-negative mutants were grown in D-LA-MM for 24 h in a shaker. Three cultures that showed the highest OD_{420} were retained as DC8212, DC8248 and DC8261.

Adaptive evolution of *E. coli* strain DC8261 and DC*bglX*

DC8261 was serially transferred every day into 5 mL of fresh D-LA (0.4%, w/v)-kanamycin MM in a 125 mL Erlenmeyer flask using 1% inoculum. Due to the high cost of analytical grade sodium D-LA salt, after 27 days, the medium volume was changed to 1 mL in 13 x 100 mm test tubes and the cultures were incubated at 37°C on a rotator at approximately 70 rotations per minute. After incubation for 12 h to 24 h, the culture was transferred to fresh

medium at a starting OD_{420} of 0.08 and this was continued for several serial transfers. A stock of the culture was made after every ten transfers or every time a significant improvement on cell yield at 24 h could be observed. After 70 passages, the culture was streaked on 0.5% D-LA MM agar and a colony that developed earlier than others was picked, cultured and named DC826170. Another strain, DC*bglX*, was transferred 30 times in 2% LD-LA MM and an adapted strain named DC*bglX*30 was isolated. Even though DC*bglX* was transferred in MM containing both D-LA and L-LA, DC*bglX*30 still retained the L-LA-negative phenotype.

Removal of D-LA from Hydrolyzed PLA Material

D-LA Consumption Rate as a Function of DC*bglX* Cell Density

To assess of the relationship between the D-LA consumption rate and the initial cell density of the microbial biocatalyst engineered to remove D-LA from PLA syrup, the following experiment was performed using DC*bglX*. *E. coli* strain DC*bglX* inoculum was generated by culturing the strain aerobically in 1 L (2.8 L Fernbach flask) of 20 g/L LD-LA MM (37°C, 250 rpm). Cells were harvested at mid-exponential phase of growth, centrifuged for 15 min at 5,000 g at room temperature, and resuspended in 30 mL of 20 g/L LD-LA MM. Cells were then used to inoculate 35 mL of 20 g/L LD-LA MM (250 mL flask) at an OD_{420} of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0. After inoculation, flasks were incubated at 37°C in a rotary shaker operating at 250 rpm. At different time periods, 1 mL of culture was sampled to measure the OD_{420} as well as to measure the total [LA] and [D-LA] by HPLC and chiral HPLC respectively. D-LA consumption rates were calculated as the slope of the plot of [D-LA] over time. Experiments were conducted in triplicates.

D-LA Removal Using Hydrolyzed PLA Material

Inoculum preparation

E. coli strains DC8212, DC8148, DC8261, DC826170 and DC*bglX*30 were each used in separate D-LA purification experiments. DC8261 inoculum was generated by culturing the strain in 50 mL of 10 g/L glycerol MM. Inocula for DC826170 and DC*bglX*30 were cultured aerobically in 1 L of 20 g/L LD-LA MM. Cultures were incubated at 37°C in a rotary shaker operating at 250 rpm. Cells were harvested at mid-exponential phase of growth by centrifugation for 15 min at 5,000 g at room temperature, washed once in half their volume of MM lacking carbon source, and resuspended in 5 to 30 mL of MM without C-source. These washed cells were used to inoculate 20 mL of MM containing PLA syrup at an initial LA concentration (total LA) of 20 g/L at an OD_{420} of about 8.0.The culture in a 250 mL Erlenmeyer flask was incubated aerobically at 37°C in a shaker (250 rpm).

An initial cell density of OD_{420} of 6.0 was used in experiments conducted with strains DC826170 and DC*bglX*30. In these experiments, 35 mL of medium containing increasing amount of LA (250 mL flask) were inoculated, and incubated at 37°C in a rotary shaker operating at 250 rpm. DC826170 culture was used to perform D-LA removal experiments of syrups at concentrations of 27, 34, 41, 58, 81, 99 and 105 g/L LA. A concentration of 126 g/L LA-MM was used when the strain tested was DC*bglX*30. Also, 1 mM or 2 mM betaine was added to the cultures to alleviate potential osmotic effect that high [LA] may cause. Experiments conducted with DC*bglX*30 were triplicated.

PLA syrup preparation

PLA syrup used in D-LA removal experiments by strains DC8212, DC8248 and DC8261 was obtained by melting “Kangaroo-Express” soft-drink cups at 160°C for 135 min. It contained about 5% D-LA and 95% L-LA as determined by HPLC analysis. PLA syrup used for the

purification of D-LA by strains DC826170 and DC*bglX*30 originated from PLA bead grades 4032D and 3052D, respectively. The syrups from these two PLA grades contained a D-LA fraction of about 5% and 7.5% of the total LA, respectively. Prior to use, pH of the PLA syrup was adjusted to 7.0 by direct addition of NaOH pellets.

Analyses and calculations

During the D-LA removal experiments, approximately 1 mL was sampled at different time periods. Cell density as OD_{420} was measured using a spectrophotometer (Beckman model DU640, USA). Total LA was measured by HPLC and D-LA fractions were evaluated by HPLC equipped with a chiral chromatographic column. As needed, viability was assessed by plate count of the *E. coli* strain on LB agar medium.

D-LA removal with air sparging

The apparatus used in these experiments is depicted in Figure 2-1. Briefly, an aquarium pump (Aqua culture, model no. 0079285405133, China) was connected to a 1 L filtering flask containing 600 mL of sterile water. The filtering flask was connected to manifold that can support aeration of up to 8 cultures simultaneously through a glass sparger immersed into the culture. The entire apparatus (except the pump) was autoclaved prior to use and a sterile filter (0.22 μm pore size, Whatman, Piscataway, NJ, USA) installed between the pump and the filtering flask ensured that the air pumped into the system was free of contaminants. The pump was used at a capacity of 1.2 L/min. Screw clamps installed on the flexible tubing connecting each sparger allowed regulation of air flow to be visually equal in each culture tube (about 0.2 L/min). Culture tubes (25 x 200 mm) containing 25 mL of medium with 135 g/L of total LA were inoculated with strain DC*bglX*30 at an initial OD_{420} of 6.0.

Insight into the L-LA Metabolism of *Escherichia coli*

Biochemistry of L-LDHs

Cell free extract preparation

Regardless of the strain used, cell extract for LDH assay was prepared as follows. One liter of minimal medium containing sodium 10 g/L DL-LA was inoculated at 1% with an overnight culture grown in the same medium. Cells were grown in 2.8 L Fernbach flasks and agitated at 250 rpm at 37°C. Cells were harvested at an OD_{420} of about 2 (mid-exponential phase of growth) by centrifugation at 4,200 xg for 15 min, washed once with MM lacking carbon source, and resuspended in about 30 mL of 50 mM HEPES buffer at pH 8.0. The cells were incubated with lysozyme (0.3 mg/mL) and EDTA (10 mM) for 30 min at 37°C with mild agitation. Spheroplasts formation was verified by microscopy was used to ensure cell wall removal. As seen on Figure 2-2A, the majority of the cells are round shaped cells and about 30% of the cells appeared intact after lysozyme treatment. RNase, DNase (0.1 mg/mL each) and $MgCl_2$ (10 mM) were then added to the mixture and returned to 37°C for another 30 min. The preparation was sonicated for a total of 3 minutes on ice with intermittent cooling. After a subsequent low speed centrifugation (120 xg, 5 min, 4°C) to remove cell debris and unbroken cells, the supernatant was retained as the crude extract. Sonication treatment allowed an efficient break down of the spheroplasts into vesicles of smaller diameter (Fig. 2.1B). Small membrane vesicles obtained after sonication is expected to be inside-out to accommodate the large ATP synthase protruding from the inner membrane into the cytoplasm of the cells (39). Total protein was assayed by the BCA method with bovine serum albumin as standard (147).

Enzyme assays

L-LDH was assayed at room temperature by the addition of 40 mM L-LA into a 1.5 mL cuvette containing between 10 to 50 µL of crude extract, 100 µM DCPIP and 50 mM buffer in a

1.0 mL reaction volume (119). L-LDHs activity in cell extracts prepared from strains J7 and DC*lldR* was determined using 50 mM MES-Tricine buffer at pH 6.5. L-LDH activity in the crude extracts from DC8255, DC82551 and DC*ykgD* were determined using HEPES-CAPSO buffer at pH 9.5, 10.0 and 9.5 respectively. L-LA oxidation was monitored at 600 nm as reduction of DCPIP, and concentration of oxidized DCPIP was calculated using the extinction coefficient for DCPIP of 19.1 $mM^{-1}.cm^{-1}$ at this wavelength (126).

Pyruvate accumulation

In order to determine that the product of the L-LDH reaction was indeed pyruvate, a reaction mixture was set up as described above and incubated at room temperature. Every 5 min or when the reaction mixture turned white due to the reduction of DCPIP, an additional 100 μM of DCPIP was added to the mixture. Occasionally, more crude extract was added, as needed. This way, around 3 mM of DCPIP was reduced. To stop the reaction and precipitate the proteins, a few drops of concentrated H_2SO_4 were added to the cuvette and the mixture was centrifuged for 5 min at 16,000 xg. The supernatant was subjected to HPLC and the product of the reaction was determined.

Localization of L-LDHs in *E. coli*

To localize the LDHs in the cell, crude extracts were centrifuged at 100,000 g for 2 h. Supernatant was retained as the soluble fraction and the pellet resuspended in 3 mL of 50 mM of HEPES buffer pH 8.0 constituted the membrane fraction of the cells. Each fraction was assayed for L-LDH activity as described above, using 50 mM HEPES buffer at pH 8.0.

Construction of Genomic Libraries and *recA* Mutants

Three plasmid libraries were used throughout this study. Standard genetic methods were used for the isolation of DNA and plasmids, digestion with restriction enzymes and ligation (135). Enzymes were purchased from New England BioLabs (Ipswich, MA) and used as directed

by the vendor. Strain DC8261 *recA*53 mutant was constructed (DC8261*RecA*) by conjugation as described elsewhere (18).

Plasmid pUC18 based plasmid library was generously provided by L.O. Ingram (University of Florida) (188). Average chromosomal DNA insert size in this library is between 2 and 4 kb.

Plasmid pACYC184 based library made from the genomic DNA of *E. coli* strain W3110 wild type was constructed by Frank Healy using plasmid vector pACYC184. Average length of the inserts in this library is about 6 kb.

Plasmid pCC1-BAC based genomic library was constructed using DNA purified from *E. coli* strain DC82551c strain (Δ*lldD*, Δ*ykgE*). Genomic DNA was partially digested with endonuclease *Sau3AI*. Fragments of 1.5 to 5 kb in length were purified from agarose gels and ligated into alkaline phosphatase-treated *BamHI* sites of plasmid pCC1-BAC (Epicentre, Madison, WI). Ligation products were transformed into electroporation-competent *E. coli* strain EPI3000 (Epicentre, Madison, WI). Blue white identification indicated that about 85% of the clones contained an insert (white colonies). More than 15,000 colonies were pooled and used to prepare a master library of plasmid DNA, used to transform DC8261c and DC8261*recA*.

About 120 to 240 ng of plasmid DNA from each library was electroporated into strain DC8261 and/or DC8261*recA* and plated on LB-chloramphenicol medium (pACYC and PCC1-BAC based libraries) or LB-ampicillin (pUC18 based library). Colonies were "replicated" onto MM with L-LA containing the appropriate antibiotic and incubated at 37°C for 48 to 72 h. When pCC1-BAC library was used, colonies from Lerechloramphenicol medium were also "replicated" onto L-LA MM containing 5 μM of IPTG. L-LA$^+$ clones were streaked onto L-LA MM and LB agar medium with appropriate antibiotic to confirm growth of the clone. Upon

verification of the desired phenotype, an isolated colony was picked from the rich medium, resuspended in 20 μL of sterile water, and the resuspension was used as DNA template for PCR using primers annealing upstream and downstream of the insertion site on the plasmid (Table 2-2). Plasmid DNA was sequenced to identify the genomic content of the insert DNA.

F' Complementation

Complementation analysis with *E. coli* F' elements was carried out as described previously (97) using the F' kit from the *E. coli* Genetic Stock Center. Crosses in liquid medium were performed with log-phase donor and recipient (DC8261) cells grown aerobically in LB medium to an OD_{420} of about 1. Donor and recipient cells were mixed in a culture tube at a ratio 10:1 respectively, and incubated at 37°C for 30 min with gentle rotation (about 50 rpm on a rotator) to allow gene transfer. A total of 10^8 recipient cells were conjugated with 10^9 F' donor. Cells were then plated on 0.3% L-LA MM supplemented with kanamycin and incubated at 37°C for 48 to 72 h.

Conjugation of DC8261 with Various Hfr Strains

Three Hfr strains were used to transfer DNA into strain DC8261c (178) (Table 2-1). Conjugations were performed as follows. Donors and recipient cells were grown in LB until mid-exponential phase of growth. Then, 10^9 CFU of DC8261c were mixed with 10^8 donor cells. Matting step was performed at 37°C under mild agitation (50 rpm) for 30 minutes. Culture tubes were vortexed for at least 30 sec to break the pairs and dilutions were plated on L-LA tetracycline agar medium.

L-LDH Activity Observed on Native Gel

Sample preparation

Ten mL of cell free extract prepared as described above was centrifuged at 100,000 x g for 2 h. The top 8 mL of the supernatant and the bottom 2 mL, which looked darker in color,

were collected separately. The sample containing the bottom 2 mL was then centrifuged at 150,000 g for another 3 h. The bottom part of the supernatant that was slightly above the pellet (diffuse material) with L-LDH activity was used in native gel electrophoresis without further treatment.

Native gel electrophoresis: PAGE

Non-denaturing gels contained 7.5% w/v polyacrylamide (acrylamide 30%, bisacrylamide 0.8%), and 2% triton X-100. Samples were subjected to electrophoresis in 25 mM Tris and 192 mM glycine buffer (pre-chilled). Approximately 35 μL of sample was loaded in each well. Immediately following electrophoresis at 4°C, overnight, the gel was bathed in 50 mM HEPES-CAPSO buffer, pH 9.0 containing 1.5 mM DCPIP. Once the gel turned dark blue and checked for no apparent lactate independent reduction of DCPIP, the solution was diluted ten times in 50 mM HEPES-CAPSO buffer at pH 9.0 and 50 mM L-LA was added. The gel with the assay mixture was gently rocked until white discoloration due to L-LA-dependent reduction of DCPIP could be observed on the gel. The white band on the gel was precisely cut and the proteins in the gel slice were identified after trypsin digestion and LC-MS/MS by the Interdisciplinary Center for Biotechnology Research at the University of Florida

Table 2-1. Plasmids and strains used in this study

Plasmid or strain	Relevant characteristics	Source or reference
Plasmids		
pCP20	FLP$^+$, λ ci857$^+$,λ p_R Rep^{ts},Ap^R,Cm^R	(15)
pKD46	*bla* P_{BAD} *gam bet exo* pSC101 *ori*TS	(20)
pKD4	*bla* FRT *kan* FRT	(20)
pCA24N::*lldD*	pCA24N carrying *lldD* from *E. coli* K-12 AG1	(84)
F143	F' plasmid of KL711, *relA1*	(97)
F104	F' plasmid of KL723	(97)
F128	F' plasmid of E5014	(97)
F254	F' plasmid of KL719	(97)
Strains		
W3110	F$^-$ λ$^-$ *rph-1* INV(*rrnD*, *rrnE*)	ATCC 27325
Top10	F$^-$ λ$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80*lacZ*ΔM15 Δ*lac*X74 *nupG recA1 ara*D139 Δ(*ara-leu*)7697 *gal*E15 *gal*K16 *rpsL*(Str^R) *end*A1	Invitrogen
MG1655	F$^-$ λ$^-$ *ilvG- rfb-50 rph-1*	ATCC 700926
KL711	F$^+$ *thi$^-$, his$^-$, ura$^-$, trp$^-$*	(97)
KL723	F$^+$ *thi$^-$, arg$^-$, his$^-$*	(97)
E5014	F$^+$ *thi$^-$*	(97)
KL719	F$^+$ *thi$^-$, met$^-$, trp$^-$, leu$^-$*	(97)
BW6166	Hfr *glgP721::Tn*10 *thi$^-$*	(178)
NK6051	Hfr *purK79::Tn*10 *thi$^-$*	(178)
BW6163	Hfr *zed-977::Tn*10 *thi$^-$*	(178)
J7	JM107 Δ*ldhA* Δ*tcdE* Δ*pflB*	(Lab collection)
DC825	J7 Δ*dld*::*kan*	This study
DC8255	J7 Δ*lldD*::*kan*	This study
DC82551	DC8255 Δ*ykgE*::kan	This study
DC82552	DC8255 Δ*ykgF*::kan	This study
DC82553	DC8255 Δ*ykgG*::kan	This study
DC8212	DC82551 UV treated (L-LA$^-$)	This study
DC8248	DC82551 UV treated (L-LA$^-$)	This study
DC8261	DC82551 UV treated (L-LA$^-$)	This study
DC8261*RecA*	DC8261 Δ*RecA*	This study
DC826170	DC8261 transferred 70X in MM D-LA	This study
DC*lldR*	DC8261 Δ*lldR*::kan (*lldD*$^+$)	This study
DC*bglX*	DC826170 Δ*BglX*::kan	This study
DC*ykgD*	DC8261 Δ*ykgD*::kan (*ykgE*$^+$)	This study
DC*bglX*30	DC826170X transferred 30X in MM LD-LA	This study
DC825*ykgEFG*	DC825 Δ*ykgEFG*::kan	This study

Table 2-2. Primers used in this study

Primer name	Sequence 5' to 3'
F-*dld*-out	ggatggttgccgaataaa
R-*dld*-out	cctgcgtacctggattgaa
F-*dld*-in	cacgctctattcgctgga
R-*dld*-in	gctcataccactcggtgtcgtta
F-*lldD*-out	gcaatcctcagtgaagcata
R-*lldD*-out	cggtgtcgtttcagagtga
F-*lldD*-in	gccgttcctgttccactat
R-*lldD*-in	gcaatcatacgcacgacat
F-*ykgE*-out	cggtggtttgggcttt
R-*ykgE*-out	gcgggtagcgtcttctt
F-*ykgE*-in	cgtaagctgggagtgaagga
R-*ykgE*-in	ggctcatcaacacttcagca
F-*ykgF*-out	cgatgtaggtgccagttt
R-*ykgF*-out	cctccagcctcgtgtca
F-*ykgF*-in	cgaacgtctgggctatga
R-*ykgF*-in	gcaggcgtagggtaaatctt
k1	cagtcatagccgaatagcct
k2	cggtgccctgaatgaactgc
F-*ykgG*-kan	atgcggcaagctggtttatcaatggcggcaaaacaccgtgtaggctggagctgcttcg
R-*ykgE*-kan	ggctcatcaacacttcagcaatatgcatcactttcatatgaatatcctcctta
F-ykgE-kan	ggcacgagactccgtgctgctactggaaaaactcggcgtgtaggctggagctgcttcg
F-*ykgD*-out	gcacgcacaaaatctgtgtg
F-*lldR*-out	tctgatctgttgctggttgc
F-*bglX*-out	ccagtgacagcaactgatcc
F-lldD-kan	gcattcgagggagaaaaacgcatgattgtttccgcagtgtaggctggagctgcttcg
R-LldD-kan	tgccgcattccctttcgccatgggagccatcatatgaatatcctcctta
F-lldP-kan	ctgcaagccaccgcagcagaagaaattggcgtctcgtgtaggctggagctgcttcg
F-pACYC-*Bam*	ctatcgactacgcgatcatg
R-pACYC-*Bam*	cggtgatgtcggcgatatag
F-M13	gtaaaacgacggccagt
R-M13	aacagctatgaccatg
Tn5-F	gggtgcgcatgatcctctagagt

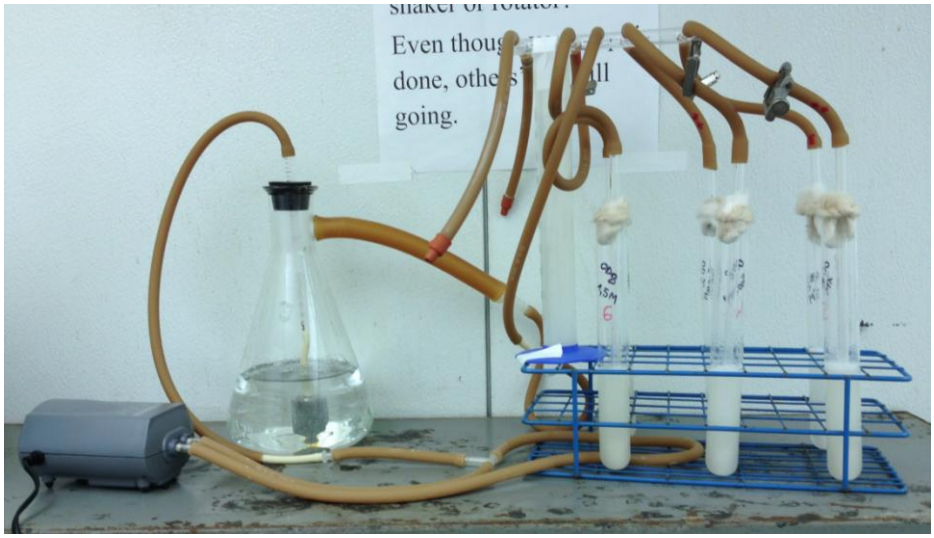

Figure 2-1. Culture apparatus used for D-LA removal from PLA syrup with sparged air

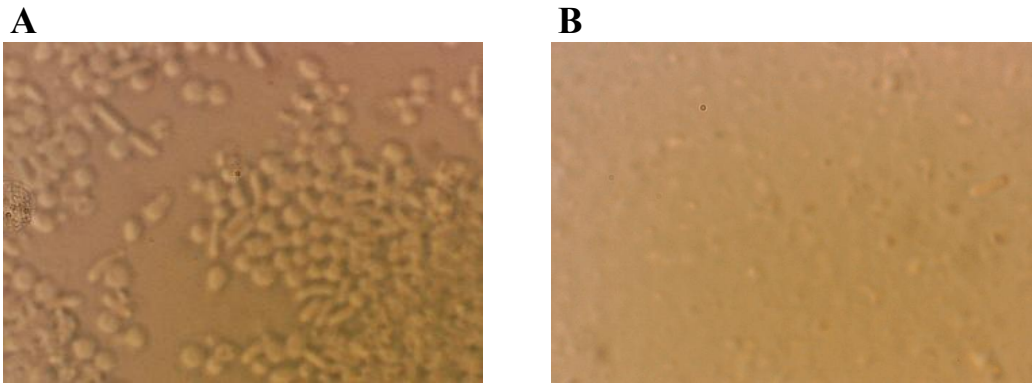

Figure 2-2. Pictures of spheroplasts of DC8255 cells (A) and vesicles after 3 minutes of sonication (B). Magnification: 1000 X.

CHAPTER 4
RESULTS AND DISCUSSION

PLA Hydrolysis to LA

PLA Hydrolysis in Water

Hydrolysis of two different PLA beads (grades 3052D and 4032D) and a consumer product (PLA-based cups) were performed at 160°C with water and the L-LA released was determined by HPLC. Since the hydrolysis profiles of all three PLA were similar (Figure 3-1), subsequent experiments were conducted using beads grade 4032D, as the crystallinity of this polymer is slightly higher than the other beads. The consumer grade plastic cup was not used due to the need for further processing (size reduction). The PLA beads were treated in water at 140°C, 150°C and 160°C and the amount of LA released was determined (Figure 3-2). Lactic acid was detected in the medium after a short lag at all three temperatures, and the duration of this lag time was dependent on the temperature of incubation (1.33 h, 2 h and 3.15 h at 160°C, 150°C and 140°C, respectively) (Figure 3-2A). During the lag period, the beads started to soften due to heat followed by swelling of the beads indicating water intake. Apparently, water molecules diffused into the amorphous regions of the beads as suggested by Tsuji et al. (170) as seen by a slight increase (less than 10% by weight) in the solid fraction weight that reached its maximum by the end of the lag phase. Following the lag, LA release was exponential and the rate of LA production increased with increasing temperature (0.382 /h at 140°C, 0.664 /h at 150°C and 0.989 /h at 160°C). The rate of LA release under the experimental condition followed a first order kinetics and all the acquired data fit a modified Gompertz model (equation 3-1) (Figure 3-2B). The model curve fitted the data set with a sum of the square errors R^2 equal to 0.0034 for the 150°C treatment and the model also allowed for the determination of the lag phase duration and maximum rate of hydrolysis.

By mid-exponential phase of LA accumulation, the beads have completely melted and the remaining solid material was opaque and white. At the end of hydrolysis of the PLA beads, no solid material was present and the syrup was clear with a slight yellow color with a density ranging from 1.162 at 140°C to 1.191 at 160°C. The amount of time required for almost complete hydrolysis of PLA was also dependent on the temperature and the final lactic acid titer was 191 (±10.6), 197 (±1.91) and 182.5 (±2.99) mmoles of LA from the 160°C, 150°C and 140°C treatment, respectively. These titers represent a yield of 92% (160°C), 95% (150°C) and 88% (140°C) of LA from PLA beads. The lower yield at 140°C could be due to incomplete hydrolysis of LA oligomers at this temperature at the time the experiment was terminated.

Similar hydrolysis profiles of PLA pellets were also reported by Tsuji *et al.* in the temperature range of 180°C to 350°C (163). In a 15 min hydrolysis time used in their study, the LA yield was 65% (220°C), 75% (260°C), 80% (300°C) and 90% (350°C). The results presented in Figure 3-2 shows that 95% of LA yield can be achieved at a significantly lower temperature of 160°C although the time required was longer than the 15 min incubation used by Tsuji, et al. (148).

Siparsky and co-workers suggested that continued accumulation of LA during hydrolysis could support an autocatalytic increase in the rate of PLA hydrolysis (143). To test this possibility, a hydrolysis profile was obtained at 160°C in the presence of equal amounts of PLA beads and LA syrup obtained from a previous experiment (pH 2.0; [LA], 460 mM) and compared to the one in which PLA beads were hydrolyzed in water. The hydrolysis profiles of both samples were similar (data not presented). Additionally, a PLA hydrolysis profile in sulfuric acid (160 mM) also did not exhibit any detectable difference in lag phase duration or the rate of lactic acid release. Siparsky et al. hydrolyzed PLA in the melt using an acetonitrile/water mixture

and this could account for the reported autocatalytic PLA hydrolysis profile (143) that was not observed in a water-based hydrolysis of PLA beads presented here (Figure 3-2). Also, the good fit of the Gompertz model to the hydrolysis profile did not suggest an autocatalytic increase in PLA hydrolysis rate.

The PLA pellets used in the hydrolysis experiments represent the raw material used by manufacturers to produce commodity plastic items distributed on the market. PLA bead grade 3052D represents the PLA typically sold to the food industries for producing packaging material for their food products. PLA grade 4032D is also used by the food industry, but mainly as films. It is possible that the hydrolysis behavior of plastic items made from these PLA beads may differ from the raw PLA used in this study. To test this possibility, soft-drink cups obtained from Kangaroo™ gas station were cut into pieces of about 2 cm^2 and subjected to hydrolysis with water at 160°C. The hydrolysis profiles obtained for the soft-drink cups were comparable to the profiles obtained for the two grades of PLA beads, as seen in Figure 3-1.

PLA Hydrolysis in an Alkaline Solution

PLA is hydrolyzed to LA in water at a rate that increases with temperature (Figure 3-2A). As presented above, PLA hydrolysis is not influenced significantly by the presence of either lactic acid or sulfuric acid. However, base has been reported to enhance PLA hydrolysis (183) although the effect of base on hydrolysis is yet to be described. In order to evaluate the effect of NaOH on hydrolysis of PLA beads, thermochemical hydrolysis profile of PLA was determined at 160°C (Figure 3-3).

In the presence of NaOH, hydrolysis profiles of PLA beads were biphasic (Figure 3-3A). There was an immediate release of LA that reached the maximum of about 17 mmoles in the presence of 15 mmoles of NaOH after 15 min at 160°C and this amount represents about 8% of the theoretical maximum (Figure 3-3B). This was followed by complete hydrolysis of PLA

(Figure 3-3A). In this experiment, the ratio of added [NaOH] to the PLA concentration was 0.07 mol/mol. A similar alkali-dependent initial burst of LA was also observed at 60°C (Figure 3-3B) but not at room temperature. At both 60°C and 160°C, this initial phase of LA release was within 15 min after the canisters were inserted in the pre-heated oven (Figure 3-3B), and the solid PLA still present in the canister retained its beads shape. When PLA was hydrolyzed with water at temperatures as high as 160°C, this initial phase of LA production seen in presence of NaOH was not detected (Figure 3-1, 3-2 and 3-3). These results suggest that added alkali directly interacts with the amorphous regions of the polymer, especially at the surface of the bead, and this requires heating of the beads to expose these regions to solvent. This is in agreement with the observation that the solid beads of slightly smaller diameter can be retrieved from the canisters after 15 min of heat treatment and also with previously reported surface erosion of PLA under alkaline conditions (167).

Increasing the [NaOH] in the reaction increased the amount of LA produced during this phase of PLA hydrolysis (Figure 3-4A). Higher concentration of NaOH also reduced the lag duration before the second phase of hydrolysis started (Figure 3-4B). Apparently, one molecule of OH^- reacts with one stereocenter of the polyester to release one molecule of LA. In this experiment, the correlation between LA release and [NaOH] was about 0.9. Since at 160°C, higher [NaOH] led to an overlap of the two phases of PLA hydrolysis (Figure 3-3B and 3-4A), the actual LA concentration released during the first phase could not be accurately determined. To overcome this limitation, 60°C was used to evaluate the first phase of alkali-dependent PLA hydrolysis since at this temperature, no significant additional LA release was observed over a 4 days period besides the initial release of LA by the base. At 60°C as well as at 160°C, a direct correlation between the initial [NaOH] in the reaction and the amount of LA released from the

PLA during the first phase can be observed (Figure 3-4A). On a molar basis, the correlation between [NaOH] and the amount of LA released into the solution was 0.72 at 60°C. The difference in the molar ratio between the two temperatures, i.e., 0.72 mol/mol at 60°C and 0.9 mol/mol at 160°C, could be related to temperature-dependent melting of the beads that exposed additional amorphous regions of the bead to base attack. Since 60°C is not high enough to completely melt the PLA beads within the four hour period of this experiment, NaOH access is restricted to the amorphous layer on the bead surface that is not stoichiometrically equal to the base concentration, especially at higher [NaOH].

After this initial base-catalyzed release of LA, a short lag in LA release was observed at 160°C. This lag duration varied depending on the concentration of NaOH added to the reaction (Figure 3-4B) and also to the temperature of incubation (Figure 3-4C). However, it should be noted that base addition only shortened the lag phase before the remaining PLA hydrolysis started and it did not significantly alter the biphasic profile of the PLA hydrolysis. This is in agreement with a previous report on PLA hydrolysis conducted at temperatures over 160°C that also showed a shortening of the lag phase by NaOH (183).

Immediately following this lag, the remaining PLA beads were hydrolyzed to LA in an exponential manner until the total LA yield reached about 90% of the theoretical value, as seen previously with water-based hydrolysis (Figure 3-2A and 3-3A). The rate of hydrolysis in the second phase of the reaction with added NaOH was slightly higher (1.23 /h) compared to water-based hydrolysis (0.99 /h). This slightly higher rate of hydrolysis could be a consequence of surface erosion of the beads during the base attack that reduced the size of the remaining beads, suggesting that the bead diameter may be a component in determining the hydrolysis rate (167).

Activation Energy of PLA Hydrolysis to LA

Addition of base to the hydrolysis reaction did not significantly change the activation energy of hydrolysis (19.6 kcal/mol with NaOH and 17.2 kcal/mol with water alone) (Figure 3-5). One possible explanation for the slightly higher activation energy obtained in the presence of NaOH is that the base initially helps hydrolyze the amorphous regions of the beads leading to an increase in activation energy for hydrolysis of the remaining more organized crystalline parts of the beads. This would be in agreement with the previously reported higher energy to achieve hydrolysis of crystalline PLA (167). These values for activation energy of hydrolysis of PLA beads are close to the 19.9 kcal/mol obtained for hydrolysis of PDLLA microcapsules and 20.0 kcal/mol for PLLA microcapsules in the range of 21°C to 45°C (101). However, values obtained in this study differ from the 12.2 kcal/mol reported for temperatures ranging from 180°C to 250°C; this lower value probably represents the activation energy required to break down the polymers into oligomers of smaller Mw as opposed to activation energy necessary for release of LA (171).

Effect of Particle Size of PLA on Hydrolysis

The results presented above suggest the reactivity of NaOH with the PLA beads (2 mm diameter) may be limited by the surface area of the beads. It is possible that increasing the surface area accessible to the hydroxyl ions would increase the amount of LA released per mole of NaOH added. To test this possibility, the 2 mm diameter PLA beads were ground, sifted through a bouillon strainer and subsequently reacted with NaOH at 60°C (Figure 3-6).

In this set of experiments, 5 g of ground PLA (70 mmoles) were added to 25 g of aqueous solution containing 90 mmoles of NaOH (Figure 3-6A). At this ratio of NaOH to PLA, the hydrolysis of PLA was monophasic and reached a maximum yield of about 90% within four hours. At [NaOH] below this value, the quantity of base added was limiting and the amount of

total LA recovered was proportional to the initial amount of base added (Figure 3-6B). When ground PLA was hydrolyzed, 0.97 (±0.028) mole of LA per mole of NaOH was obtained as opposed to a ratio of 0.82 (±0.0034) obtained for the 2 mm diameter PLA beads. This increase in the molar ratio shows that the total surface area of the polymer accessible to hydroxyl ions limits the amount of LA that can be released under such conditions. It is also possible that grinding the beads to a powder led to partial decrystallization of the polymer accounting for the monophasic hydrolysis profile at 60°C and the higher molar ratio. It is interesting to note that with the 2 mm PLA beads, the LA released to base ratio at 60°C increased to 0.82 in this experiment compared to 0.72 seen before (Figure 3-4A). This is apparently due to the PLA to base ratio at the beginning of the experiment and the time of sampling: 17% PLA (w/w) in this experiment (Figure 3-6) compared to 50% PLA (w/w) in the previous experiment (Figure 3-4A) and a time of sampling from 4 hours to 24 hours.

Racemization of Lactic Acid during Hydrolysis

In the recovery of LA for reuse in the polymer industry, it is important that the LA produced by hydrolysis of PLA remains true to the original optical purity of the PLA. To investigate the isomeric stability of LA, PLA grade 3052D that contains a reported 4% of D-isomer was used in this experiment. During hydrolysis with water, trace amount of LA was released during the first hour before the exponential increase in LA (Figure 3-7). During this period, the LA released was enriched in D-LA reaching as high as 20% of the total released LA. As the total LA concentration started to increase, the D-LA fraction of the total declined to a final ratio of about 7.8% (Figure 3-7). Similar results were also obtained during PLA hydrolysis at temperatures lower than 160°C (Table 3-1), or by addition of limiting amounts of NaOH (Figure 3-7B). This higher than anticipated D-LA content (about 5-8%) in the syrup was

unexpected since it has been reported by others that racemization was not observed during heat treatment of PLA beads below 200°C (163, 171, 183).

The increase in the D/L ratio could arise from limited racemization of LA during production of the beads (4) or during hydrolysis. To distinguish between the two alternatives, LA syrup obtained after PLA hydrolysis in water at 160°C was mixed with equal amount (w/w) of either water or 1 M NaOH and heated to 160°C for 2 h. Also, pure L-LA in water or in 1 M NaOH was treated the same way. Results of these experiments show that the D/L ratio of either syrup or pure L-LA did not change significantly upon treatment in presence of water or NaOH at 160°C (Table 3-2). Based on these results, it can be concluded that the observed higher than expected D/L ratio (Figure 3-7; Table 3-1) is apparently introduced during the preparation of the PLA beads and not during thermohydrolysis in water or in limiting NaOH. However, racemization of LA during hydrolysis of PLA could not be ruled out although free LA was not significantly altered in this experiment.. Racemization of free L-LA did occur when the NaOH is present at stoichiometrically higher concentration compared to the PLA and heated to 160°C (Table 3-2). Similar increase in racemization of LA in the presence of excess KOH and heat has been reported by Lockwood et al. (96).

Even though NaOH in limiting or stoichiometrically equal amounts did not appear to affect racemization during hydrolysis (Table 3-1 and Figure 3-7), base must be used with caution as excess NaOH remaining after hydrolysis could lead to racemization of the product. Ideally, the process chosen to hydrolyze PLA polymer should not increase the D-LA content of the product above that of the starting polymer to minimize the need for separation of the two enantiomers before L-LA can be reused (172). This can be achieved by judicial application of alkali, time and temperature in the thermochemical hydrolysis of PLA for reuse.

Engineering *E. coli* for Removal of D-LA from Hydrolyzed PLA

Toxicity Induced by LA and PLA Syrup on the Growth of Wild Type *E. coli*

Since almost all PLA material found on the market include small amount of D-LA or other co-polymers, purification of the bulk L-LA is a critical factor in the overall process before the recovered L-LA can be reused for production of PLA. This can be achieved either by expensive purification processes (58) or biologically using microbes that can selectively remove the minor component of the PLA-syrup, such as D-LA. Although all PLA polymers are made almost exclusively from lactic acid or other biodegradable compounds, during their production, the polymers could acquire some toxic compounds that may prevent successful bio-based purification of the PLA syrup. Before genetically engineering an *E. coli* strain to selectively remove D-LA from the PLA syrup, assessment of the toxicity of the PLA syrup is necessary. *Escherichia coli* is known to use both L- and D-LA as sole carbon sources for aerobic growth. To assess the potential toxicity of PLA syrup, growth of *E. coli* strain W3110 in minimal medium containing PLA syrup was evaluated (Figure 3-8).

E. coli strain W3110 was grown in mineral salts minimal medium containing increasing amounts of PLA syrup corresponding to a LA concentration ranging from 4 to 110 g/L. As a control, the same experiment was conducted using pure Na-L-LA as a sole carbon source. The highest specific growth rate was recorded for the lowest [LA] used, i.e.,4 g/L, regardless of the source of the lactic acid (Figure 3-8). However, the specific growth rate of 0.5 /h observed for Na-L-LA was higher than the growth rate of 0.4 /h when LA originated from PLA syrup. For [LA] lower than 35 g/L, growth rates observed with Na-L-LA were consistently 15% higher on average than the growth rates measured for cultures using melted PLA. This result suggests that additional compound(s) present in the syrup have a negative effect on the metabolism of the cell affecting its growth rate. Even though the growth rates recorded using PLA syrup were lower

than that of the cultures containing Na-L-LA, the cells reached the same final OD_{420} for both carbon sources (data not shown). Regardless of the LA source, a sharp decrease in the specific growth rate was observed with LA concentrations higher than 27 g/L. The decrease reported was probably due to the high solute concentrations and osmotic effect (17). At [LA] higher than 42 g/L and 56 g/L in cultures containing PLA syrup and Na-L-LA, respectively, growth of the organism was not observed during the course of the experiment that lasted 27 h. Overall, wild type *E. coli* was able to grow using PLA syrup as a sole carbon source. In spite of the slight decrease in the growth rate observed when PLA is used, *E. coli* is a satisfactory candidate for metabolic engineering in order to selectively remove D-LA from hydrolyzed PLA material.

Isolation of *E. coli* Mutant Lacking L-LDH Activity

The first step in engineering *E. coli* to selectively remove D-LA from hydrolyzed PLA material is the construction of a strain that is unable to utilize L-LA as a carbon source. The two genes encoding known L-lactate dehydrogenases in *E. coli* (*lldD* and *ykgE*) were deleted with the expectation that the double mutant will be defective in oxidation of L-LA without any change in its D-LA metabolism (Figure 3-9). Unexpectedly, the double mutant, strain DC82551, still grew in mineral salts medium with L-LA as sole carbon source. These results indicate that there is at least one or possibly more additional protein(s) with L-LDH activity produced by *E. coli*. In order to obtain an *E. coli* mutant that is unable to grow on L-LA as sole source of carbon, random insertions of Tn5 transposons into the genome of DC82551 using a EZ-Tn5 kit was constructed (Epicentre). Using the double mutant as the recipient of the mutated DNA, only 500 mutants were obtained due to low transformation efficiency of DC82551. None of the mutants had a L-lactate-minus phenotype (Figure 3-9). To increase the total number of transposon mutants, mutated DNA was electroporated into *E. coli* strain NEB Turbo electrocompetent cells. The obtained transposon mutants were pooled (>10,000) and phage P1 was grown on this pool of

mutants. The P1 library was used to transduce the tetracycline-resistance gene into the *lldD, ykgE* double mutant, strain DC82551. About 1200 transductants were tested for growth on L-LA in MM. Three tranductants did not to grow on L-LA. The genomic DNA from these three mutants was extracted and sequenced using the Tn5-F primer, provided by Epicentre. In all three mutants, the transposon was inserted in the same gene, i.e.,*gltB* gene, encoding the large subunit of glutamate synthase the enzyme that converts L-glutamine and alpha-ketoglutarate into two molecules of L-glutamate (108). To evaluate the putative role of *gltB* in L-LA metabolism, the *gltB* gene in the double mutant was deleted using the "Keio" collection of deletion mutants. All 32 transductants tested were found to be L-LA positive suggesting that the *gltB* gene has no detectable role in L-LA metabolism. It is unclear why the transposon-induced *gltB* mutation negatively affected L-LA metabolism in the *E. coli* double mutant. A possibility is that the transposon insertion followed by transduction into the double mutant caused additional genetic alteration in the chromosome leading to the L-LA-minus phenotype. These strains were not further studied.

Chemical mutagenesis using ethylmethane sulfonic acid also failed to yield a L-LA-minus mutant.

UV light has long been used as a mutagen of bacterial and eukaryotic genomes and the mechanism of this process is well studied (36, 46, 88). Strain DC82551 was mutated using UV and among an approximately 4,000 colonies screened, 20 mutants were found to be defective in L-LA utilization ($L\text{-}LA^-$, Glu^+, $D\text{-}LA^+$, $Succ^+$) (Figure 3-9 and Figure 3-10). These 20 mutants were tested in liquid medium with L-LA as sole C-source and their growth rate and cell yield with D-LA were determined. Three mutants that yielded the highest cell density after 24 h of growth in minimal medium with D-LA as a sole carbon source were retained and named

DC8212, DC8248 and DC8261 (Figure 3-10). The kanamycin gene cassette in *ykgE* gene (derived from the "Keio" deletion mutant) was cured from each of the three strains as described in the Material and Methods section, to generate DC8212c, DC8248c and DC8261c.

Lactic acid is transported in *E. coli* by two known gene products encoded by *lldP* and *glcA* (115). Only a mutant lacking both transporters failed to accumulate LA in the cell. When *ykgE*$^+$ was transduced into the UV-induced L-LA-minus DC8261 mutant, the resulting transductants (*ykgE*$^+$, expression no other L-LDH) grew on lactate at about the same rate as an *lldD* mutant (DC8255, Table 3-3), suggesting that lactate transport is unaffected in strains DC8212, DC8248 and DC8261 (Figure 3-9). This was further confirmed by transducing *lldD*$^+$ into the DC8261 strain.

Growth of one such transductant; carrying a deletion in *lldR* with kanamycin-resistance gene insertion DC*lldR*) is presented in Figure 3-11. When cultured in liquid L-LA MM, the maximum growth rate of strain DC*lldR* was comparable to the growth rate observed for DC8255 and DC82551 parent strains (Table 3-3). Transformation of strain DC8261, with plasmid pCA24N that contains the coding sequence of *lldD* gene (pCA24N::*lldD,* Table 2-1) under the control of an IPTG-inducible promoter (84) restored growth on L-LA. These results are in agreement that the third mutation that eliminated growth of *E. coli* on L-LA is not in any of the gene(s) contributing to L-LA transport.

Purification of PLA Syrup by DC8212, DC8248 and DC8261

Purification of the PLA syrup requires a strain that is L-LA-minus but has high rate of D-LA metabolism. To determine which strain could remove D-LA from a culture broth most efficiently, strains DC8212, DC8248 and DC8261 were grown in glycerol MM, harvested at mid-exponential phase of growth and washed as described in the Material and Method section. Growing the cells using glycerol as a carbon source should not influence the level of D-LDH

activity, as suggested in two previous studies (40, 113). A minimal medium containing 20 g/L PLA syrup with a composition of 10 mM of D-LA and 210 mM of L-LA was inoculated at an OD_{420} of 8.0. Strains DC8212 and DC8261 were able to metabolize all the D-LA in the medium. Strain DC8248 only consumed about 50% of the D-LA added to the medium in about 4 days and the [D-LA] remained constant thereafter (data not shown). Consequently, DC8248 was not studied further. It took five days for DC8212 and DC8261 to completely remove the 0.9 g/L D-lactate added to the medium. The large molar excess of L-LA present might have hampered with the ability of the *E. coli* strains to rapidly metabolize D-lactate in the medium. As expected, both strains did not metabolize L-LA. Specific D-LA removal rate recorded for DC8261 was slightly higher than for DC8212, i.e., 0.004 and 0.003 $g_{D\text{-}LA}/(g_{DW}.h)$. Consequently, DC8261 was retained for further experiments.

Adaptive Evolution of *E. coli* Strain DC8261 in D-LA MM and Optimization

Serial transfers of DC8261 in D-LA MM were performed to improve the growth rate of the bacterium in 3 g/L D-LA MM. The growth rate after transferring the strain 70 times (DC826170) had a specific growth rate of 0.29 /h, which is about 50% higher than for the starting strain (DC8261) that had a specific growth rate of 0.20 /h (Table 3-3 and Figure 3-9).

In order to improve further the growth of strain DC826170 on D-LA as a sole carbon source, *dld* structural gene was introduced into the genome. The *dld* gene codes for a D-LDH in *E. coli* and is responsible for the oxidation of D-LA into pyruvate under aerobic condition (129). This gene was previously deleted as it was shown that D-LDH in *E. coli* exhibits a minor L-LA dependent reduction of MTT (38) (Figure 3-9). Transduction of Δ*bglX*::*kan*, a neighboring gene to *dld*, into strain DC826170 allowed restoration of the native copy of the *dld* gene coding for a D-LDH in *E. coli*, resulting in creating strain DC*bglX*. BglX is a periplasmic β-D-glucosidase that is not predicted to influence the aerobic D-LA metabolism in *E. coli* (185). Surprisingly,

restoring the expression of this second D-LDH in strain DC826170 did not increase the growth rate of the resulting strain on D-LA MM (data not shown). DC*bglX* was then transferred every day in LD-LA MM and an adapted strain named DC*bglX*30 exhibited a maximum growth rate of 0.36 /h (Td of 1.92 h) in D-LA MM (Table 3-3 and Figure 3-9). This represents an improvement of about 25% over the growth rate of DC826170, and 80% improvement over the growth rate of the parent strain DC8261. Even though *dld* gene encodes a LDH that was shown to possess the ability to oxidize L-LA in vitro (38), DC*bgl*X and DC*bgl*X30 still lacked the ability to grow on L-LA as a sole carbon source (Table 3-3).

D-LA Consumption Rate as a Function of Cell Density

The PLA syrup purification experiments described above were conducted at high cell density (OD_{420} of 8.0) and high concentration of PLA syrup. It is possible that the D-LA removal rate of similar or higher value could be obtained in a culture started at a lower cell density to increase O_2-availability per cell. To assess the relationship between the D-LA removal rate and the starting culture density, the following experiment was performed. Strain DC*bglX* was cultured in 20 g/L LD-MM and harvested at mid-exponential phase of growth, centrifuged and used to inoculate 20 g/L LD-LA MM at various OD_{420}; 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 (Figure 3-12). At starting cell densities up to 4.0 (OD_{420}), D-LA removal rate increased with increasing cell density, suggesting that the amount of cells was a limiting factor during this phase. At initial cell densities of above 4.0 (OD_{420}), D-LA removal rate remained constant and at about 0.55 $g_{D\text{-}LA}$/(L.h) suggesting that the cell density was in excess.

The increase in D-LA removal rates as a function of cell density exhibited two linear curves of different slopes; a rapid increase up to a cell density of 1.0, and a relatively lower increase in the D-LA removal rate between 1.0 and 4.0 (Figure 3-12). At lower initial cell densities, the cultures grew at a higher growth rate compared to the high cell density cultures.

This is in agreement with the fact that growing cells do metabolize the C-source in the medium at a higher rate than non-growing or slow-growing cells. As inoculation density increased, D-LA and/or oxygen became limiting, limiting growth but supporting only maintenance at the expense of D-LA. These results show that a balance between metabolic activity and cell density is essential to achieve the highest rate of D-LA removal from the PLA syrup. Under the current experimental condition, this appears to be about 4 OD_{420} units.

Purification of PLA Syrup by DC826170 and DCbglX30

Strain DC826170 was selected after transferring DC8261 into D-LA MM for about 700 generations, which increased the growth rate of the evolved strain by about 50% over the growth rate of the starting strain DC8261 in the same medium. The ability of strain DC826170 to remove D-LA in PLA syrup was tested in shake flasks containing 35 mL of minimal medium containing 28, 33, 48, 65, 80 and 85 g/L of total LA. These media were inoculated at an initial cell density of 6.0 (OD_{420}) to ensure that the cells were in excess under the conditions of this experiment. Strain DC826170 completely removed all the D-LA in cultures started with 28, 33, and 48 g/L of PLA-derived LA in about 66, 67 and 71 h, respectively (data not shown). The specific rates of removal of D-LA from these cultures were 0.014, 0.021 and 0.025 $g_{D\text{-}LA}/(g_{DW}.h)$, respectively. The specific rate of D-LA removal by the evolved strain DC826170 was about 6.5-fold higher than the starting strain of the metabolic evolution, D8261 (0.004 $g_{D\text{-}LA}/(g_{DW}.h)$).

These results suggest that the [D-LA] has a positive effect on the rate of D-LA removal from the PLA syrup reaching a maximum at 50 g/L of total LA from PLA syrup. As the concentration of PLA syrup increased above 50 g/L of LA, the rate of removal of D-LA declined, probably as a consequence of inhibitory compounds present in the PLA syrup (Figure 3-8). During the experimental period of 110 h, only about 12, 13 and 20% of the D-

LA present in the medium was removed from cultures that started with 65, 80 and 85 g/L of PLA syrup, respectively.

Continued metabolic adaptation of strain DC826170 for growth in the presence of 20 g/L of DL-LA combined with restoration of *dld*$^+$ genotype led to strain DCbglX30 (Figure 3-9). Growth rate of this strain on D-LA was about 30% higher than strain DC826170 (Table 3-3). Interestingly, strain DCbglX30 was able to efficiently metabolize all the D-LA in PLA syrup at a total LA concentration as high as 126 g/L in about 40 h (Figure 3-13). Apparently, adaptive evolution in the presence of 20 g/L DL-LA accumulated enough beneficial mutations to counteract part of the inhibitory effects of high [LA]. At 126 g/L of LA from PLA syrup, the specific rate of D-LA removal by strain DCbglX30 was 0.114 $g_{D\text{-}LA}/(g_{DW}.h)$ with a volumetric rate of D-LA removal of 0.25 $g_{D\text{-}LA}/(L.h)$. This represents a significant improvement over strain DC826170 that was limited to a maximum of 50 g/L LA from PLA syrup. When the total LA concentration was increased to 200 g/L, even strain DCbglX30 could only remove about 50% of the D-LA present in the syrup in about 24 h, and remained constant afterwards.

The D-LA removal experiments detailed above were all conducted in shake flasks agitated at 250 rpm for optimum aeration of the culture. Because D-LA oxidation is oxygen dependent, it is possible that oxygen is a limiting factor in the shake flasks with high cell density. To test this possibility, air was sparged into the cultures actively removing D-LA from PLA syrup. In this experiment, concentration of LA used was 135 g/L and the cultures were started at an OD_{420} of 6.0. Under such condition, strain DC*bglX*30 metabolized the entire pool of D-LA present in the culture broth in about 24 h, which is 10 h earlier than in shake flask agitated at 250 rpm (Figure 3-13). D-LA removal rate was about 50% higher when air was sparged than in shake flask, i.e.,0.363 and 0.250 $g_{D\text{-}LA}/(L.h)$, respectively.

Overall, this study shows that it is possible to engineer *Escherichia coli* to selectively remove the D-LA present in PLA syrup. Adaptive evolution, genetic manipulation as well as process modification to supply higher rate of oxygen supply are ways to increase tolerance to high lactic acid concentration in the PLA syrup and D-LA removal rate. Further adaptive evolution in the presence of PLA syrup with high [LA] is expected to further enhance the ability of strain DC*bglX*30 and its derivatives to purify PLA syrup. The use of a microbial biocatalyst to purify a steric mixture of LA has not been described before. Such a bioprocess alleviates the requirement for expensive equipment necessary to perform this task by abiological means.

Insight into the L-LA Metabolism of *Escherichia coli*

Phenotype of DC8255 on L-LA MM

Strain DC8255 lacks the three lactate dehydrogenases known to date, *i.e.,* proteins coded by *lldD*, *dld* and *ldhA* (40, 104, 129) (Figure 3-9). In minimal medium containing L-LA as sole carbon source, strain DC8255 grew aerobically and with limiting oxygen. Specific growth rate of strain DC8255 in MM with L-LA was 0.27 /h under oxygen-limitation condition whereas the growth rate of the parent strain (J7) was 0.38 /h, representing a 39% decrease due to the *lldD* mutation (Table 3-3). However, strain DC8255 reached the same cell yield as strain J7 (data not shown). The L-lactic acid specific consumption rate, calculated during the exponential phase of growth was 33% lower in DC8255 than in the wild type *E. coli*, i.e,12 $g_{L\text{-}LA}/(g_{DW}.h)$, compared to 18 $g_{L\text{-}LA}/(g_{DW}.h)$ respectively. This indicates that while the L-LDH encoded by the *lldD* is important for L-LA utilization, *E. coli* is also producing additional L-LDHs that are contributing to L-LA metabolism. In order to confirm the presence of multiple L-LDHs in *E. coli*, the *lldD* deletion was also transduced into other *E. coli* wild type strains, W3110 and MG1655. As seen with strain DC8255, these *lldD* mutants also grew in minimal medium with L-LA as a sole carbon source, confirming the presence of additional L-LDHs in *E. coli.*

Ykg as an LDH

Pinchuck et al. (123) reported that *E. coli ykgEFG* operon, when introduced into a lactate-minus mutant of *Shewanella oneidensis* MR-1, restored growth on L-LA suggesting that this operon encodes an L-LDH activity. To further evaluate this *ykg* encoded L-LDH activity, *ykgE* was deleted in strain DC8255, giving strain DC82551 (Figure 3-9). Since *ykg* operon contains three genes, *ykgF* and *ykgG* were also deleted individually yielding strains DC82552 and DC82553, respectively (Table 2-1). All three double mutants (*lld* and *ykg*) still grew in L-LA minimal medium although at a lower growth rate compared to the *lld* mutant, and the cell yield of the three double mutants on L-LA was also reduced by about 23±7.5% in comparison to the parent strain DC8255 (data not shown). An *lld* mutant that also lacks all three genes of the *ykg* operon (DC825*ykgEFG*) still grew in L-LA minimal medium. Based on comparative genome analysis (27), it was determined that the *ykgE* encoded product likely possesses the dehydrogenase activity, and therefore, strain DC82551 was used in all further experiments (Figure 3-9).

After UV treatment of DC82551, DC8261 was isolated upon its L-LA-minus phenotype (Figure 3-9). This strain possesses multiple mutations, including *lldD* and *ykgE*. As observed previously, the reintroduction of a native copy of *lldD* structural gene into the genome of DC8261 restored the ability of the strain to grow on L-LA as a sole carbon source (Figure 3-9 and Figure 3-10). Similarly, *ykgE* gene sequence was reintroduced into the genome of DC8261c by cotransduction of a kanamycin marker adjacent to *ykgE* gene, creating DC*ykgD* strain (Figure 3-9). After selection on LB-kanamycin agar medium, transductants were streaked onto L-LA-kanamycin agar medium. Interestingly, all the transductants screened grew well on L-LA medium by 48h incubation at 37°C (Figure 3-11). This result strongly suggests that *YkgE* is an L-LDH, allowing growth of the organism on L-LA MM. When cultured in L-LA liquid MM, the

maximum growth rate of DC*ykgD* strain was comparable to the growth rates of DC8255 and DC82551, *i.e.,*0.28 /h as opposed to 0.27 /h and 0.30 /h respectively (Table 3-3).

Third L-LDH in *E. coli*

The results presented in the previous section suggest that *E. coli* has at least three proteins with L-LDH activity. The LldD was the first L-LDH described in *E. coli* (25, 119). Based on heterologous complementation and comparative genomic analysis, YkgEFG constitutes a second enzyme complex oxidizing L-LA in the cell. Growth of an *E. coli* strain lacking both *lldD* gene and *ykgEFG* operon (Table 3-3; strain DC82551) in L-LA minimal medium and the inability of mutant strain DC8261 to grow on L-LA suggests the presence of, yet to be identified, one or more additional gene(s)/operon(s) encoding L-LDH activity. This third L-LDH activity is referred to as X, encoded by the gene *x*.

Biochemical Properties of L-LDHs in *E. coli*

Cellular localization of L-LDHs and identification of electron acceptor for activity

LldD has been described to be a membrane bound protein and its activity can be measured by coupling the oxidation of L-LA to the reduction of MTT or DCPIP (40, 120). Therefore, crude extracts of strain DC8255 ($LldD^-$, $YkgE^+$ and X^+) was used to localize the remaining L-LDH activities in the cell and also to identify an electron acceptor able to couple lactate oxidation. Among the various electron acceptors tested; NAD^+, $NADP^+$, ferricyanide, MTT and 2,6-dichlorophenol-indophenol (DCPIP), only MTT and DCPIP were reduced by the cell extract in the presence of L-LA. DCPIP was selected over MTT since MTT requires phenazine methosulfate (PMS) as an intermediate electron carrier.

Irrespective of the bacterial strain tested (strain J7, DC*lldR*, DC*ykgD* or DC82551), pyruvate was identified by HPLC to be the product of the L-LDH reaction in the presence of DCPIP as electron acceptor.

Spheroplasts of strain DC8255 (*lldD*) had 0.03 unit of L-LDH activity and this activity increased by almost two-fold upon sonication that reduced the vesicle size and generated "inside-out" vesicles (Figure 2-2, Material and Methods) (39). Using the membrane vesicles collected after centrifugation at 100,000 x g, 92% of the remaining L-LDH activity was detected in the vesicles (Figure 3-14). These results suggest that the two remaining L-LDH activities (Ykg and X) in the *lldD* mutant also appear to be membrane associated.

Kinetic properties of the three L-LDHs

In order to determine the kinetic characteristics of the three L-LDHs, *E. coli* strains that produced only one of the three enzymes were submitted to enzyme activity assays (Figure 3-9; DC*lldR*, DC*ykgD* and DC82551).Also, crude extracts of J7, DC8255 and DC8261 were prepared and L-LDH activity assays were performed. Activities measured in crude extracts prepared from strains J7 and DC*lldR* were the highest. These two strains produce LldD protein in the cell, suggesting that LldD is the most active L-LDH in the cell (Table 3-4). Specific activity of YkgE measured in membranes from cells that produced only the Ykg-derived L-LDH was about half of the LldD activity and the activity recorded for X alone represented about 23 % of the LldD activity. Silencing Ykg and X from being expressed did not diminish the Lld activity indicating that the Lld –LDH is the primary enzyme in the wild type cell. Removing the Lld-LDH by deletion of the gene could have elevated the level of the other two enzymes and in the wild type cell the activity of these two alternate L-LDHs could be significantly lower. Since the all three mutants producing only one of the three enzymes grew at about the same specific growth rate (Table 3-3), apparently loss of one or more of the L-LDHs is compensated by the other although the level of enzyme activity of these mutants may vary significantly (Table 3-4). Activity profiles at different pH values for the three L-LDHs are presented in Figure 3-15. The Lld had a pH optimum for activity of about 6.5 using DCPIP as electron acceptor (Figure 3-15A). This

optimum pH value differs from the pH value of 8.5 reported by Futai and Kimura (40). One explanation may rely on the different electron acceptor they used in their study; another explanation may be that, in the present study, the activity was measured on vesicles and not on isolated protein. This observation has been made for other proteins such as *N. meningitis* D-LDH, also a membrane associated oxidoreductase, which exhibited an optimum pH of 7.0 in vesicles and an optimum pH of 8.0 when the assay was performed using purified protein (32).

As seen in Figure 3-15B and 3-15C, the optimum pH recorded for YkgE and X were more basic, i.e., 9.4 and 10.0 respectively. A better comparison of their pH profile is depicted in Figure 3-16. DC8255 expresses both YkgE and X whereas DC*ykgD* and DC82551 express solely YkgE or X respectively (Figure 3-9). DC8255 pH profile exhibits a peak at 9.5 as well as a shoulder at pH around 10, which may represent YkgE activity, and X activity respectively (Figure 3-16).

The initial velocities and double reciprocal plots obtained for L-LDH activity measured in DC82551 (X^+) with increasing concentrations of L-LA and DCPIP are presented in Figure 3-17. Velocity curve obtained when varying L-LA provided a profile characteristic of a Michaelis Menten curve (78), and corresponding double reciprocal plot allowed to determine a *K*m for L-LA of 316 μM for the third L-LDH activity of *E. coli* (Figure 3-17A and Table 3-5) (93). Apparent *K*m of the LldD enzyme for L-LA was 88 μM which is in agreement with the value of 120 μM reported elsewhere (40). Apparent *K*m for L-LA for Ykg of 640 μM was the highest of the three L-LDH activities in *E. coli* (Table 3-5).

Apparent *K*m for DCPIP determined for LldD, YkgE and X were 44, 70 and 500 μM respectively. These results show that the some of the enzyme activities were not determined at saturating concentration of one of the substrates, DCPIP, especially the activity of X when the

DCPIP concentration was set at 100 μM due to its intense color in the oxidized form. The lower activity of the X-LDH reported in Table 3-4 could arise from the difficulty in assaying the enzyme using DCPIP as electron acceptor.

Based on physiological, biochemical and genetic analysis; *E. coli* appears to possess three L-LDHs activities with distinct characteristics. All three L-lactate oxidoreductases are membrane associated and catalyze the oxidation of L-LA to pyruvate that supports growth of the organism under aerobic conditions.

Attempts to Map the Third L-LDH

Complementation of L-LA minus phenotype using plasmid libraries

In the first set of experiments to map the gene encoding the X LDH activity, strain DC8261c was transformed with a cloned *E. coli* DNA library (Figure 3-9). None of the plasmid containing transformants grew on L-LA MM. Since the X-L-LDH being a membrane associated enzyme, it is possible that a potential complementing plasmid due to its high copy number (plasmid pUC18 was used as a vector) was toxic to the cell. To overcome this possibility, a lower copy number plasmid library was used, i.e.,pACYC184 based library. This plasmid allows about 20 to 40 copies to be maintained in *E. coli* cells as opposed to an average of 500 copies with pUC based plasmids. About 10,000 transformants were tested for growth on L-LA. Few small colonies that appeared on L-LA MM with chloramphenicol were selected and tested further. However, none of these transformants grow in liquid culture in the same medium. Plasmids extracted from these putative clones upon transformation back into strain DC8261 also failed to support growth on L-LA. Insert DNA in these plasmids, analyzed by agarose gel electrophoresis or by colony-PCR using pACYC primers (Table 2-2) was never larger than about 200 nt long suggesting significant rearrangement of the insert DNA in these plasmids. The small insert found in these plasmids was sequenced. The chromosomal region surrounding these insert

DNAs insert sequence (± 3 kb) in the genome did not lend to identification of a plausible L-LDH candidate based on the presence of an oxidoreductase and/or flavin-binding motif.

Considering the putative rearrangements that occurred, it was possible that the initial poor growth on L-lactate is due to zygotic induction of the gene upon plasmid entry (7, 35, 67, 181). The pACYC-based plasmid enters the cell, and expression of the plasmid-borne genes starts, allowing the production of L-LDH. Because of the number of plasmid in the cell and the fact that L-LDH is a membrane-associated protein, the production of too high levels of such protein disrupts the organization of the membrane, cell growth ceases. However, the initial production of the enzyme led to L-LA oxidation and allowed the strain to divide few times. As the cells divided, a dilution effect of the L-LDH present in the membrane have occurred, which allowed the daughter cells to divide as well, even though these cells did not produce any new L-LDH. This way, a colony appeared on L-LA-chloramphenicol agar medium, but the cells do not carry, in fact, a gene coding for an L-LDH.

The "zygotic induction" likely explains why a colony could be seen on the original L-LA MM replica-plate, but could not be subcultured in the same medium. Nonetheless, the toxicity induced by high level of L-LDH triggered recombinases to excise the piece of DNA that caused the toxicity in the cell. Therefore, a *recA* derivative of DC8261 was constructed (18) (DC8261*recA*), to minimize recombination L-LA$^+$ transformants obtained in this new genetic background (DC8261*RecA*) could be subcultured on the same medium. DNA insert in plasmids isolated from seven transformants were sequenced. The insert DNA in all 7 plasmids contained the *lldD* gene. As a result, a plasmid library was created using DC82551 genomic DNA lacking *ykgE* and *lldD* genes. Additionally and to avoid potential toxicity induced by the copy number of the plasmid, a single copy plasmid was used. A total of over 10,000 chloramphenicol resistant

transformants were screened for growth on L-LA as a sole carbon source. No L-LA$^+$ clones appeared on agar medium, whether or not IPTG was added (5 μM) (Figure 3-9). The results presented above suggests the possibility that more than one gene is involved in the third L-LDH activity observed in *E. coli*. Therefore, a different genetic approach was adopted, consisting of conjugating DC8261 (L-LA-) with various F' and Hfr *E. coli* strains.

F' complementation and conjugation of DC8261 with Hfr *E. coli* strains

The L-LA minus strain DC8261, was conjugated with various F' donor strains to identify a region of the *E. coli* K-12 chromosome that would complement the L-LA minus phenotype of the mutant (Figure 3-9). The exconjugants containing the following plasmids grew on L-LA MM agar medium: F143, F104, F128 and F254 (Figure 3-18). F104, F128 and F254 harbor the *ykgE* gene. Interestingly, F143 complemented the L-LA$^-$ phenotype of DC8261, though at a significantly lower frequency than that of the other plasmids. F143 plasmid is not known to include any identified L-LDH, and the results presented here suggest that this region contains a gene(s) involved in the aerobic L-LA metabolism of *E. coli*. The 14 other F' strains tested did not lead to any obvious L-LA$^+$ phenotype.

To confirm these results, three selected Hfr strains were used in conjugation experiments (Table 2-1). Hfr strain BW6166 point of origin is located at 91.46 min on the *E. coli* chromosome and a Tn10 transposon is inserted about 13 min upstream of the point of insertion (76.78 min). Therefore, BW6166 can insert the *lldD* gene in the early stages of conjugation, and select for a tetracycline marker. NK6051 and BW6163 points of origin are located at 96.80 and 65.00 min respectively, and harbor a Tn10 insertion at 11.87 and 43 minutes respectively. Therefore, the regions transferred by Hfr strains NK6051 and BW6163 contain *ykgE* and the region equivalent to F143 episome of the F' strain KL711, respectively. All three conjugations allowed some level of growth on L-LA MM. Out of the three, BW6163 x DC8261 gave the best

frequency of recombination with more than 10^6 L-LA$^+$ CFU obtained (1% frequency). NK5061 x DC8261c gave about 250 colonies (0.00025%) and BW6166 x DC8261 gave about 6000 colonies on L-LA MM plate (0.006%). In summary, F143 seems to harbor gene(s) that can complement L-LA$^-$ phenotype of DC8261 corresponding to the third L-LDH. This result was also confirmed by the conjugation of Hfr BW6163 with DC8261c.

Consequently, corresponding portion of the *E. coli* K-12 chromosome was screened for potential dehydrogenases that were individually tested in a series of transductions (Table 3-6). Among the potential genes, three putative flavoproteins were identified (*ygcU, ygcQ, yqcA*). Deletions of all these genes listed in Table 3-6 in DC82551c did not yield any L-LA-minus phenotype indicating that these oxidoreductases did not contribute to L-LDH activity (figure 3-9). As an alternative approach, genes about 30 kb apart in the entire region corresponding to the F143 plasmid (58 to 70 min) were selected and P1 were grown on the corresponding "Keio" deletion mutants (Table 3-7). These P1 phages were used to transduce DC8261c (L-LA$^-$). Table 3-7 gives the number of kanamycin resistant transductants obtained for each transduction performed. None of the transductants grew on L-LA-kanamycin medium, suggesting that more than one gene in this region may be required to obtain a L-LA$^+$ phenotype. This experiment further suggests that these genes are not cotransducible by P1 phage.

It is possible that the genes needed for growth on L-LA as sole carbon source involve a dehydrogenase as well as accessory proteins and a regulatory protein. The L-LA$^+$ phenotype of DC82551 appears more complex than expected. Nonetheless, this strain is able to oxidize L-LA for aerobic growth on L-LA as a sole carbon source.

Attempts to identify the third L-LDH by LC/MS

Since the genetic approach failed to identify the gene encoding the X-LDH, a biochemical approach was attempted (Figure 3-9). This involved identification of the X in native

polyacrylamide gels by activity staining (Figure 3-19) followed by identification of the component proteins by LC/MS after trypsin digestion. Membrane fraction was solubilized with Triton X-100 in the gel during electrophoresis. A L-LA dependent white band characteristic of the reduction of DCPIP appeared in the gel when soaked in reaction buffer after electrophoresis. The white band representing the third L-LDH activity as well as other proteins with similar electrophoretic migration was removed and processed for LC/MS analysis. The results obtained after trypsin digestion, LC/MS-MS separation and peptide identification were compared with *E. coli* proteome to identify the various proteins at the location in the gel. These results revealed a multitude of proteins present in the excised gel. Some of the identified proteins with unknown function could potentially serve as a oxidoreductase catalyzing L-LA oxidation. This was based on the following criteria: peptides exhibited a flavin motif, or a dehydrogenase putative function, or the peptide belonged to an inner surface protein of unknown function (Table 3-8). Among the genes encoding the identified proteins, *ygbJ* and *yphG* are located in the chromosomal region carried by F143 F' element that complemented the L-LA minus phenotype of DC8261. Two other candidates for the third L-LDH are *ygaF* and *visC*. Transducing the null mutation of each of these genes (from "Keio" mutant collection) into the double mutant (*lldD, ykgE*) did not yield an L-LA$^-$ minus phenotype, except for *visC*. When a visC deletion mutation was transduced into the double mutant, 30% of the transductants that contained Δ*visC*::*kan* did not grow on L-LA. If the VisC is encoding the third L-LDH, deletion of this gene is expected to yield 100% of L-LA-minus mutants. This transduction result suggests that at least two separate genes are contributing to the third L-LDH activity and deletion of both these genes are needed for abolishing the third L-LDH activity in *E. coli*. Apparently, these two genes are unlinked in the chromosome. Identification of these two or more genes contributing to the third L-LDH activity as well as

unraveling the anticipated complex interaction among these two gene products is part of a continuing future study.

Although the proteomic study failed to clearly pin point the protein(s) responsible for the third L-LDH, similar experiment using membranes that contained the Ykg-L-LDH did lead to identification of the protein in the tryptic peptide pool (Figure 3-9). Purification of the third L-LDH from a large amount of detergent-solubilized membranes using conventional protein purification methods followed by trypsin digestion and LC/MS-MS separation of the peptides is expected to identify the nature of the protein complex responsible for L-LDH activity along with the gene(s) encoding it. This will be the starting part of a future study towards establishing the L-lactic acid metabolism in *E. coli* and by extension in other bacteria.

Table 3-1. D-LA content of syrup obtained after hydrolysis of PLA at various temperatures

	Water			1 M NaOH		
Temperature (°C)	Time* (h)	(D/total)-LA (%)	LA yield (%)	Time* (h)	(D/total)-LA (%)	LA yield (%)
160	2.1	6.4	92.0	1.9	4.7	98.1
155	2.8	6.3	92.4	2.0	6.3	96.1
150	3.7	6.8	95.0	2.5	6.0	95.9
140	5.5	8.3	88.0	4.1	5.4	96.5
130	15.6	6.4	94.7	17.0	6.4	97.5
120	50.7	5.6	94.7	40.7	6.0	94.2

This experiment was conducted with PLA beads grade 4032D
* Time of sampling

Table 3-2. Effect of base on racemization of LA

	(D/total)-LA (%)	
Treatment	0-time (stdev)	2 h (stdev)
PLA syrup + water	6.26 (0.80)	6.46 (0.90)
L-LA + water	UD	UD
PLA syrup +1M NaOH	6.58 (0.69)	14.64 (0.48)
L-LA + 1M NaOH	UD	9.02 (1.25)

UD, undetectable amount of D-LA
PLA syrup from bead grade 4032D or pure L-LA solution was treated at 160°C.
Values in parenthesis represent standard deviations.

Table 3-3. Growth rates of relevant *E. coli* strains in minimal medium containing various lactic acid isomers

Strain	Relevant characteristics	Phenotype on L-LA	Specific growth rate (1/h) D-LA (4 g/L)	LD-LA (8 g/L)	L-LA (4 g/L)
J7	*lldD*$^+$, *ykgEFG*$^+$, *x*$^+$	+	0.25	0.41	0.38
DC8255	*ykgEFG*$^+$, *x*$^+$	+	0.17	0.27	0.27
DC82551c	*x*$^+$	+	0.14	0.27	0.30
DC*lldR*	*lldD*$^+$, *lldR*	+	0.20	0.26	0.27
DC*ykgD*	*ykgEFG*$^+$, *ykgD*	+	0.19	0.29	0.28
DC8261c	no L-LDH	-	0.20	0.25	NG
DC826170c	no L-LDH	-	0.29	0.34	NG
DC*bglX*30	no L-LDH	-	0.36	0.36	NG

NG: No detectable growth
Growth rates are in 1/h. Cultures were grown at 37°C in a shaker (250 rpm) in mineral salts medium with indicated carbon source.
x represents the third, yet to be identified, L-LDH activity

Table 3-4. L-LDH activities of *E. coli* mutants defective in various L-LDH activities

Strain	Relevant mutation	Active L-LDH	Activity (U/mg protein)	% total
J7	none	LldD, Ykg, X	0.13 (±0.016)	100
DC*lldR*	Δ*ykgE*, x	LldD	0.14 (±0.016)	108
DC8255	Δ*lldD*	Ykg, X	0.06 (±0.010)	49
DC*ykgD*	Δ*lldD*, x	Ykg	0.05 (±0.008)	38
DC82551	Δ*lldD*, Δ*ykgE*	X	0.03 (±0.0013)	23
DC8261	Δ*lldD*, Δ*ykgE*, x	None	UD	0

UD, undetectable; <0.01 unit
U, μmol of reduced DCPIP per minute
X represents the third, yet to be identified, L-LDH activity
Values in parenthesis represent standard deviations

Table 3-5. Affinity of different L-LDH to L-LA in *E. coli*

Strain	Relevant mutation	Active L-LDH	L-LA *K*m (mM)
J7	none	LldD, Ykg, X	0.096 (±0.012)
DC*lldR*	Δ*ykgE*, x	LldD	0.088 (±0.036)
DC8255	Δ*lldD*	Ykg, X	0.753 (±0.074)
DC*ykgD*	Δ*lldD*, x	Ykg	0.640 (±0.113)
DC82551	Δ*lldD*, Δ*ykgE*	X	0.316 (±0.04)

X represents the third, yet to be identified, L-LDH activity
Values in parenthesis represent standard deviations

Table 3-6. Potential L-LDH gene candidates located between 55 min and 65 min on the *E. coli* chromosome

Gene	Locus (min)	Annotation in *E. coli* W3110
ygbJ	61.63	Predicted dehydrogenase, Rossmann-fold domain
ygcO	62.33	Predicted 4Fe-4S cluster-containing protein
fucO	63.18	Lactaldehyde reductase
ygcN	62.30	Hypothetical protein
ygcR	62.39	Putative electron transfer flavoprotein
ygcU	62.45	Putative FAD containing dehydrogenase
yqcA	62.96	Flavoprotein
ygcQ	62.37	Hypothetical protein

Table 3-7. Transduction of DC8261c with P1 grown on various "Keio" mutants

Marker Transduced	Strand	Distance (kb)	Number of transductants LBK	LLAK
iscS::kan	-	(58 min) 0	76	0
yfhK::kan	-	28	199	0
yfiQ::kan	+	29	75	0
yfjD::kan	+	26	47	0
ypjF::kan	+	27	59	0
proW::kan	-	29	175	0
ascB::kan	+	34	240	0
iap::kan	+	34	319	0
ygcF::kan	-	27	235	0
sdaC::kan	+	24	200	0
ptrA::kan	-	26	180	0
yqeH::kan	+	29	250	0
ssnA::kan	+	31	175	0
gcvP::kan	-	26	228	0
yggP::kan	-	27	316	0
pppA::kan	+	35	200	0
yghS::kan	-	(68 min) 24	250	0

Transductants were selected at 30°C on LB kanamycin agar medium and "replicated" to L-LA kanamycin agar medium, and incubated at 37°C for a total of 4-5 days.

Table 3-8. List of potential L-LDHs identified from LC/MS analysis

Gene	Molecular weight (kDa)	Operon	Locus (min)	Annotation
ygbJ	139	*ygbJK*	62.99	Dehydrogenase
yphG	7	no	59.09	Hypothetical protein
yajO	36	no	0.08	Oxidoreductase, aldo/keto
visC	26	no	67.07	FAD oxidoreductase
ybiC	39	no	18.98	Lactate malate dehydrogenase
qor	35	no	93.06	Quinone oxidoreductase
ydbC	31	*ydbCD*	33.35	Oxidoreductase
ygaF	46	*ygaTF*	61.43	FAD oxidoreductase

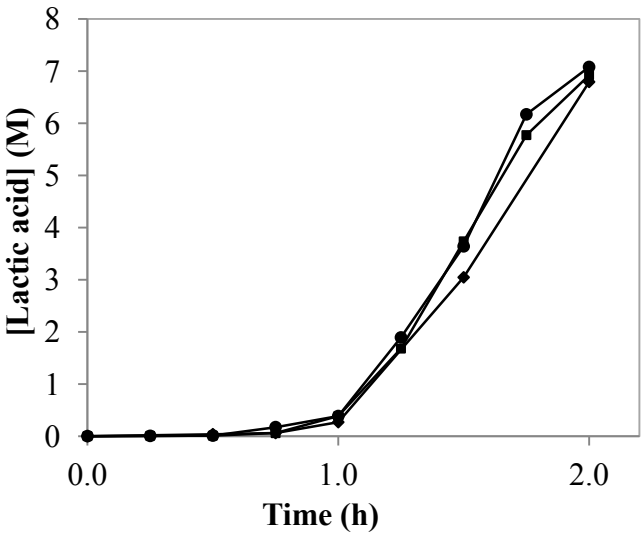

Figure 3-1. LA recovery profiles of various PLA materials in water at 160°C. LA released in the surrounding liquid obtained for PLA beads grade 3052D, •, grade 4032D, ■, and 2 cm^2 pieces of soft-drink cups, (obtained from Kangoroo ™ gas station) ◆.

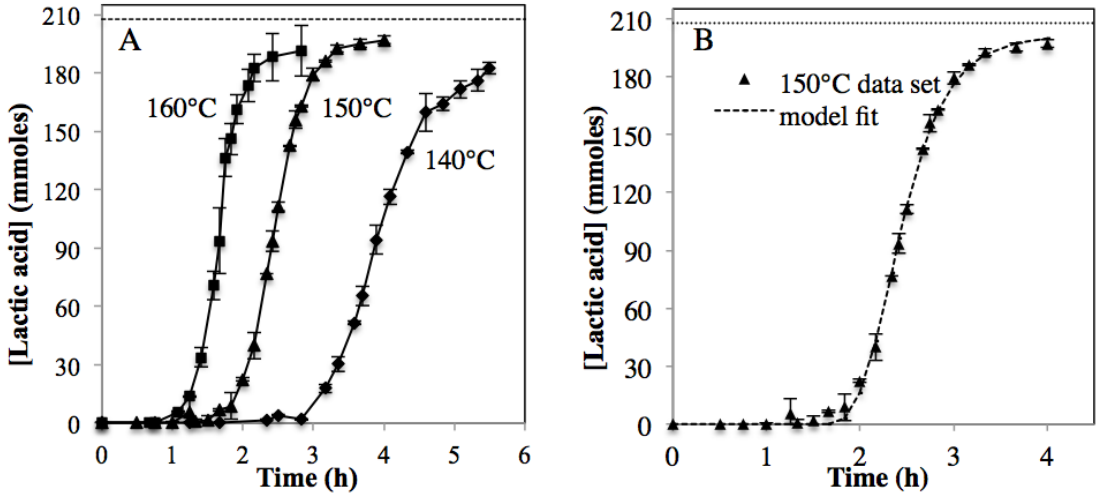

Figure 3-2. Hydrolysis profiles of PLA at different temperatures. (A) LA recovery at 140°C, 150°C and 160°C. PLA and water were 15 g each. (B) LA recovery profile at 150°C. Dashed line was derived from Gompertz model predicted LA production profile (Eq. 1). In (A) and (B), the dotted lines located at the top represent the theoretical maximum LA concentration (207.63 mmoles) that can be obtained from 15 g of PLA.

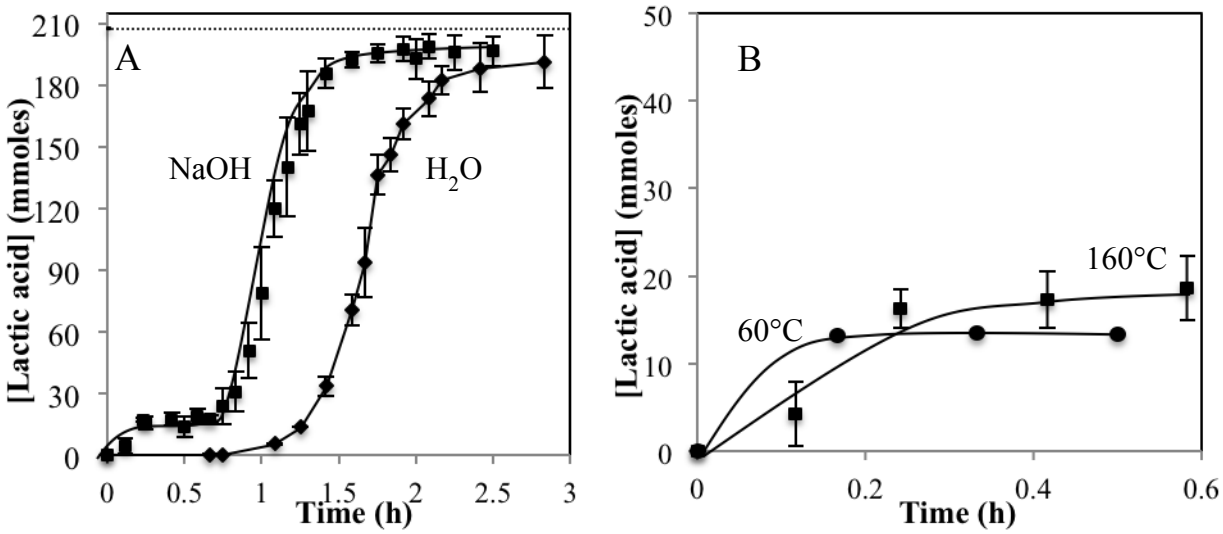

Figure 3-3. Hydrolysis profiles of PLA in water or 1 M NaOH at 160°C. The NaOH solution used in this experiment corresponds to 15 mmoles of OH- in the reaction. (A) The dotted line at the top corresponds to the theoretical yield of LA (207.63 mmoles) that can be obtained from 15 g of PLA. (B) Initial release of LA at 60°C and 160°C under alkaline conditions.

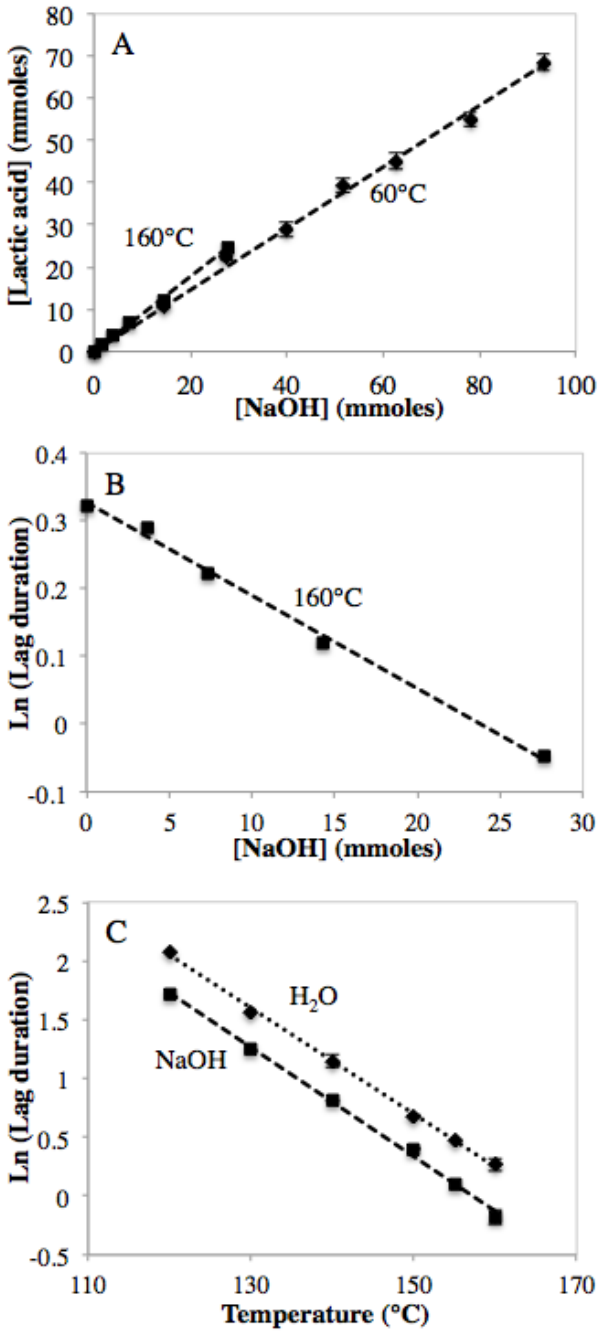

Figure 3-4. Relationship between the amount of LA recovered during the first phase of PLA hydrolysis and the initial concentration of NaOH at different temperatures. (A) LA recovered after 15 min at 160°C, ■ (R^2 = 0.99877) and 4 h at 60°C, ◆ (R^2 = 0.99680) as a function of initial NaOH concentration. (B) Natural logarithm of the lag duration (h) as calculated using Equation 1 as a function of initial NaOH concentration (R^2 = 0.99575). (C) Natural logarithm of the lag duration (h) as calculated using Equation 1 as a function of temperature (R^2 were above 0.99).

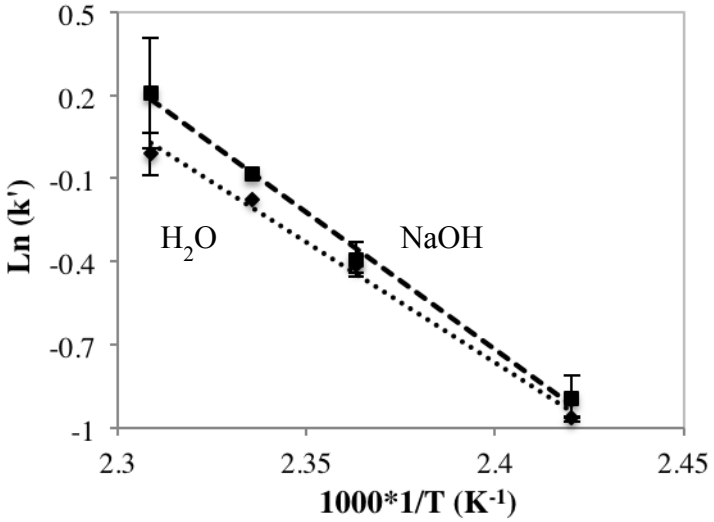

Figure 3-5. Arrhenius plots for the hydrolysis rate constant k' obtained with water or 1 M NaOH aqueous solution.

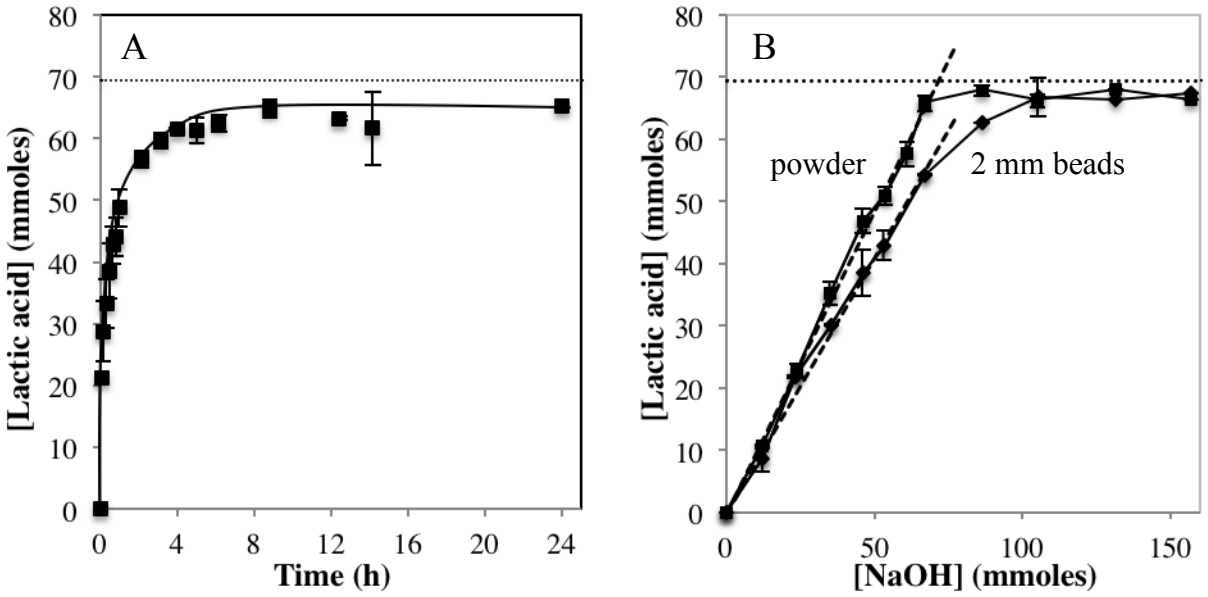

Figure 3-6. Effect of PLA particle size and NaOH concentration on LA recovery. In both Figures, the dotted line at the top represents the theoretical yield of LA (69.4 mmoles) (A) LA obtained at 60°C for ground PLA (17% (w/w)) with 90 mmoles of NaOH. (B) Coarse PLA powder and 2 mm polished beads were heated at 60°C for 24 h and the total LA released is presented as a function of the initial NaOH concentration (17% PLA (w/w)). (R2 values on the linear portion of the curves were above 0.995 for both treatments). See Methods section for other details.

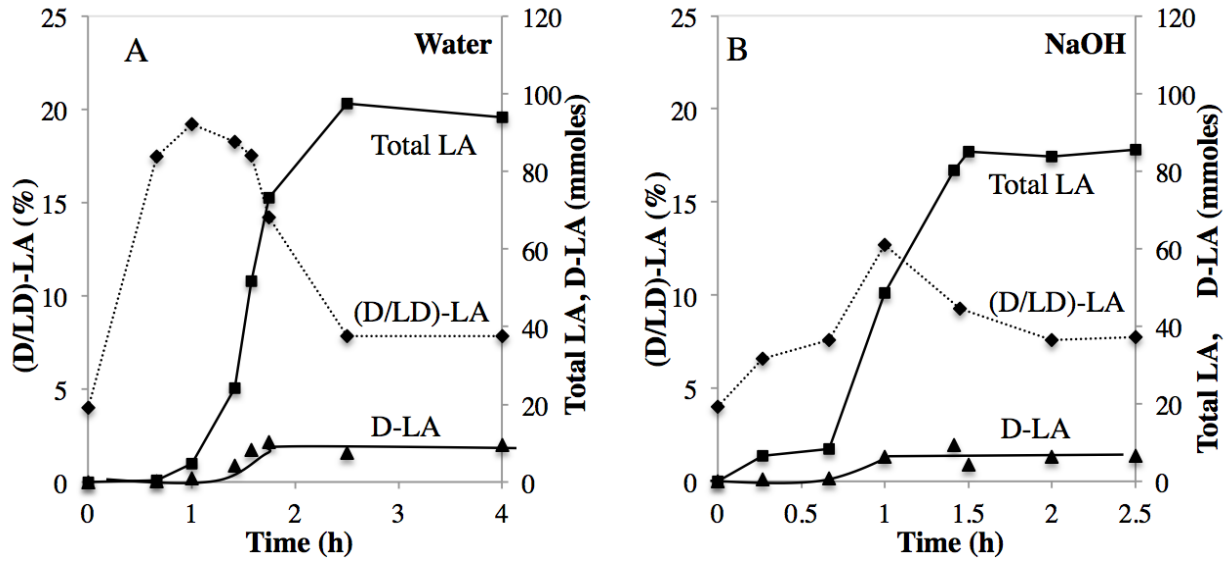

Figure 3-7. Optical purity of LA released during the hydrolysis of PLA beads (grade 3052D) at 160°C. (A) Hydrolysis of PLA beads with water. (B) Hydrolysis of PLA beads with 1 M NaOH.

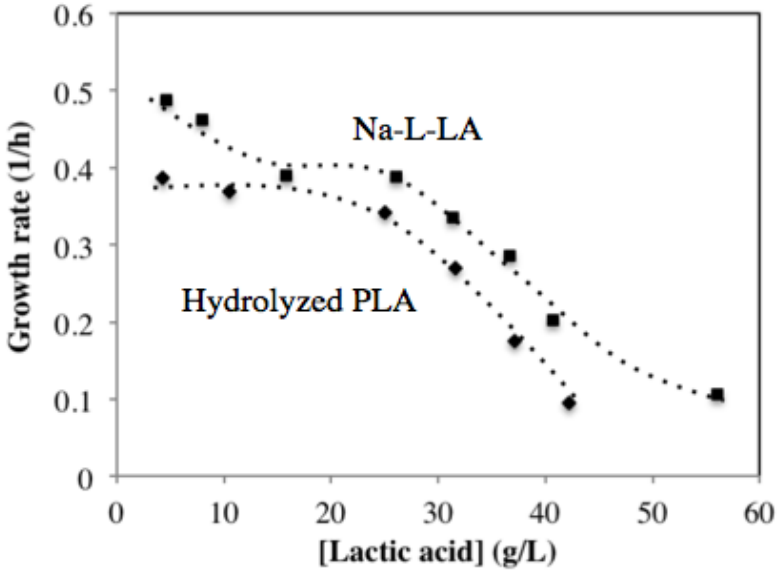

Figure 3-8. Growth rate of *E. coli* strain W3110 in increasing concentration of either Na-L-LA (■) or hydrolyzed PLA syrup (◆). Experiment was conducted in minimal medium with lactic acid as sole carbon source (35 mL in 250 mL flask). Flasks were agitated at 250 rpm on a shaker at 37°C.

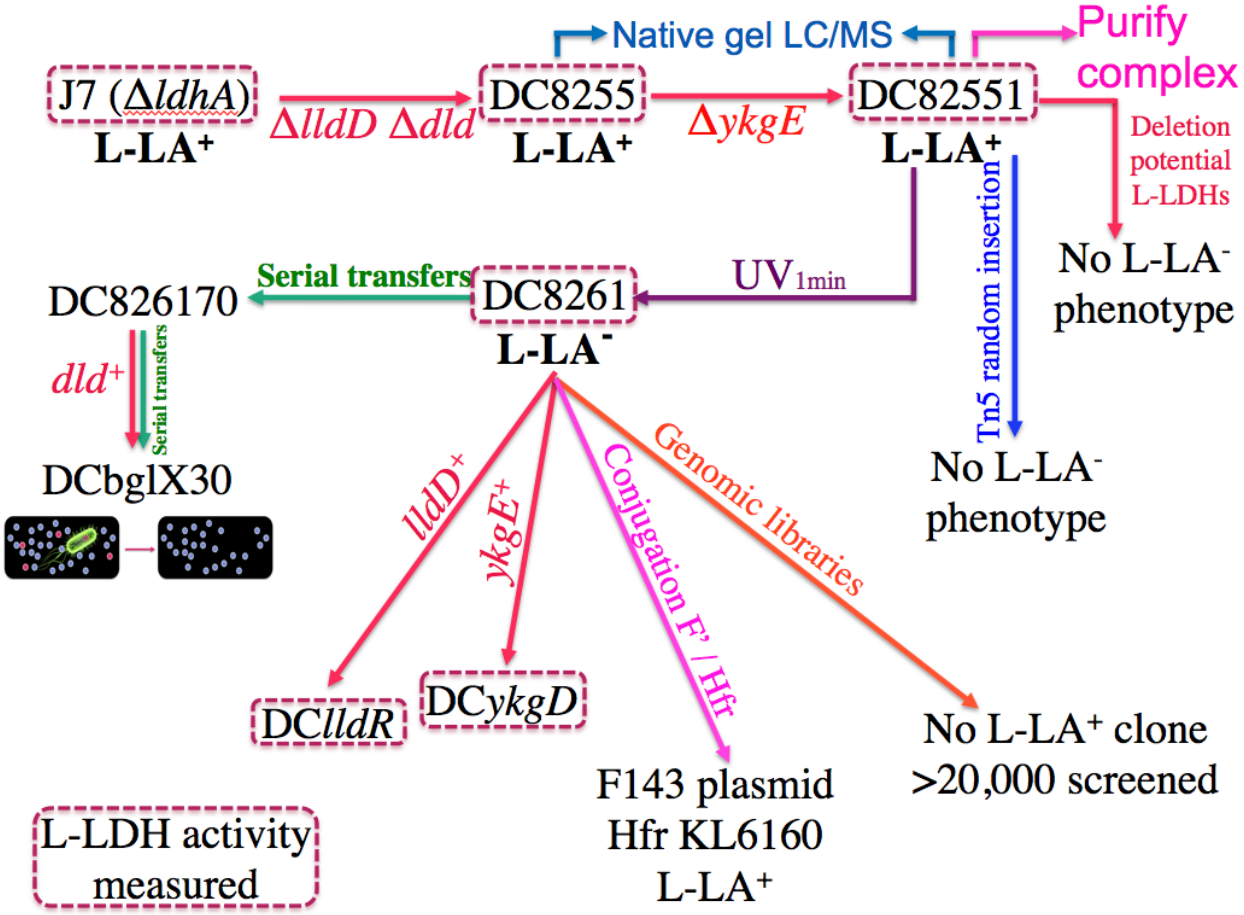

Figure 3-9. Roadmap of the different strains constructed throughout the present study. Red arrows represent genetic manipulations performed by transduction.

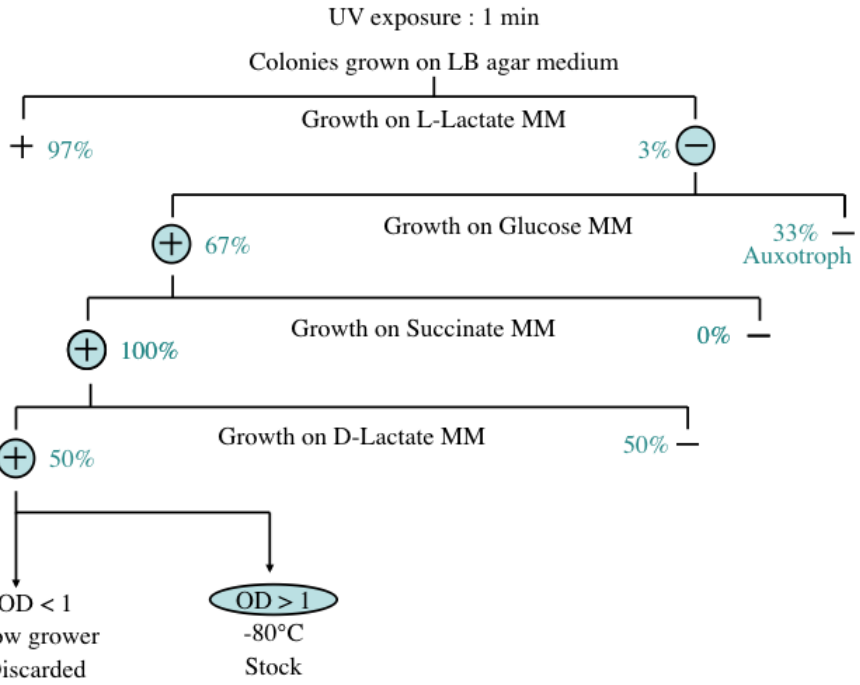

Figure 3-10. Screening steps following UV exposure of *E. coli* strain DC82551. OD_{420} was determined at 24 h.

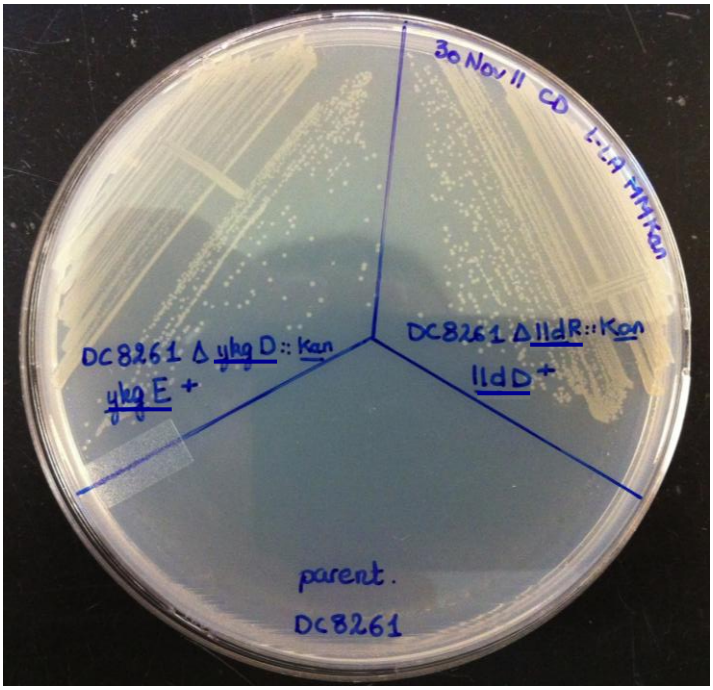

Figure 3-11. Growth of strains DC8261, DC*lldR* and DC*ykgD* on 3 g/L L-LA MM-kan after 48 h of incubation at 37°C.

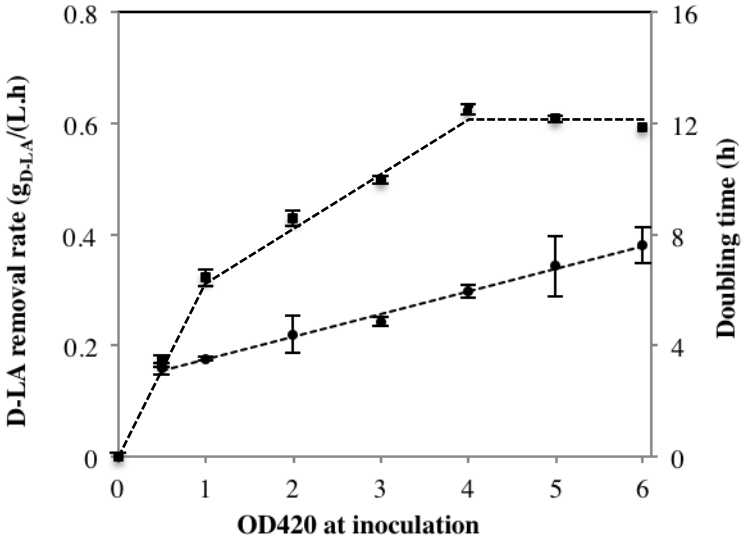

Figure 3-12. D-lactic acid consumption rates (■) and doubling time (◆) of *E. coli* strain DC*bglX* as a function of initial cell density. The experiment was conducted in 35 mL of minimal medium (250 mL flask) and 20 g/L of synthetic lactic acid syrup (1:1 of L-LA and D-LA). Flasks were agitated at 250 rpm on a shaker at 37°C.

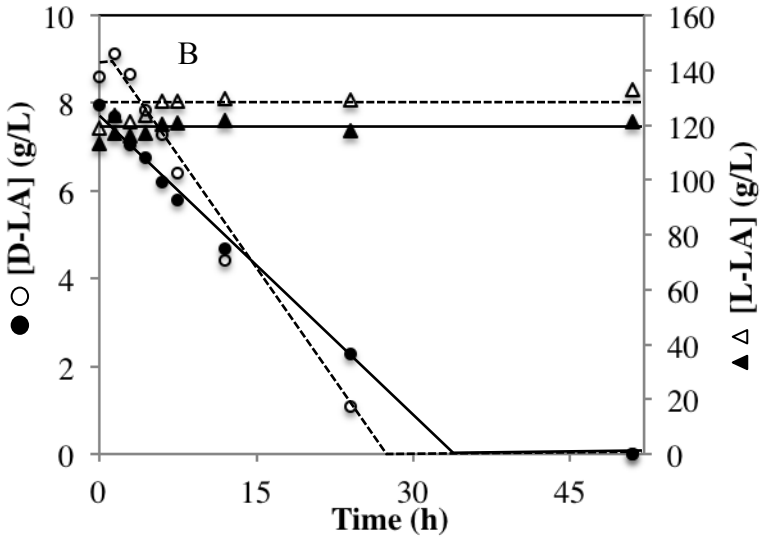

Figure 3-13. D-LA removal from PLA syrup by *E. coli* strain DC*bglX*30. D-LA removal from PLA syrup with 125 g/L total LA at 37°C in shake flasks (35 mL in 250 mL flask) agitated at 250 rpm (filled markers) or with air sparged through the culture (empty markers).

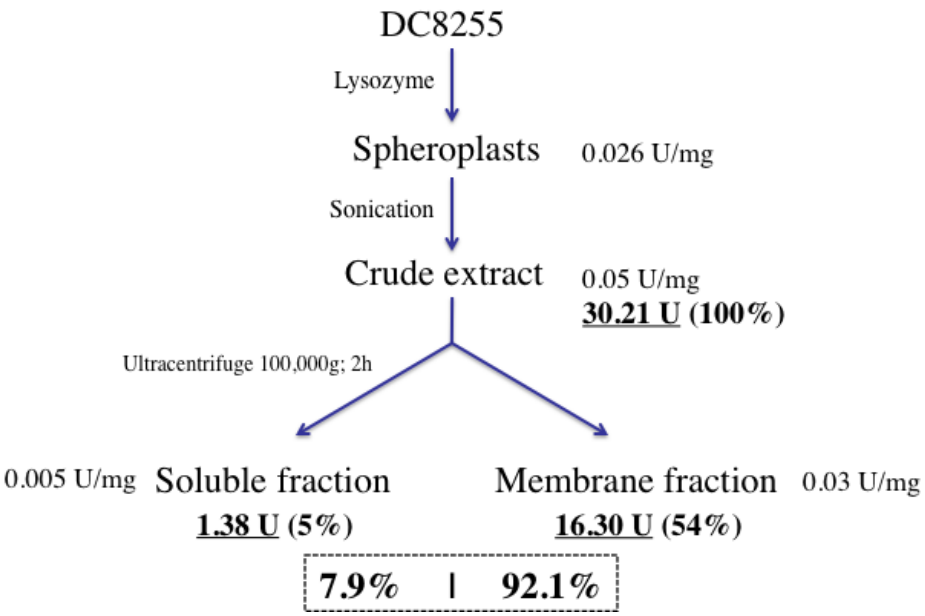

Figure 3-14. L-LDH activity of various fractions during membrane preparation from *E. coli* strain DC8255. Enzyme activity was measured in 50 mM HEPES buffer pH 8.0. U represents one unit of L-LDH activity (µmol of DCPIP reduced per min). Specific activity is listed next to each fraction; underlined values represent total activities and values in the dotted box represent proportion of total activity retrieved in the soluble and membrane fractions.

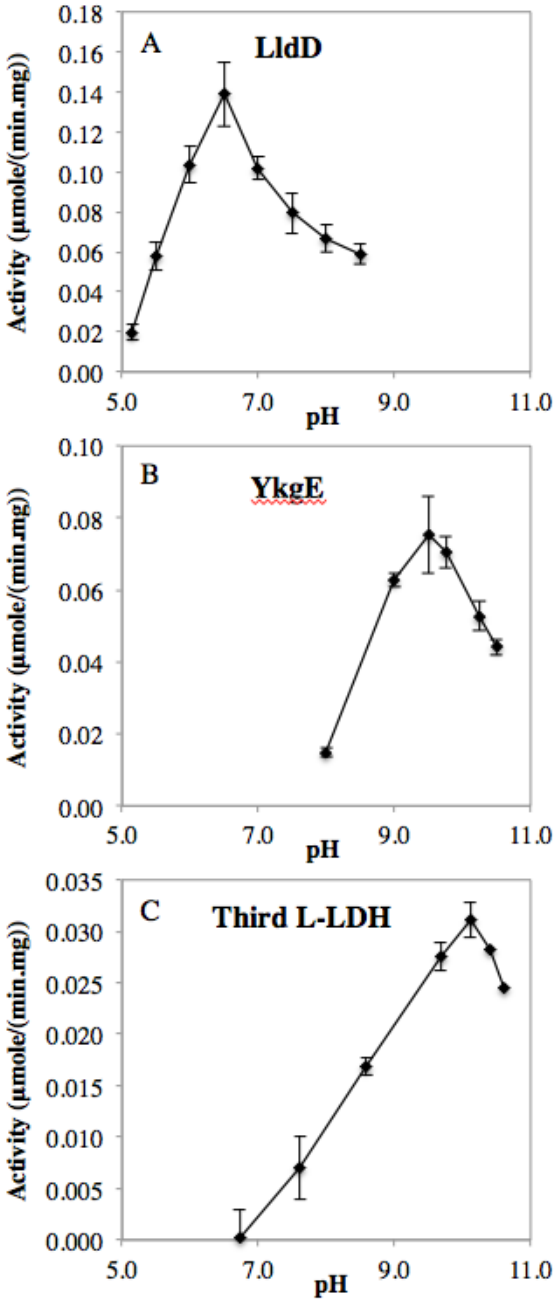

Figure 3-15. pH profiles of the three different L-LDH activities in *E. coli.* Enzyme activity was measured in isolated membranes using 50 mM MES-Tricine buffer (A), or HEPES-CAPSO buffer (B) and (C).

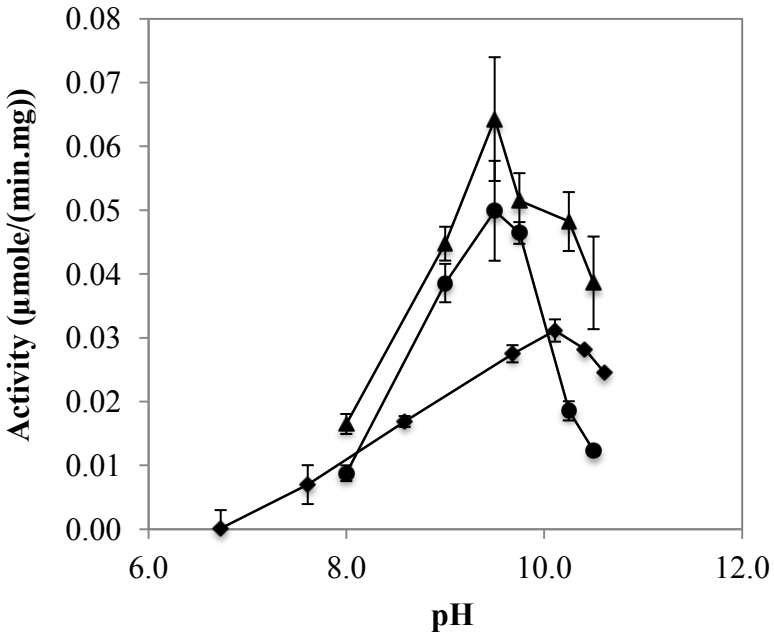

Figure 3-16. L-LDH activity pH profiles of membranes with multiple L-LDH activities. pH profiles for E. coli strains DC8255, ▲; DCykgD, ●; and DC82551, ◆. Strain DC8255 contains both YkgE and the third enzyme whereas DCykgD and DC82551 contain only YkgE or the third enzyme, respectively.

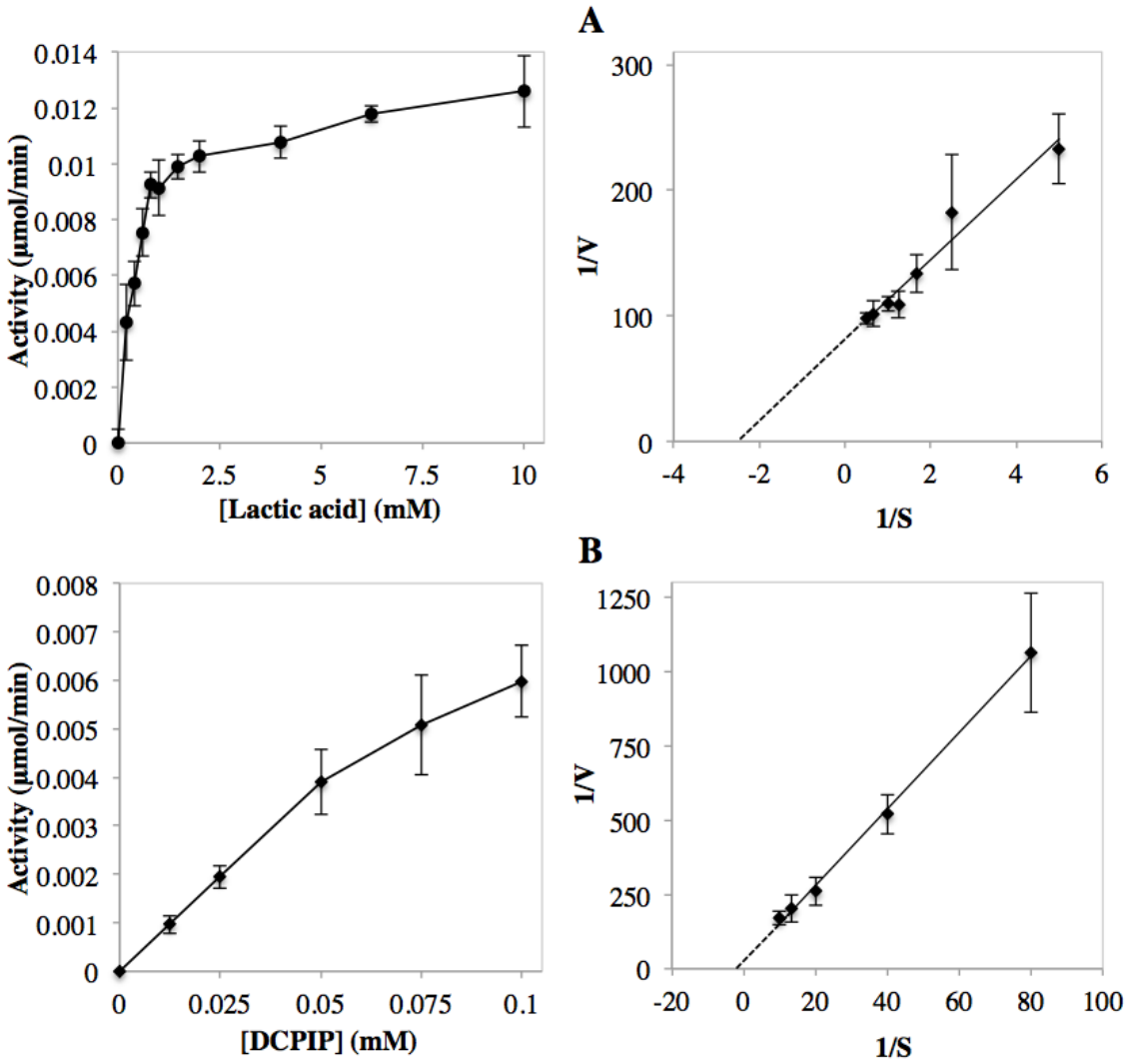

Figure 3-17. Enzyme activity as a function of substrate concentration using membranes from *E. coli* strain DC82551 that contains only the third L-LDH activity.

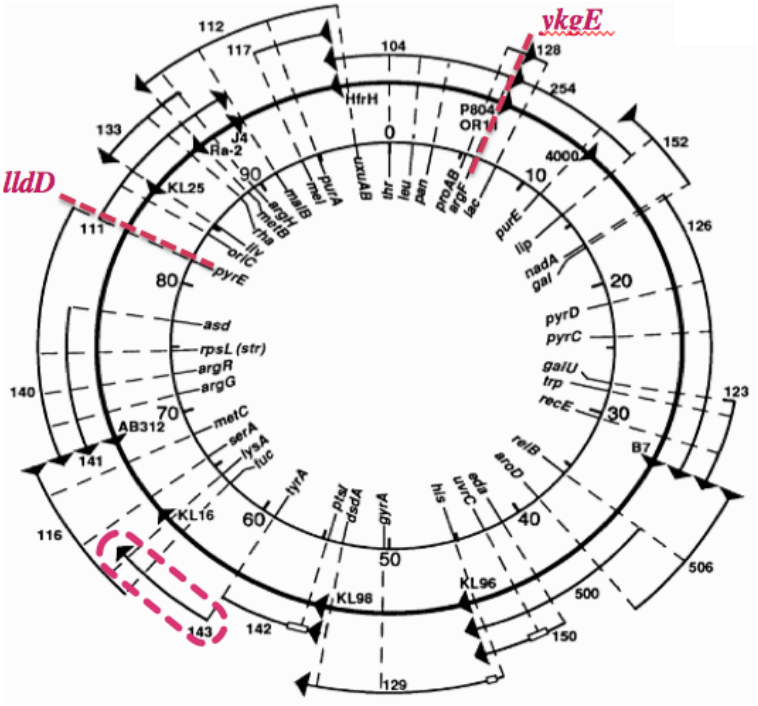

Figure 3-18. Genetic map of *E. coli* K-12 showing approximate chromosomal regions carried by different F-prime elements. Each F' is represented by an arc which has a narrow head drawn to show the point of origin of the ancestral Hfr strain(see inner circle). The dashed black lines, which extend radially from the genetic markers on the outer circle, indicate the known termini of the F-prime elements. Known deletions are indicated by narrow rectangles. *lldD* and *ykgE* gene loci are represented and the bold dashed lines represents the F' factor that complemented the L-LA minus phenotype in DC8261.

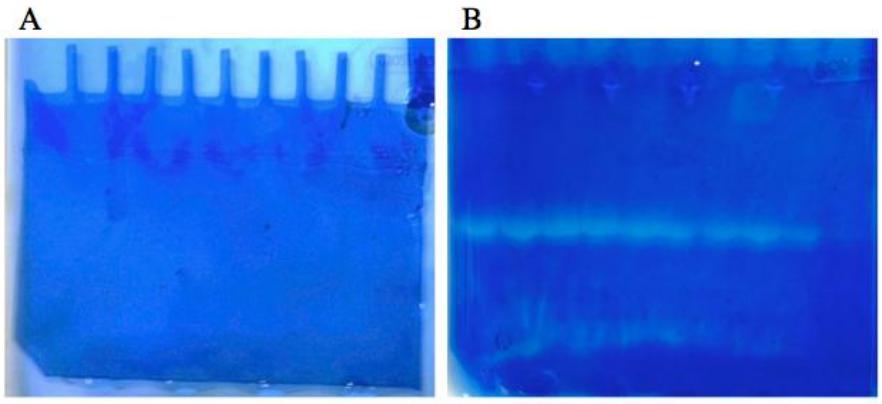

Figure 3-19. Native PAGE gels stained with DCPIP, without L-LA (A), and with L-LA (B). 2% triton-100 was added to the gel and migration was performed at 4°C to conserve activity.

CHAPTER 5
CONCLUSION

Polylactic acid polymer (PLA), although biodegradable, persists in nature upon disposal due to various constraints. Thermochemical hydrolysis of the PLA-based plastics is an effective method of recycling them at the end of life to generate the constituent monomer, lactic acid (LA) that can be reused to produce PLA. Hydrolysis of PLA beads in water occurs in two stages; swelling and melting of the polymer followed by release of LA from the molten plastics. Temperature-dependent release of LA from PLA beads in water follows apparent first order decay kinetics after a short lag and a modified Gompertz equation can explain the overall process. In the presence of limiting amount of NaOH, a base concentration-dependent immediate release of LA, apparently from the amorphous regions of the beads was detected. Bulk of the lactic acid production followed after a short lag until a maximum of about 95% of the theoretical yield of LA was reached. The rate of hydrolysis of PLA was higher in the presence of NaOH compared to water alone and is dependent on particle size. Racemization of released LA was not detected during hydrolysis in water or with limiting amount of NaOH. However, molar excess of NaOH during PLA hydrolysis led to racemization of LA. Overall, the results presented in this dissertation show that thermochemical hydrolysis of PLA-based plastics in the presence of limiting amount of base is an effective and rapid method of recovering LA for reuse as chemical feedstock.

A novel bio-based process was developed for purification of the PLA syrup obtained after hydrolysis. The D-LA removal is achieved at 37°C under aerobic conditions using an engineered *E. coli* that lacks L-LDH activity. No D-LA was detectable after this process step, suggesting that the L-LA obtained after downstream purification can be redirected into the PLA production process. The use of a biological system for the purification of diastereoisomers has

never been described and constitutes a promising economical way of obtaining technical grade L-LA from post-consumer PLA items.

Finally, this study led to uncovering new features of the aerobic L-LA metabolism in *E. coli*. *E. coli ykgEFG* operon encodes a second L-LDH, in addition to the previously reported *lldD*-encoded L-LDH. The Ykg-LDH is also localized in the membrane and the activity of this complex in crude extract was about 2-fold lower than the activity of LldD. Additionally, a third L-LDH activity was also detected in a mutant lacking both *lldD* and *ykgE* and the gene(s) encoding this third LDH activity is yet to be identified. This third L-LDH activity in the cell, even though lower than for the other two LDH activities, is sufficient to support growth of *E. coli* on L-LA.

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BIOGRAPHICAL SKETCH

Diane Chauliac earned her bachelor's degree in biology ("Ecole Préparatoire aux Grandes Ecoles" section Biology) in 2004. She received her engineering degree in biotechnology in 2007 from the University of Luminy at Marseilles, France. After working as a visitor scientist for Merck & Co (New Jersey, USA), Diane joined the doctoral program of the Microbiology and Cell Science Department at the University of Florida in 2009.

D. Chauliac has been the recipient of a scholarship since the start of her PhD. While pursuing her degree, D. Chauliac worked as a teaching assistant for the department of microbiology and Cell Science during her first scholar year. Additionally, Diane helped three undergraduate students and three graduate students in their own researches, leading to publications independent of her PhD project (44, 156) and an additional manuscript is under in the writing. During her PhD, Diane was able to publish her work relating to the hydrolysis of PLA and titled "Thermohydrolysis kinetics of PLA beads in aqueous solution".