University of Alberta

Bio-Transformation of Fatty Acids

by

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Dedicated to the endless patience of

my respected mentors,

my beloved children and husband

Abstract

This research focused on the bioconversion of unsaturated fatty acids to hydroxy fatty acids, which are platform chemicals of biological and industrial significance. Oleic, linoleic and linolenic acid are liberated in the soap stock of plant oil refineries as free fatty acids and were used as model substrates for bioconversion. The thesis selected microorganisms, and developed methodology for the rapid and enhanced enzymatic transformation of these unsaturated fatty acids to hydroxy fatty acids. The protocols established can be extended to derive value added compounds from the by-products of plant oils.

The first objective was to identify bacteria with the ability to produce hydroxy fatty acids, and to increase the product turnover. GC-MS analyses indicated the transformation of oleic acid to 10-hydroxystearic acid by *Pseudomonas aeruginosa*, *Lactobacillus plantarum*, *L. sanfranciscensis*, *L. reuteri*, *L. sakei*, and *Bifidobacterium bifidum* BB12. Linoleic and linolenic acid were converted to 10-hydroxy-12-octadecenoic acid, 13-hydroxy-9-octadecenoic acid and 10, 13-dihydroxystearic acid by lactobacilli but not by *P. aeruginosa*. Maximum transformation rate was observed from crude cell extract and the activity of a hydratase enzyme was indicated.

The second objective was to develop a single step method for the production of coriolic acid from linoleic acid with lipoxygenase (Lox). A process for single step formation of coriolic acid was achieved with a 70% or higher yield from free and immobilized Lox using cysteine as reducing agent containing 2 mmol/L and 100 mmol/L initial linoleic acid. Immobilized Lox was re-used 10

times with 64% yield from 2 mmol/L linoleic acid and 3 times with 40% yield from 100 mmol/L linoleic acid. A comparable activity of free Lox was observed in reactions performed at a 5 mL and 1 L scale.

The dissertation provides practical approach for the biotransformation of unsaturated fatty acids to hydroxy fatty acids by using the specific enzymes from bacterial cell extracts or by commercially available enzymes. The nature of products depends upon the substrates, source of enzyme and transformation conditions. The products thus generated hold the potential to be used as bioactive compounds for food, pharmaceutical and industrial purposes.

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List of Abbreviations

BLAST	Basic local alignment search tool
BSA	N, O-bis-(trimethyl silyl) acetamide
C18:3	Linolenic acid
C18:2	Linoleic acid
C18:1	Oleic acid
C17:0	Heptadecenoic acid
CI-GC-MS	Gas chromatography-mass spectrometery chemical ionization
cP	Centipoise
CYP	Cytochrome P450
cys-HCl	Cysteine Hydrochloride
DAD	Diode array detector
DAM	Diazomethane
eV	Electronvolt
FAD	Flavin adenine dinucleotide
FID	Flame ionization detector
GC-MS	Gas chromatography-mass spectrometery
HFAs	Hydroxy fatty acids
HPOD	Hydroperoxy octadecenoic acid
HPLC	High performance liquid chromatography
10-HSA	10-Hydroxystearic acid
12-HSA	12-Hydroxystearic acid
LAB	Lactic acid bacteria

LB medium	Lauria Bertani medium
Lox	Lipoxygenase
mDa	Milli Dalton
MIC	Minimum inhibitory concentration
$[M+H^+]$	Molecular weight plus a proton
$[M-H]^-$	Molecular weight minus a proton under negative ion mode
[M-OTMS]	Molecular weight minus Oxy-trimethyl silyl
mMRS medium	Modified DeMan Rogosa Sharpe medium
m/z	Mass to charge ratio
$NaBH_4$	Sodium borohydride
OTMS	Oxy-trimethyl silyl
psi	Pounds per square inch
$Si(CH_3)_3$	Trimethyl silyl
THOA	12,13,17-trihydroxy-9(*Z*)-octadecenoic acid
THODeA	9,12,13-trihydroxy-10(*E*)-octadecenoic acid
TLC	Thin layer chromatography
TOD	7,10,12-trihydroxy-8(*E*)-octadecenoic acid
UFAs	Unsaturated fatty acids

Chapter 1

Bio-Transformation of Fatty Acids

1.1 Background

Fatty acids from plant and animal origin, or from agricultural waste, constitute a vast renewable resource. This renewable resource can be used to supplement the declining natural petroleum reserves, allowing for the production of oleochemicals and oil-based industrial products such as plasticizers and urethanes (Sensoz *et al*. 2000). Deriving material applications for renewable resources such as vegetable oils is advantageous due to their minimal carbon dioxide emissions, inherent biodegradability and low toxicity (Xia and Larock 2010). It has been estimated that 15% of all soybean oil from 2001 to 2005 was consumed in industrial processes (Xia and Larock 2010). A reason for the increased utilization of lipids in industrial processes has been the ability to tailor the products according to specific needs. Their applications are outlined below. For example, fatty acids liberated in the waste streams of natural oil refineries can serve as a natural alternative source for making specialty chemicals of vast industrial significance *e.g*., pharmaceuticals, food additives, flavour compounds, polymer precursors and natural fungicidal agents (Naughton 1974).

Soybean and canola oils are rich sources of unsaturated fatty acids (UFAs), while palm oil mainly contains saturated fatty acids in the form of lauric, myristic and palmitic acid (Luo *et al*. 2010, Misra and Murthy 2010). In western Canada, total canola production was 12.56 million metric tones for the year 2008 (Anonymous: Canadian Grain Commission). UFAs accumulate during the refining process of canola and soybean oil in the soap stock and deodorizer distillate. The free fatty acids liberated in soap stock of canola oil are comprised of 18 to 19% unsaturated fatty acids (Table 1.1). The free fatty acids from canola oil or from the by-products of these plant oil refineries can be transformed into industrially important compounds using biological systems (Hou 2000).

Table 1.1. Fatty acid composition of canola oil and soap stock of canola oil (Durant *et al*. 2006).

Fatty acid	Canola oil	Soap stock
Oleic acid (C18:1)	61%	15.57%
Linoleic acid (C18:2)	21%	3.31%
Linolenic acid (C18:3)	9-11%	0.24%

This biological transformation of UFAs into valuable chemicals and materials would generate products from the waste stream of plant oil processing operations which are rich in UFAs. Soap stock is currently being used as an animal feed additive, particularly in pig and poultry diets. Addition of 1% soybean soap stock to the poultry diet brings about an increase of approximately 1.7 g in the weight of a broiler over a period of 1-7 weeks (Pardio *et al*. 2001). In feed applications, the soap stock is used directly and no value added materials are derived from it. However, fatty acids removed from the soap stock could provide a sustainable source of important industrial raw materials after undergoing chemical or biological transformation. Various functional groups in UFAs offer sites for chemical and biological modification. Particularly, the formation of hydroxy fatty acids is important in this respect, as addition of hydroxyl groups at unsaturation sites on UFAs converts them into platform chemicals for various applications (Naughton 1974, Hou 2000).

The aim of this work was to develop a green technology capable of bio-transforming pure UFAs in the form of oleic, linoleic, and linolenic acid as model compounds. Efforts were devoted to investigating and developing potential bio-transformation routes leading to hydroxy fatty acids from UFAs. After the progress with model compounds, the UFAs, which are produced as waste products, can be used for the generation of renewable and cost-efficient platform chemicals in the form of hydroxy fatty acids.

1.2 Introduction

This chapter outlines the relevant literature pertaining to the practical applications of specialty fatty acids known as hydroxy fatty acids (HFAs). The focus is on applications of selectively hydroxylated fatty acids, as these fatty acids were the primary target for this study. An overview of HFAs in various areas including food, biomedical research, pharmaceutical, and chemical synthetic industry is described, followed by naturally existing sources of HFAs and their industrial significance. In the later sections, chemical synthesis of hydroxy fatty acids with implications on complex procedures, lower yields, and purity of final products is compared with biotransformation using whole cell microbial systems utilizing different enzymatic pathways, and purified enzymes. Among various enzyme systems for producing specialty chemicals, emphasis has been placed on a soybean enzyme, lipoxygenase. The mechanism of action of lipoxygenase is described in order to develop an insight into the secondary reactions leading to the formation of undesirable products. An understanding of the mode of action of lipoxygenase is also required to develop a stable high yield enzyme system. Additionally a literature review about immobilization procedures developed to date for this enzyme is presented in a tabulated form with special reference to the formation of a relatively unstable primary product in the form of hydroperoxy octadecenoic acid.

1.3 Bio-transformation of unsaturated fatty acids (UFAs)

Modification of the alkyl chain of fatty acids for the introduction of new functionality can be performed through chemical methods or through biological systems (i.e. microbes or purified enzymes). The enzymatic reactions have the advantage of stereo and regio-specificity, lower energy demand, and decreased by-product and waste generation, which also curb environmental concerns (Hasan *et al*. 2009, Gunstone 2003, Schmid *et al*. 2001).

The specific desired modification of fatty acids depends on their final usage *e.g*. as a nutritional, food grade or industrial end product. Current modifications in food systems are usually focused on the levels of saturated and

unsaturated fatty acids (Kris-Etherton 2010). However, for industrial purposes, a more elaborate range of modifications of chain length, position of double bonds and variation of functional groups is often used to attain the required attributes. Introduction of hydroxyl groups on fatty acid chains makes them more polar, viscous and reactive. Currently, commercial polyols are mostly produced from petroleum-based monomers (Lligadas *et al*. 2010). In the following sections, a review of pertinent literature regarding the applications of HFAs, their natural sources and different approaches for introducing hydroxyl functionalities into the UFA chain to form HFAs will be described.

1.4 Uses of hydroxy fatty acids (HFAs)

HFAs and their derivatives are multifunctional molecules used in pharmaceuticals, cosmetics, paints and coatings, lubricants, food, and cosmetic industries (Naughton 1974). They are useful intermediates in the synthesis of industrial grade fine chemicals. Various HFAs, categorized according to their applications in relevant areas are described in the following sections.

1.4.1 HFAs as industrial raw materials

Hydroxy fatty acids are intermediates for the formation of polyurethane, polyesters, waxes, resins, coatings, paints and enamels (Weber *et al*. 1995). Fatty acids from natural oils can be used as raw materials for polyurethane production for which multiple hydroxyl groups are required. Castor oil contains a large amount (94%) of ricinoleic acid, chemically which is, 12-hydroxy-9(*Z*)-octadecenoic acid (Barnes *et al*. 2009a, b). The presence of hydroxyl group on ricinoleic acid has made it an attractive raw material for the chemical industry over the past century for the production of lubricants, polyurethane building blocks, castings, resins, elastomers, urethane foams, and interpenetrating networks (Barnes *et al*. 2009a). In addition to unsaturated HFAs, saturated HFAs such as 10-hydroxystearic acid and its derivatives have been described as ingredients of multipurpose industrial grade greases (Hagemann and Rothfus 1991, Kuo and Levinson 2006). The synthetic routes for the preparation of these grease thickeners are described by Kenny *et al*.

(1974a, b). Hydroxy stearates can be applied in the form of oil resistant, water-proof leather coatings and for the formation of roll leaf foils (Naughton 1974). HFAs with di- and tri-hydroxy substitutions have been reported from microbial transformation of oleic acid, ricinoleic acid and linoleic acid. The use of di- and tri-hydroxy fatty acids in the formation of microemulsions and as additives in various commercial preparations has been reported (Kuo and Lanser 2003, Hou 1999).

1.4.2 HFAs in the food industry

HFAs are structurally analogous to naturally occurring HFA, ricinoleic acid and can find applications in the formation of flavouring agents and drugs (Naughton 1974). Lactones and conjugated linoleic acid can also be derived from HFAs (Alewijn *et al*. 2007). γ-Decalactones and δ-dodecalactones impart peach and coconut flavours to various food products respectively (Serra *et al*. 2005). The sweet, fruity and fatty flavour of these lactones in malt whisky were reported to be produced by microbial processes originating from the hydration of UFAs, using 10-hydroxystearic acid as a precursor (Wanikawa *et al*. 2000, Waché *et al*. 2003). Conjugated linoleic acid formation has been demonstrated using ricinoleic acid as a precursor in a biological transformation reaction employing *L. plantarum* and through chemical transformation of ricinoleic acid (Ando *et al*. 2004, Villeneuve *et al*. 2005). Additionally, HFAs for food-grade applications can be produced from "natural" feedstocks available as fatty acids from the by-products of plant oil refineries thus meeting the rising demand for "natural" flavours and aromas (Serra *et al*. 2005).

1.4.3 Bio-active HFAs

Biologically active HFAs have antifungal activities and are secreted by plants as a stress response (Kato *et al*. 1983). Microbially produced 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid has been shown to possess antifungal activity against fungi affecting wheat, potato, and cucumber plants (Hou and Forman 2000). Various antifungal HFAs are summarized in Table 1.2. Antibiotic activity of microbially produced 7,10-dihydroxy-8(*E*)-octadecenoic acid has been

Table 1.2. Anti-fungal activity of hydroxy fatty acids.

Hydroxy Compound	Substrate (producer organism)	MIC mg/L	Indicator strain	References
2-hydroxy-C12:0	- (*L. plantarum*)	5	*P. roqueforti*	Sjogren *et al*. 2003
3-hydroxy-C10:0		25		
3-hydroxy-C11:0		10		
3-hydroxy-C12:0		25		
7,10,12-trihydroxy-C18:1$^{\Delta 8}$ (TOD)	Ricinoleic acid (*P. aeruginosa* PR3)	5	*Pyricularia grisea*	Kuo *et al*. 2001
12,13,17-trihydroxy-C18:1$^{\Delta 9}$ (THOA)	Linoleic acid (*B. megaterium*)	200	*Erysiphe graminis*	
12,13,17-trihydroxy-C18:1$^{\Delta 9}$ (THOA)	Linoleic acid (*B. megaterium*)	200	*Puccinia recondite*	Hou and Forman 2000
12,13,17-trihydroxy-C18:1$^{\Delta 9}$ (THOA)	Linoleic acid (*B. megaterium*)	200	*Phytophthora infestans*	
13-hydroxy-C18:2$^{\Delta 9,11}$	(Chemically prepared)	63	*Pyricularia oryzae*	Kobayashi *et al*. 1987
8(*R*)-hydroxy-C18:2$^{\Delta 9,11}$	(*Laetisaria arvalis*)	20	*Pythium ultimum*	(Bowers *et al*. 1986)
13-hydroxy-C18:2$^{\Delta 9,11}$	(Chemically prepared)	32	Rice blast fungus	(Kato *et al*. 1984)
9,12,13-trihydroxy-C18:1$^{\Delta 10}$ (THODeA)	(*Colocasia antiquorum*)	50	Black-rot fungus (*Ceratocystls fimbrzata*)	(Masui *et al*. 1989)
Hydroxy fatty acids (mono-,di-, & tri-)	Linoleic acid (*P. aeruginosa*)	32	*Penicillium funiculosum*	(Martin-Arjol *et al.* 2010)

THOA: 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid

TOD: 7,10,12-trihydroxy-8(*E*)-octadecenoic acid

THODeA: 9,12,13-trihydroxy-10(*E*)-octadecenoic acid

MIC: Minimum inhibitory concentration

suggested against various food borne pathogenic bacteria, plant pathogenic bacteria and *Candida albicans* (Hou 2000, 2008). Antibacterial activity of HFAs such as coriolic acid, a 13-hydroxy analogue of linoleic acid, and dimorphecolic acid toward the growth of *Bacillus subtilis*, *Staphylococcus aureus* and

Micrococcus flavus has been reported (Mundt *et al*. 2003). The growth of apotato tuber infecting bacterium *Erwinia carotovora* T29 was found to be inhibited by the application of 9-hydroxy-octadecenoic acid and coriolic acid (Kimura and Yokota 2004). Moreover, very low doses of coriolic acid, with LD 50 values in the range of 5-10 ppm, against saprophytic nematode *Caenorhabditis elegans* have also been described (Stadler *et al*. 1994).

In the area of biomedical research, HFAs in the form of tetrahydrofuranyl fatty acids, and 8-oxo-9,10-dihydroxy-9(*Z*)-octadecenoic acid derived from the microbial transformation of linoleic acid, and isolated from an edible mushroom, *Hericium erinaceum*, respectively, have been shown to possess cytotoxic activities against cancer cells (Hou 2008, Kawagishi *et al*. 1990). A synthetic hydroxy derivative of oleic acid, 2-hydroxy-9(*Z*)-octadecenoic acid (Minerval®), exhibited anticancer activities in animal models of cancer cells (Martinez *et al*. 2005, Llado *et al*. 2010). Potential use of 2-hydroxy-9(*Z*)-octadecenoic acid and other related compounds as anticancer and hypotensive drugs has been described in a patent disclosure (Marquez *et al*. 2004). Apart from its antimicrobial activity, coriolic acid acts as an autocrine and paracrine lipid mediator and as a calcium specific ionophore (Rao *et al*. 1986).

Coriolic acid is produced in mammals when linoleic acid is oxidized by either 15-lipoxygenase or cyclooxygenase. These oxidative reactions lead to the formation of 13-hydroperoxy octadecadienoic acid (Kuhn 1996). These hydroperoxy compounds are reduced to coriolic acid in the presence of a selenium-containing glutathione peroxidase (Kuhn 1996). This endogenous coriolic acid has been shown to play a role in relaxing preconstricted arterial rings, indicating its vasoactive properties by mediating direct influx of Ca^{+2} into the smooth muscles (Stoll *et al*. 1994). *In vitro* cytotoxic effects of coriolic acid and other hydroxylated isomers of coriolic acid have been mentioned against numerous human cancer cell lines (Li *et al*. 2009, Shureiqi *et al*. 2003). Biological activity of coriolic acids as anti-bacterial and anti-cancer compound and its role as an anti-inflammatory agent warrants the use of coriolic acid for future biomedical research and development (Aisen *et al*. 1985, Gautam and

Jachak 2009). Pharmacological studies necessitate a direct and simple approach for pure isomeric preparation of coriolic acid and other bioactive HFAs.

1.5 Natural sources of HFAs

The major source of naturally occurring HFA is ricinoleic acid (12-hydroxy-9(*Z*)-octadecenoic acid) from the seeds of *Ricinus communis* (castor bean) (Barnes *et al*. 2009b, Severino *et al*. 2010). However, the cultivation of castor oil plant has been discouraged due to the prevalence of a toxic protein, ricin in castor seed. Ricin is a naturally occurring potent cell poison with potential to be used as a bioterrorism weapon (Frigerio and Roberts 1998, Pinkerton *et al*. 1999, Audi *et al*. 2005). Castor cake is toxic to animals and is not used as feed until specially treated by steam or alkali, which is known to destroy the toxic ricin (Barnes *et al*. 2009a). As a result, finding unique methods for the formation of natural HFAs has drawn substantial attention. Castor oil containing 12-hydroxy-9(*Z*)-octadecenoic acid, was regarded as a strategic material critical for national defence due to its application for the formation of a wide range of lubricants and greases for military equipment (US congress 1991). Furthermore, ricinoleic acid from castor oil is a source of hydroxy fatty acid for industrial scale production of a wide variety of technical products *e.g*. sebacic acid, undecylenic acid, polyols for polyurethanes, detergents and antifungal agents (Powell 2009, Teomim *et al*. 1999). Ricinoleic acid is generally regarded as safe for over-the-counter use as a stimulant laxative and is applied in the cosmetic industry as an emulsion stabilizer, surfactant, occlusive and viscosity enhancing agent in skin cleaning and conditioning preparations, deodorants and fragrances (Johnson Jr. 2007).

Castor oil is a major export commodity of India, China and Brazil, and its price has been highly volatile over the last decade (Panwar *et al*. 2010). Presence of toxic allergens on the seed castor plant, along with price and availability constraints are the driving factors in the search for substitute methods for generating hydroxy fatty acids (Panwar *et al*. 2010). As a result, discovery and development of new methods to convert fatty acids, librated in the waste stream of plant oil refineries offer a potential source of locally generated HFAs. Various

natural sources of functionalized fatty acids from plant sources and their principal applications are summarized in Table 1.3.

In addition to ricinoleic acid, lesquerollic acid (14-OH -C20:$1^{\Delta 11}$) from *Lesquerella fendleri*, a C-20 homologue of ricinoleic acid, is another source of naturally occurring mono-hydroxy fatty acid. The seed contains 52% oil in the form of lesquerollic acid (David *et al*. 1993).

Table 1.3. Natural sources of hydroxy fatty acids.

Plant Species (% age of fatty acid in seed) **Common name**	Functionality (Fatty acid) symbol	Applications
Riccinis communis (90) **Castor bean**	Hydroxy (ricinoleic acid) 12-hydroxy-C18:$1^{\Delta 9}$	Plasticizer, cosmetics, medicine[A]
Lesquerella fendleri (55) **Fendler's bladderpod/ Lesquerella**	Hydroxy (Lesquerolic acid) 14-hydroxy-C20:$1^{\Delta 11}$	Lubricants, plastics[B]
Crepis palestenia (60) **Hawk's-beard** *Euphorbia lagascae* **Vernin spurge**	Epoxy (vernolic acid) 12-13-epoxyC18:$1^{\Delta 9}$	Resins, coatings[A], photocuring[C]

A: (Murphy 2006)

B: (David *et al*. 1993)

C: (Metzger and Bornacheur 2006)

1.6 Chemical synthesis of HFAs

Industrially useful fatty chemicals (e.g. hydroxy and keto acids) for use in surfactants, lubricants, cosmetic and fragrances are derived from fats and oils through chemical processes. Direct synthesis of only one isomer is not possible by chemical means, and as a result, an extensive downstream purification is often required. One multistep method for the chemical synthesis of HFAs involved acidification of olefinic acids (e.g. oleic or erucic acid) with sulphuric acid and then hydrolysis of the acidulated product in various steps to result in a mixture of hydroxystearic acids (Tomecko and Adams 1927). Another method for producing HFAs with double bond functionality along the carbon chain (11-hydroxy-9(*Z*)-octadecenoic acid and 8-hydroxy-9(*Z*)-octadecenoic acid) was found to require extensive purification due to undesired side reactions (Crombie and Jacklin 1957). Chemical synthetic routes for the preparation of coriolic acid have also been described elsewhere (Yadav *et al*. 1992, Bloch and Perfetti 1990).

1.7 Microbial transformation of unsaturated fatty acids to HFAs

In addition to naturally occurring and chemically synthesized HFAs, microbial transformations of unsaturated fatty acids results in the conversion of UFAs to HFAs. This occurs through stereospecific microbial enzymes which typically result in the formation of enantiomerically pure products. Compounds with multiple-substituted hydroxyl groups at double bond sites offer a wide range of applications. The large-scale exploitation of microbially produced hydroxy fatty acids is currently not in practice due to the high cost of production, limited understanding of their interactions and oxidative instability due to the presence of unsaturations (Hou 2005). A summary of microbial species with potential to biotransform oleic acid and linoleic acid to various HFAs is provided in Table 1.4 and Table 1.5 respectively. In the following sections a brief overview of whole cell microbial transformations of unsaturated fatty acids to HFAs by the mechanism of hydration, hydroxylation and lipoxygenation is described.

Table 1.4. Bacterial strains using oleic acid as substrate for producing HFAs.

Organism	Product	Yield	References
Pseudomonas sp.	10-hydroxy-C18:0	14%	(Wallen *et al*. 1962)
Nocardia cholesterolicum	10-hydroxy-C18:0	>90%	(Davis *et al*. 1969)
Corynebacterium spp.	10-hydroxy C18:0 & 10-KSA	9.1% & 22%	(Seo *et al*. 1981)
R. rhodochrous	10-hydroxy-C18:0 & 10-KSA		(Litchfield and Pierce 1986)
S. cerevisiae	10-hydroxy-C18:0	45%	(Elsharkawy *et al*. 1992)
Selenimonas ruminantium	10-hydroxy-C18:0		(Hudson *et al*. 1995)
Enterococcus faecalis	10-hydroxy-C18:0		
Sphingobacterium thalpophilum	10-hydroxy-C18:0	40%	(Kuo and Levinson 2006)
Staphylococcus spp.	10-KSA	>90%	(Lanser 1993)
Flavobacterium spp. DS5	10-KSA		(Hou 1994b)
P. aeruginosa strain PR3	7,10-dihydroxy C18:1$^{\Delta 8}$	>80%	(Hou and Bagby 1991)
P. aeruginosa NRRL BD-23258	7,10-dihydroxy C18:1$^{\Delta 8}$	81%	(Kuo and Nakamura 2004)
P. aeruginosa NRRL BD-18602	7,10-dihydroxy C18:1$^{\Delta 8}$	76%	
Pseudomonas sp.42A2	7,10-dihydroxy C18:1$^{\Delta 8}$ & 10-hydroperoxy-C18:1$^{\Delta 8}$	13% 28%	(Guerrero *et al*. 1997)
Stenotrophomonas maltophilia	3-hydroxy-C14:2$^{\Delta 5}$	-	(Weil *et al*. 2002)

10-KSA: 10-ketostearic acid

Table 1.5. Bacterial strains using linoleic acid as substrate for producing HFAs.

Organism	Product	Yield	References
R.rhodochrous	10-hydroxy-C18:1$^{\Delta 12}$	22%	(Litchfield and Pierce 1986)
Nocardia cholesterolicum	10-hydroxy-C18:1$^{\Delta 12}$	71%	(Koritala and Bagby 1992)
Flavobacterium spp. DS5	10-hydroxy-C18:1$^{\Delta 12}$	55%	(Hou 1994a)
L. acidophilus	10-hydroxy-C18:1$^{\Delta 12}$	65%	
L. casei	10-hydroxy-C18:1$^{\Delta 12}$	43%	(Kishimoto *et al.* 2003)
L. paracasei	10-hydroxy-C18:1$^{\Delta 12}$	91%	
L. rhamnosus	10-hydroxy-C18:1$^{\Delta 12}$	40%	
L. plantarum	10-hydroxy-C18:1$^{\Delta 12}$	59%	
		10%	(Yamada *et al.* 1996)
Stenotrophomonas nitritireducens	10-hydroxy-C18:1$^{\Delta 12}$	96%	(Yu *et al.* 2008)
Enterococcus faecalis	10-hydroxy-C18:1$^{\Delta 12}$	22%	(Hudson *et al.* 1998)
	13-hydroxy-C18:1$^{\Delta 9}$	14%	
Geotrichum candidum	9 & 13-hydroperoxy-C18:2		(Perraud *et al.* 1999)
Penicillium sp.	9, 10 & 13-hydroperoxy-C18:2		(Perraud and Kermasha 2000)
Clavibacter sp. ALA2 named as *B. megaterium* (Hou *et al*. 2005)	12-,13-,17-trihydroxy-C18:1$^{\Delta 9}$	35%	(Hou *et al.* 1998)
Stenotrhomonas maltophilia	3-hydroxy-C14:2$^{\Delta 5,8}$		(Weil *et al.* 2002)

1.7.1 Microbes utilizing a hydratase enzyme system

Hydratase enzymes are characterized by their ability to add hydroxyl groups across double bonds in fatty acid chains, where the oxygen is derived from water instead of molecular oxygen (Volkov *et al*. 2010). The formation of 10-hydroxystearic acid from oleic acid in 14% yield from *Pseudomonas* sp. (NRRL B-2994) through an intracellular enzyme was first described by Wallen *et al*. (1962). Later experimentation verified the stereospecific nature of transformation in an anaerobic environment and the hydroxyl oxygen was found to be derived from water (Wallen and Davis 1969). *Nocardia cholesterolicum* NRRL 5769 converted oleic acid under anaerobic conditions to l0-hydroxystearic acid in greater than 90% yield (Davis *et al*. 1969). Use of deuterium oxide and oxygen 18-labelled water confirmed water to be the source of hydroxyl group (Davis *et al*. 1969). The optimum conditions for this conversion were found to be a higher pH and a reaction specificity for fatty acids with *cis* unsaturation at position $C^{\Delta 9}$ (Davis *et al*. 1969). Koritala and Bagby (1992) showed that *N. cholesterolicum* also hydrated linoleic acid and linolenic acid to 10-hydroxy-12(*Z*)-octadecenoic acid (71% yield) and 10-hydroxy-12(*Z*),15(*Z*)-octadecadienoic acid with 77% yield, under anaerobic conditions. Oleate hydratase isolated from cell free extracts of *N. cholesterolicum* NRRL 5767 was found to be a tetrameric protein with a molecular weight of ~32 kDa of each subunit and a pH optimum in the range of 6.5 - 7 (Hou 1995b). Additionally, *Flavobacterium* species DS5 oxidize HFAs to ketostearic acid which may accumulate as an end product of oleic acid transformation (Hou 1994b). When linoleic acid was used as a substrate, *Flavobacterium* sp. produced 10-hydroxy-12(*Z*)-octadecenoic acid as a major product (Hou 1994a). *Flavobacterium* sp. DS5 hydratase was active only for unsaturated fatty acid substrates. The analysis of products generated by using oleic, linoleic and alpha and gamma linolenic acid (Figure 1.1) as substrates, revealed that hydratase from *Flavobacterium* species DS5(NRRL B-14859) was a C10-position specific enzyme and preferred a mono-unsaturated C18 fatty acid as substrate (Hou 1995a, b).

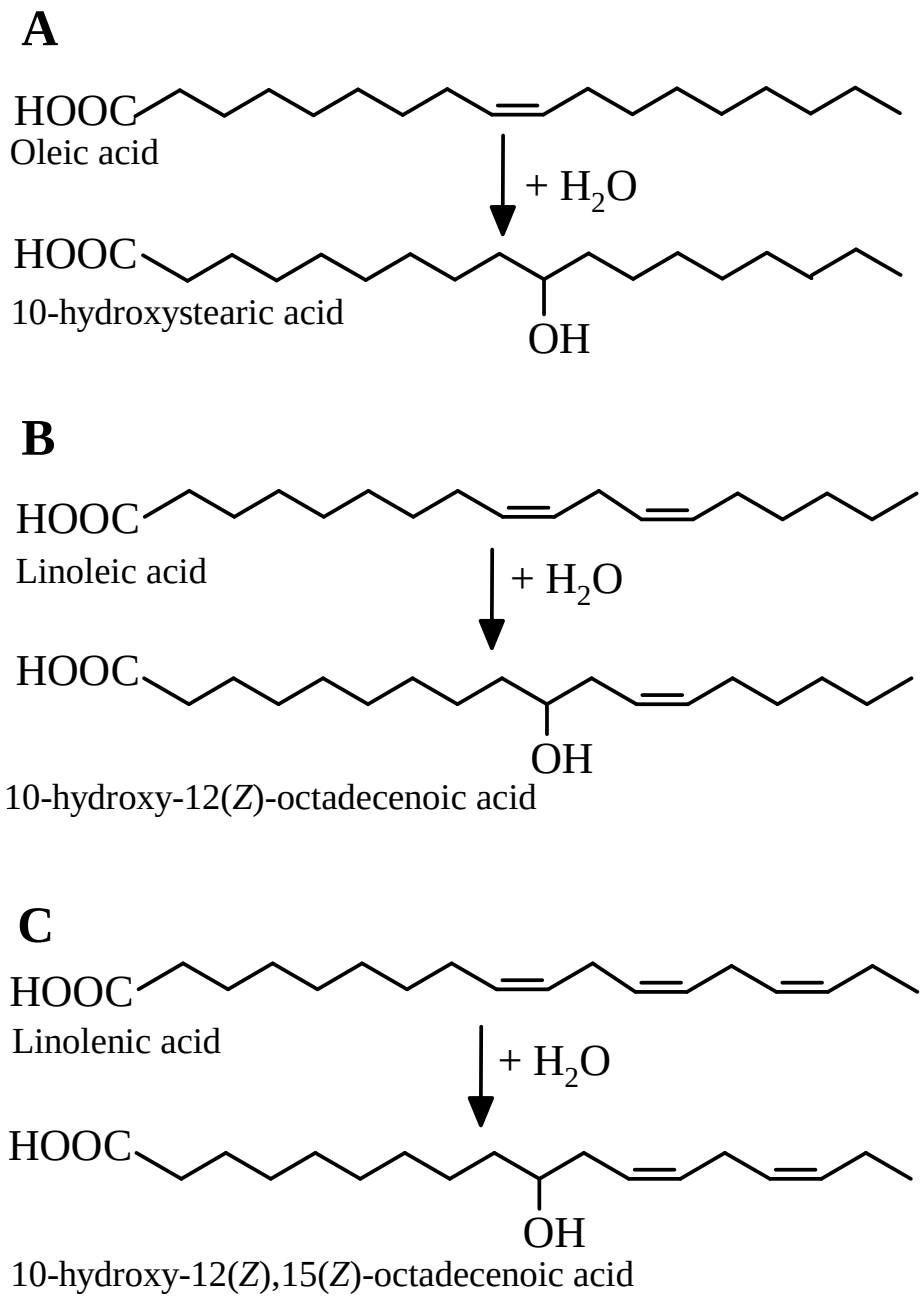

Figure 1.1. Transformation of oleic (A), linoleic (B), and linolenic acid (C) by hydratase DS5 enzyme of *Flavobacterium* sp. (Wallen *et al*. 1969, Davis *et al*. 1969).

Resting cells of *Corynebacterium* sp. S-401, isolated from soil, exhibited the capability of hydrating oleic acid to 10- ketostearic acid and 10-hydroxystearic acid in a 22.4% and 9.1% yield, respectively (Seo *et al*. 1981). A similar kind of oleic acid hydration was exhibited by *Staphylococcus* sp. with greater yield of ketostearic acid *i.e*. greater than 90% (Lanser 1993). Litchfield and Pierce (1986) demonstrated in a patent disclosure that *Rhodococccus rhodochrous* can convert linoleic acid to 10-hydroxy-12-octadecenoic acid and 10-keto-12-octadecenoic acid, whereas formation of 10-hydroxystearic acid and 10-hydroxy-12(*Z*),15(*Z*)-octadecenoic acid in response to linolenic acid exposure has been reported for *L. lactis* (Kim *et al*. 2003).

1.7.2 Bacteria using the mechanism of hydroxylation for transformations

Hydroxyl functionality can be introduced through hydroxylation of UFAs, in which hydroxyl groups are introduced into the substrate while retaining the double bond of the parent compound, however, the configuration of the parent fatty acid is altered from a natural *cis* to *trans* form. *Pseudomonas aeruginosa* PR3, utilizes hydration and hydroxylation to produce 7,10-dihydroxy-8(*E*)-octadecenoic acid with the intermediate formation of 10-hydroxy-8(*E*)-octadecenoic acid, (Hou and Bagby 1991;1992, Hou *et al*. 1991, Knothe *et al*. 1992). Therefore, the bioconversion pathway involves a hydratase, introducing a hydroxyl group at the $C^{\Delta 10}$ position and simultaneously shifting the double bond from Δ9 to Δ8 position (Hou 1999). The resulting 10-hydroxy-8(*E*)-octadecenoic acid was shown to be attacked by a hydroxylase to introduce a hydroxyl group at $C^{\Delta 7}$ (Figure 1.2A). 7,10-dihydroxy-8(*E*)-octadecenoic acid formed from *P. aeruginosa* strain PR3 was later on, in a patent disclosure, described as a plasticizer and as a source of intermediate in the synthesis of specialty chemicals (Hou 1999, Kim *et al*. 2000, Kuo *et al*. 2001). By using ricinoleic acid as a substrate, *P. aeruginosa*, in a similar pathway produced 12,13,17-trihydroxy-8(*E*)-octadecenoic acid (Kuo *et al*. 2001). Some strains of *P. aeruginosa* transformed more than 75% of the added substrate. *P. aeruginosa* strains showed very low-level activity when linoleic acid was used as

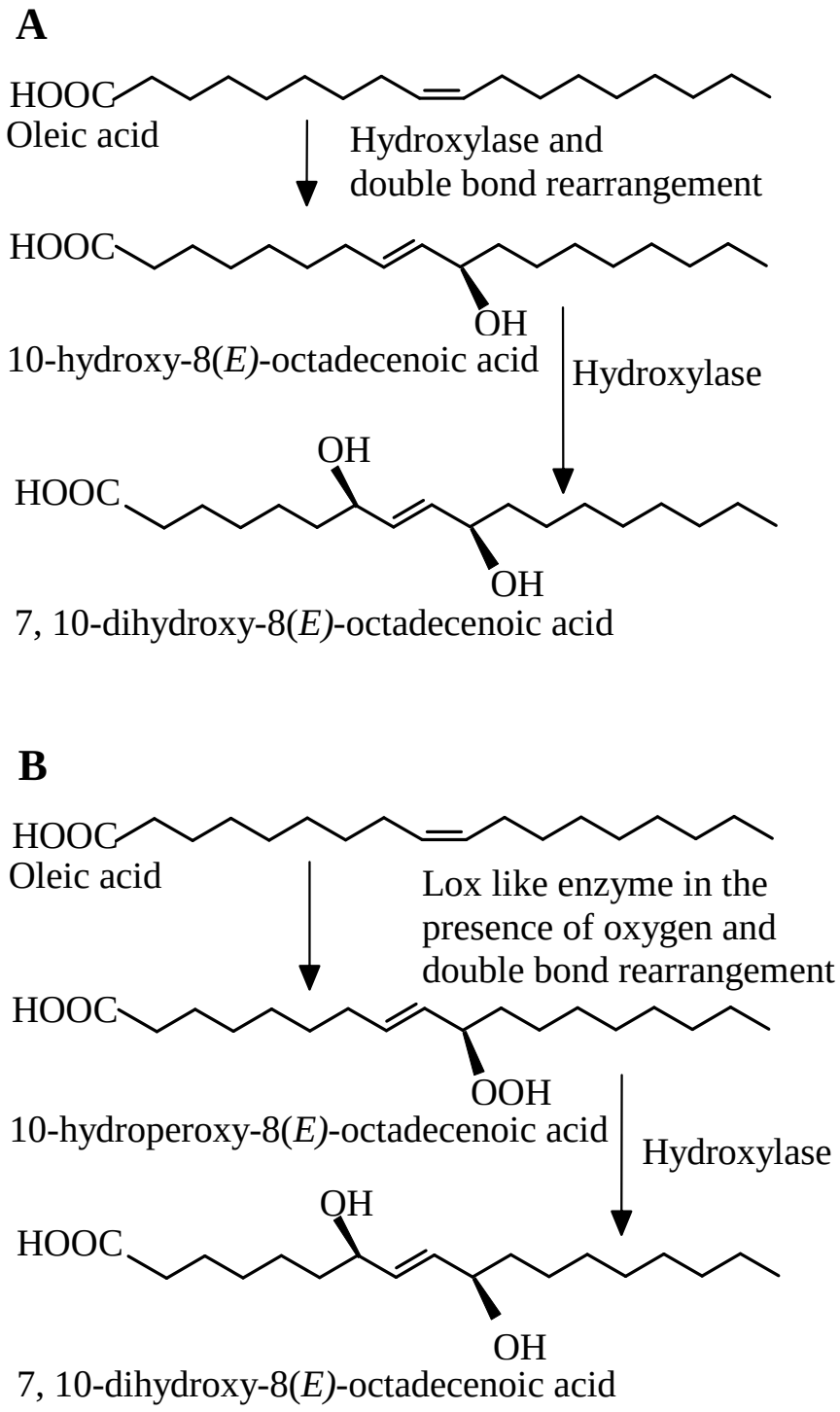

Figure 1.2. Biotransformation of oleic acid by *P. aeruginosa* strain PR3 for the formation of a dihydroxy product with intermediate formation of 10-hydroxy-8(*E*)-octadecenoic acid (A) (Kim *et al*. 2000). Transformation of oleic acid by *Pseudomonas* sp. 42A2 using lipoxygenase like enzyme for the formation of 7, 10-dihydroxy-8(*E*)-octadecenoic acid (B). This pathway produced 10-hydroperoxy-octadecenoic acid as an intermediate.

a substrate suggesting that linoleic acid might be metabolized as an energy source for the bacterium, due to the absence of any by-products (Kuo and Nakamura 2004). *Bacillus pumulis*; NRRL BD-174 & BD-226 transformed oleic acid to 15-, 16-, 17-hydroxy-8(*E*)-octadecenoic acid, whereas, 7-hydroxy-16-oxo-9(*Z*)-octadecenoic acid was formed by Bacillus strain BD-447 (Lanser *et al*. 1992, Lanser 1998).

Hydroxy fatty acids with $C^{\Delta 7}$ substitutions have been described in fungi and *P. aeruginosa* (Hou and Bagby 1991). 10-Ketostearic acid was produced when oleic acid was used as a substrate through the mechanism of hydration (Litchfield and Pierce 1986). From the above mentioned examples it can be inferred that the nature of product formed, its configuration, and yield depends upon the substrate fatty acid, individual organism and experimental reaction conditions.

The above paragraphs provide a snap shot of the wealth of literature available regarding the microbial formation of HFAs, but which do not address the potential of lactic acid bacteria for the formation of bioactive hydroxyl fatty acids (Hou 1999, Litchfield and Pierce 1986, Kuo and Hou 2001, Hou and Hosokawa 2005, Hou 2007). Literature about lactic acid bacteria in this regard is mainly dominated with references to the formation of conjugated linoleic acid and lactones for food applications, while substantiating HFA formation as intermediates in this process (Ogawa *et al*. 2005). Lactic acid bacteria can bring about the transformation of UFAs through the mechanism of hydration. Numerous lactobacilli strains transform oleic acid to 10-hydroxystearic acid, and linoleic acid to isomers of monohydroxy octadecenoic acid, *i.e*. 10-hydroxy-12-octadecenoic acid and 13-hydroxy-9-octadecenoic acid (Wanikawa *et al*. 2000, 2002, Kishimoto *et al*. 2003). The role of 10-hydroxy-12-octadecenoic acid during the transformation of linoleic acid was suggested as a precursor for the formation of conjugated linoleic acid while working with *L. acidophilus*, whereas accumulation of this mono hydroxy isomer has been reported as an end product in other studies (Ogawa *et al*. 2005, 2001, Yamada *et al*. 1996). Occurrence of a dihydroxy stearic acid in response to linoleic acid transformation was observed for *L. acidophilus* (Kishimoto *et al*. 2003). Sakata *et al*. (1986) reported the formation of 10-hydroxy-

12(*Z*),15(*Z*)-octadecenoic acid from linolenic acid (C18:3) by uncharacterized species of lactic acid bacteria under anaerobic conditions during corn silage fermentations. The formation of 10-hydroxy-12(*Z*),15(*Z*)-octadecenoic acid in response to linolenic acid exposure has also been reported for *L. lactis* (Kim *et al*. 2003). In addition to the transformation of UFAs to various HFAs, inhibitory effects of free fatty acids on certain lactic acid bacterial species in the growth medium have also been mentioned (Kim *et al*. 2003). The ability to hydrate across the $C^{\Delta 10}$ double bond appears to be most prevalent in the genera *Lactobacillius*, *Streptococcus*, *Lactococccus* and *Pediococcus*, in contrast to the $C^{\Delta 13}$ hydration route which was reported in specific strains of *Lactobacillus* and *Pediococcus* (Kishimoto *et al*. 2003). A recent study about *Streptococcus pyogenes* verified the role of an FAD containing double bond hydratase for the conversion of UFAs to HFAs (Volkov *et al*. 2010).

1.7.3 Transformation of substrate by microbial lipoxygenase or monooxygenase like enzymes

The lipoxygenase enzyme introduces a molecule of oxygen in the substrate while retaining the double bonds in a re-arranged form. The rate of reaction is specifically higher in the presence of oxygen with hydroperoxides as end products (Nanda and Yadav 2003). 7, 10-Dihydroxy-8(*E*)-octadecenoic acid was shown to be produced by *Pseudomonas* sp. 42A2 using oleic acid as substrate along with the formation of 10-hydroxy-8(*E*)-octadecenoic acid and 10-hydroperoxy-8(*E*)-octadecenoic acid (Figure 1.2B), suggesting a different enzymatic route in this strain as compared to the pathway suggested by Hou (1999) (Guerrero *et al*. 1997). Lipoxygenase like enzyme was shown to be responsible for the oxidation of fatty acid at $C^{\Delta 10}$ position with a *cis-trans* isomerisation of double bonds to produce hydroperoxides. This was a characteristic feature of Lox like enzyme, which was found to be located in the periplasmic space in the bacterial cell and required the presence of oxygen for its proper functioning (Busquets *et al*. 2004).

A comparison of biotransformation pathways for oleic acid by *P. aeruginosa* strain PR3 using hydratase/hydroxylase enzyme system and *P.*

aeruginosa strain 42A2 using lipoxygenase like enzyme system is provided in Figure 1.2A and 1.2B, respectively. *Bacillus megaterium* ALA2, previously identified as *Clavibacter* sp. ALA2 converts linoleic acid into many poly-hydroxy unsaturated fatty acids (Hou *et al*. 1998, Hou 2005). The authors mentioned 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid as a major product which appeared after purification from HPLC as a colourless oily liquid and was reported to be similar to the plant self-defence substance (Hou *et al*. 1998). Potential use of the organism, *B. megaterium* ALA2, for the formation of tri-hydroxy fatty acids from the biotransformation of linoleic acid was suggested (Hou *et al*. 1998). The role of cytochrome P450 monoxygenase like enzyme system for bringing about the above mentioned bioconversion for generating various products for specialty industrial or biomedical applications was proposed.

Microorganisms having the capability to transform fatty acids extend into eukaryotic groups as well. Yeast and fungal strains introduce hydroxyl functionality into specific locations on the long chain of UFAs (Fabritius *et al*. 1997, Metzger and Bornscheur 2006). Presence of HFAs in the form of 8(*R*)-hydroxy-octadecadienoic acid have been reported in *Laetisaria arvalis*, *Aspergillus nidulans*, *Gaeumannomyces graminis* (the takeall fungus), *Leptomitus lacteus* (the sewage fungus) and from *Magnaporthe grisea* (rice blast fungus) by the action of a heme-containing dioxygenase enzyme (Garscha and Oliw 2007, Bowers *et al*. 1986). It can be concluded that the diversity of microbial enzymes can generate a diverse range of specific products.

1.8 Purified enzymes for introducing hydroxyl functionality

Enzymatic insertion of molecular oxygen into the substrate is brought about by oxygenase enzymes (Vilker *et al*. 1999). Naturally occurring oxygenase enzymes result in the formation of HFAs, ketones, epoxides or hydroperoxides as intermediates (Bugg 2003, Smith 1989). The class of enzymes which introduce one atom of molecular oxygen into the substrate with subsequent reduction of the other oxygen atom to water are classified as monooxygenases, whereas dioxygenases transfer both atoms of molecular oxygen to the substrate (Vilker *et*

al. 1999, Bugg 2003). Three different oxygenases, namely, cytochrome P450 monoxygenases, di-iron centre oxygenases (hydroxylase), and dioxygenases have been mentioned in view of fatty acid oxygenations (Hou and Hosokawa 2005, Shanklin and Cahoon 1998). The oxygenases oxidize fatty acids through a series of reactions as in β-oxidation or require a cofactor in the form of Mn, Cu or Fe to direct the oxidative power of dioxygen for breaking the carbon to hydrogen bond and for introducing oxygen (Hou and Hosokawa 2005). The following sections contain a brief outline of oxygenases, while a detailed description related to the occurrence, products (in plant and animals), and mechanism of action of dioxygenase-lipoxygenase is presented. Lipoxygenase is commercially available in purified form and will be discussed in chapter 3 for the enzymatic preparation of coriolic acid.

1.8.1 Mono-Oxygenase Cytochrome P450 - Occurrence, products, and limitations

Cytochrome P450 monooxygenases (CYP) are heme-containing enzymes which bring about the oxidation of substrate by introducing one atom of molecular oxygen through the process of hydroxylation mediated by a reduced heme iron (Urlacher *et al*. 2004b). The mode of action of CYPs include insertion of one atom of molecular oxygen into non-activated alkyl chains or fatty acids, at their allylic positions, or at double bond locations, resulting in oxygenated and/or dibasic carboxylic acids (Budde *et al*. 2004). The products have multiple pharmaceutical industry applications and can be used for environmental and biological detoxification purposes (Goldstein and Faletto 1993, Feyereisen 1999, Budde *et al*. 2004, Urlacher and Schmid 2004a). A recent review article by Kumar (2010) not only includes various characteristics of CYPs in terms of low activity, limited stability, and their need for an expensive cofactor (NADPH) to reduce the heme iron, but also highlights various areas of current practical commercial applications of CYPs as well.

1.8.2 Dioxygenases

Dioxygenases are enzymes which catalyze reactions involving incorporation of both atoms of molecular oxygen into the substrate molecule (Bugg 2003). Lipoxygenase (Lox), or Linoleate oxygen reductase are a class of dioxygenase (Nanda and Yadav 2003). In plants, the common substrate for Lox is linoleic acid, whereas in mammals the most common substrate is arachidonic acid, which generates the leukotriene family of physiological regulators through the intermediate formation of its hydroperoxides (Bugg 2003, Smith 1989).

1.8.2.1 Dioxygenase-Lox – Natural Occurrence

Lipoxygenases (Lox) or linoleate oxygen reductase (EC 1.13.11.12) have been widely studied since their discovery in 1932 by Andre and Hou (Andre and Hou 1932). Lox is comprised of a heterogenous family of lipid peroxidising, non-heme iron containing dioxygenases, which catalyze the addition of molecular oxygen to polyunsaturated fatty acids in a stereo- and regiospecific manner (Siedow 1991, Nanda and Yadav 2003). Lox are widely distributed in plants, animals and fungi (Brash 1999, Feussner and Wasternack 2002). Lox are comprised of a single polypeptide chain with a molecular mass of 75–80 kDa in animals and 94–104 kDa in plants (Brash 1999). A higher level of Lox activity is present in legume seeds *e.g*. soybean flour, which is useful as an improver for dough rheology during bread making and for bleaching the carotenoid pigment for the commercial preparation of white bread (Goesaert *et al*. 2005). This high level of expression led to the characterization of lipoxygenase from soybean (Liavonchanka and Feussner 2006). In the cotyledon of mature soybean seed Lox occurs in the form of six isozymes termed Lox 1 to Lox 6, with high levels of expression of Lox 1, 2 and 3 (Siedow 1991). The specific features for these isozymes are mentioned in the Table 1.6. The isozymes 4, 5 and 6 are expressed during early stages of germination when the Lox 1 activity diminishes (Kato *et al*.1992). All Lox-isoforms are composed of single polypeptides that are folded into a two-domain structure (Mei *et al*. 2008). Detailed structural and functional aspects of plant and mammalian Lox have been extensively reviewed (Yamamoto 1992, Boyington *et al*. 1993, Brash 1999, Mei *et al*. 2008)

Busquets *et al*. (2004) reported the isolation of Lox-like enzyme from *Pseudomonas aeruginosa* 42A2. Enzyme activity was highest with substrates having unsaturation at the $C^{\Delta 9}$ position, with linoleic acid being the preferred substrate. The reported enzyme was active under aerobic conditions and generated hydroperoxy octadecenoic acid, hydroxy octadecenoic acid and dihydroxy octadecenoic acid products (Busquets *et al*. 2004). Lox from *P.aeruginisa* 42A2 did not have a light absorption in the visible range, suggesting lack of a heme group, a feature similar to plant and animal Lox, however it contained 0.55 moles of ferrous iron per mole of protein (Busquets *et al* 2004).

Table1.6. Isoforms of lipoxygenase from soybean for the formation of hydroperoxy octadecenoic acid (HPOD).

Lox isozymes	**pH optimum**	**Product specificity**	**Substrate**
Soybean L1	9	13-HPOD	Charged fatty acid in non-esterified form [A]
Soybean L2	6.5	9 & 13- HPOD in 50:50 ratio	Neutral fatty acids at pH < 7 [A]
Soybean L3	Broad range centered around 7	9 & 13- HPOD in 50:50 ratio	Neutral fatty acids at pH < 7 [A]
Soybean L4	6.5	9 & 13- HPOD	C18:3 [B]
Soybean L5	6.5	9 & 13 –HPOD in 85:15 ratio	C18:3 [B]
Soybean L6	6.5	9 & 13 –HPOD in 85:15 ratio	C18:3 [B]

A: (Siedow 1991)

B: (Kato *et al*. 1992)

The products of Lox mediated oxygenation reactions are hydroperoxides of *S*-configuration. In mammals, Lox are classified into 5-, 8-, 12-, 15- Lox; according to the positional specificity of archidonic acid (C20:4 $^{\Delta 5,8,11,14}$) oxygenation (Yamamoto 1992). In plants, however, the Lox are classified as 9-, or 13-Lox, due

to the positional specificity of linoleic acid oxygenation at $C^{\Delta 9}$, or $C^{\Delta 13}$ of the carbon chain backbone (Gardner 1991, Liavonchanka and Feussner 2006). Polyunsaturated fatty acids are prone to auto-oxidation and can form oxylipins due to the presence of electron rich double bonds, however the product profile of enzymatically generated oxylipins and non-enzymatic oxylipins differs (Kuhn and Thiele 1999). As specificity of the product pattern occurs for the enzymatically derived products, the non-enzymatic auto-oxidation products exist as mixtures of optical and positional isomers (Kuhn and Thiele 1999).

1.8.2.2 Mechanism of enzyme reaction and substrates

Characteristics of Lox mediated oxygenation are shown in Figure 1.3. One of the hydrogen-atoms is stereospecifically removed from the central methylene group of the linoleic acid; concomitantly, molecular oxygen is inserted into the substrate (Nanda and Yadav 2003). Double bond re-arrangement takes place to give rise to a conjugated system of *cis*, *trans* pentadiene structures (Nanda and Yadav 2003). The products of fatty acid oxygenation by Lox are hydroperoxides of octadecenoic acid and are converted into aldehydes, ketones and alcohols by further enzymatic cleavages (Nanda and Yadav 2003).

Lox from animal origin forms leukotrienes from arachidonic acid. Lox from plant origin catalyses conversion of linoleic and linolenic acids to aroma compounds (aldehydes), plant modulators of gene expression (jasmonites), or as direct deterrents to pests and pathogens (divinyl ethers) (Smith 1989, Noordermeer *et al*. 2002). Lox enzymes are also associated with undesirable off flavours during storage and processing of foods (Vancanneyt *et al*. 2001). Lox enzymes are inactivated by their products, hydroperoxides, and the depletion of oxygen (Siedow 1991). Naturally occurring strong inhibitors of Lox are catechol-containing compounds, such as nordihydroguaiaretic acid, gossypol, and propyl gallate (Serpen and Gokmen 2006). Inhibition occurs due to reduction of enzyme bound Fe^{+3} to Fe^{+2} (Kemal *et al*. 1987). Iron chelators such as Zileuton also inhibit Lox (Serpen and Gokmen 2006). The oxygen scavengers such as dihydrolipoic acid, tetrapetalone and β-carotene are natural inhibitors of Lox activity (Serpen and Gokmen 2006).

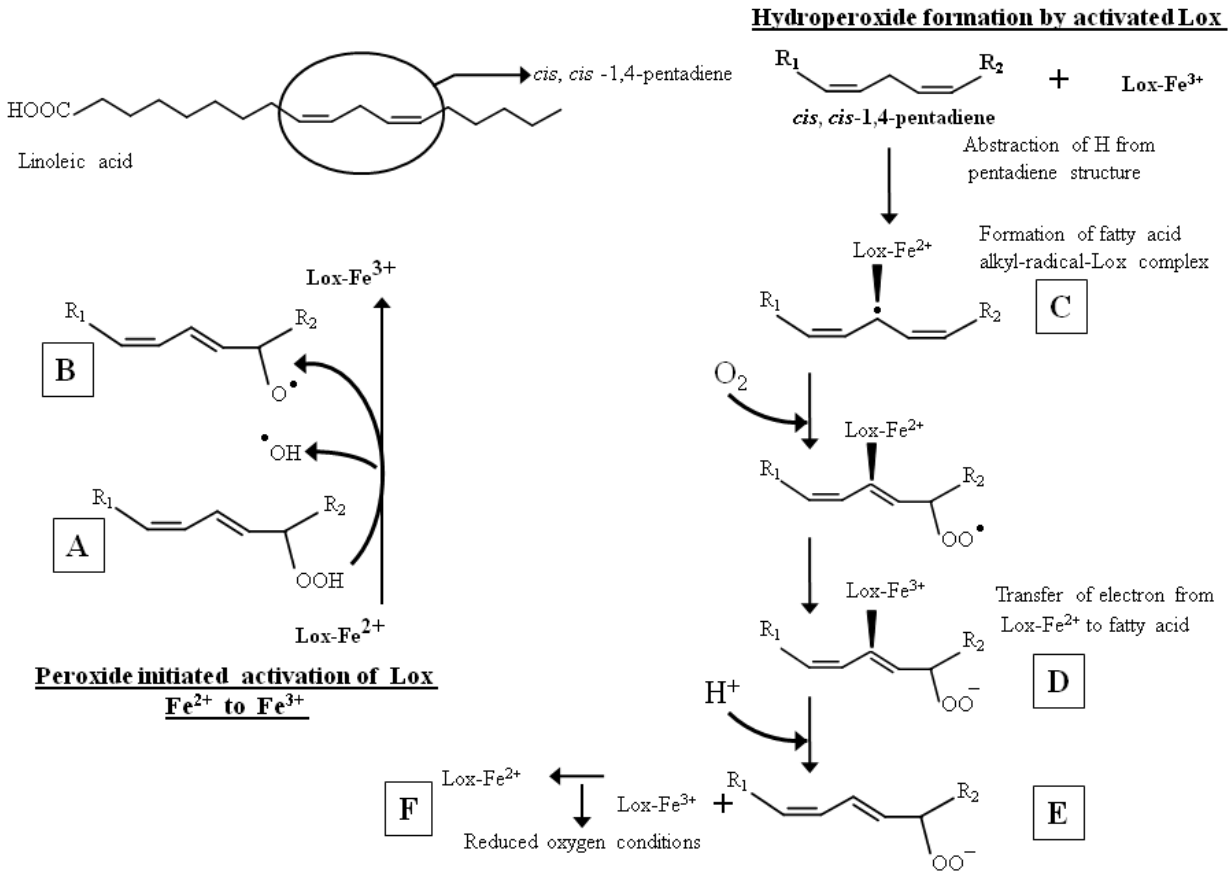

Figure 1.3. Mechanism of hydroperoxides formation by soybean Lox-1 with C18:2 as substrate. Lox-1 isoform primarily reacts with free fatty acids and produces 13-hydroperoxides from linoleic and linolenic acids. The inactive form of iron is Lox-ferrous (Fe^{2+}) [A]. Peroxide initiates the process of activation of Lox –ferrous to Lox-ferric (Fe^{3+}) [B]. Lox catalyzes the abstraction of H from methylene interrupted carbon to form fatty acid alkyl radical-Lox complex [C] and conversion of Lox-iron back to ferrous state. An electron from the ferrous iron is then donated to the peroxyl radical to form a peroxyl anion [D]. When the peroxyl anion reacts with hydrogen to form the hydroperoxides, the fatty acid is released from the enzyme [E]. Under oxygen depletion conditions, the enzyme abstracts hydrogen from the fatty acid and the iron is converted to ferrous [F]. Reaction mechanism adapted from Nawar (2004).

1.8.2.3 Immobilization of Lox

Immobilization of enzymes is achieved through the attachment of enzyme molecules to an inert and insoluble material or through the entrapment of the enzymes in a rigid matrix. Industrial biotransformation often employs immobilized enzymes to increase the stability of enzymes to withstand longer periods of storage and to allow the reusability. Many immobilized enzymes exhibit increased stability towards a range of pH, pressure and temperature conditions (Morales Borges *et al.* 2009). The utilization of immobilization techniques may also improve enzyme properties (Alduri *et al*. 1995). Immobilization of enzymes on porous supports helps prevent aggregation, and proteolysis (Alduri *et al*. 1995). The immobilization methods used for Lox include adsorption methods including attachment to Mg silicate based support talc (Battu *et al.* 1994), controlled pore glass (Santano *et al.* 2002), glutenin, oxarine acrylic beads (Pinto *et al.* 1997); entrapment in polyacrylamide gel (Piazza *et al.* 1994); and covalent attachment to carbonyl diimmidazole and oxarine group bearing commercial supports, such as Eupergit C (Chikere *et al.* 2001). Immobilization of Lox is thought to protect the iron atom and thus stabilises Lox in the presence of inhibitors, e.g. nordihydroguaiaretic acid (Pinto *et al*. 1997). Table 1.7 summarizes immobilization supports used for Lox and their stability towards aqueous, organic solvent, and supercritical carbon dioxide reaction media. The products of reaction in all these cases are hydroperoxides of linoleic acid, which therefore calls for a need to develop a system for the production of coriolic acid from immobilized Lox.

1.9 Concluding remarks

Microbial transformation of UFAs introduces hydroxyl groups in a stereospecific and regiospecific manner. Different bacteria introduce these functionalities in specific ways with little or no by-products, and give rise to a diverse range of hydroxyl species depending on the enzyme system. In contrast, chemical processes, generally suggested as simple and cost effective, lack the specificity and result in an array of isomeric compounds requiring an extensive purification process. Formation of microbial HFAs by *Pseudomonas aeruginosa,*

Table 1.7. Immobilization of Lox on different supports to produce hydroperoxy octadecenoic acid (in literature hydroxy not reported).

Support	Reaction medium	Stability	Initial concen-tration C18:2	References
Adsorption				
Talc	Aqueous 0.05 M Na phosphate buffer	Stability of immobilized enzyme	66 μM	(Battu *et al*. 1994)
Covalent bonding				
Carbonyl diimmidazole	Aqueous and organic	7 reaction cycles		(Parradiaz *et al*. 1993)
Oxarine acrylic beads	Aqueous	9 reaction cycles	0.66 mM	(Pinto *et al*. 1997)
Eupergit C	Aqueous	7 cycles	10 mM	(Chikere *et al*. 2001)
PBA-Eupergit 250L	Supercritical CO_2	Batch reactor	100 mM	(Chikere *et al*. 2000)
Eupergit C-250L	Organic solvent media	Thermo stability	4 mM	(Vega *et al*. 2005)
Eupergit C	Aqueous media with cyclodextrins		11.6 μM	(Perez-Gilabert and Garcia-Carmona 2005)
Ionic bonding				
Dowex 50W-X4-200	Ternary micellar medium	4 cycles		(Kermasha *et al*. 2002)
Entrapment				
Alginate silicate sol gel matrix	Aqueous with 100 mM deoxycholate	5 cycles	5 μM	(Hsu *et al*. 1997)
Silica gel	Aqueous	7 cycles of decreasing activity due to loss of gel	0.3 mM	(Karout *et al*. 2007)
Phyllosilicates crosslinked with TMOS	Aqueous	Storage stability and reusability	5 μM	(Shen *et al*. 1998, Hsu *et al*. 1998)
Polyacrylamide gel derivatized by glutareldhyde	Aqueous 50 mM borate buffer pH 9 at 20°C	Tubular-Bio-reactor continuous	0.035 mM	(Pinto and Macias 1996)

TMOS: Tetramethoxy orthosilicate

Bacillus megaterium, and *Rhodococccus* sp. has been extensively studied while few data are available on transformation by lactic acid bacteria. Studies of lactic fermentations emphasised the formation of conjugated linoleic acid. For the formation of HFAs from soybean Lox, a number of articles have discussed the diversity of Lox-derived products formed in biological systems (Blee 1993, Feussner and Wasternack 2002). However, the use of isolated Lox in various reaction media comprised of aqueous or organic solvents for biotechnological applications, especially coriolic acid production, is not well investigated. Furthermore, the literature about Lox generated coriolic acid for chemoenzymatic synthesis, lacks information about coriolic acid production using the immobilized enzyme in a single step process. Therefore a study was proposed to analyse the indicated gaps in previous literature for the formation of HFAs.

1.10 Hypothesis and objectives

This thesis aimed to test the hypothesis that specific hydroxy fatty acids can be produced by bio-transformation of unsaturated fatty acids and product yield can be enhanced to an industrial scale by changing the reaction parameters.

The first study dealt with microbial transformation of UFAs that leads to hydroxylation of the substrate. The first objective was to screen the bacterial species for their ability to transform UFAs. The second objective was to increase the product turnover, and identify the mechanism of this reaction.

The second study tested the hypothesis that purified lipoxygenase can be used in an immobilized form for the formation of coriolic acid from linoleic acid in a single step process. The study had two objectives, (i) the optimization of reaction conditions for the single step formation of coriolic acid, particularly by increasing the substrate concentration and (ii) the conversion with immobilized lipoxygenase. Alternate reducing agents were employed for reduction of hydroperoxides formed by Lox, and the yield of coriolic acid was compared with the two step process.

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Chapter 2

Microbial Transformation of Unsaturated Fatty Acids to Hydroxy Fatty Acids

2.1 Introduction

Various microbial enzymes hydroxylate fatty acids. The activities of lipoxygenase (Lox)-like enzymes or hydratase have been used for the transformation of UFAs to HFAs (Hou 1995a, Yamada *et al* 1996, Park *et al* 1999). Microbial transformation of unsaturated C-18 fatty acids to HFAs has been studied widely from bacterial species such as *Pseudomonas aeruginosa*, *Flavobacterium* sp., *Nocardia cholesterolicum*, and *Bacillus* sp. (Kuo *et al*. 1998, Hou 1995a, b, Hudson *et al*. 1998, Lanser 1993, Lanser *et al*. 1992). Bacterial Lox-like enzymes require the presence of molecular oxygen and metal ions for the introduction of hydroperoxy groups into unsaturated substrates (Busquets *et al*. 2004). As a result, aeration of the system was found to be the most critical step for the formation of 7,10-dihydroxy-8(*E*)-octadecenoic acid by *Pseudomonas* sp. 42A2 (Deandres *et al*. 1994). A certain optimal level of oxygen was found necessary for 7,10-dihydroxy-8(*E*)-octadecenoic acid formation (Deandres *et al*. 1994). The role of oxygen in the formation of poly-oxygenated hydroxy fatty acids was studied, and the formation of 12,13,17-trihydroxy-9(*Z*)-octadecenoic acid from the biotransformation of linoleic acid by *Clavibacter* sp. ALA2 occurred under aerobic conditions (Hou *et al*. 1998). Additionally, differences in the yield of HFAs from live bacterial culture and washed cells have been studied (Hou and Bagby 1992). A seventy percent higher yield of 7,10-dihydroxy-8(*E*)-octadecenoic acid from washed cells has been reported by Hou and Bagby (1992) in contrast to its yield from live bacterial culture (Deandres *et al*. 1994). Since Lox enzymes are activated by the conversion of Fe^{2+} to Fe^{3+}, the effect of the presence of metal ions on biotransformation of oleic and linoleic acid by *P. aeruginosa* PR3 was studied by Tsung and Nakamura (2004) who reported that addition of Mn^{2+} to the oleic acid transformation medium enhanced the yield of 7,10-dihydroxy-8(*E*)-octadecenoic acid from 63% to 89%. Another study revealed that linoleic acid transformation by *P. aeruginosa* PR3 required a certain

threshold level of Cu^{2+} and Fe^{2+} for the formation of 9,10,13-trihydroxy-11(*E*)-octadecenoic acid and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid (Kim *et al*. 2002). In all the above mentioned examples, a Lox-like oxygenation pattern has been observed for different strains of *P. aeruginosa* in UFA transformations (Hou 2005, Kuo *et al*. 2001, 1998). The characteristic feature of Lox-like oxygenations is that they require oxygen and transition metal ions as cofactors in contrast to hydratase based oxygenations.

Hydratase enzymes introduce hydroxyl functionality to the double bonds of unsaturated fatty acid alkyl chains without a requirement for molecular oxygen (Volkov *et al*. 2010). The difference between Lox-like and hydratase mediated conversions not only lies in different media requirements, but is also reflected in the structure of the final hydroxy product. Oleic acid transformation in a bacterial Lox-like system, as described above, introduces two hydroxyl groups while retaining the double bond of the parent compound. In contrast, oleic acid transformation by a hydratase introduces the hydroxyl group at the expense of the double bond (Wanikawa *et al*. 2001, 2002). Biotransformation of linoleic acid from growing cells of *Stenotrophomonas nitritireducens* to 10-hydroxy-9(*Z*)-octadecenoic acid has been reported (Yu *et al*. 2008a). For this system, 90% conversion of linoleic acid to 10-hydroxy-9(*Z*)-octadecenoic acid was observed from washed cells under anaerobic conditions (Yu *et al*. 2008b). Bioconversion was observed by cells of *S. nitritireducens* harvested in the stationary phase at a glucose deprivation stage, and maximal product yield in anaerobic conditions indicated the involvement of hydratase enzyme (Yu *et al*. 2008a).

Microaerobic conditions (containing less than 1% oxygen) have been described as favourable for the formation of hydroxy fatty acids by lactic acid bacteria (LAB), but the enzymes responsible for this conversion have not been fully characterized. Most of the hydroxy fatty acids originating from linoleic acid transformation using washed cells of LAB have been reported as intermediates during the formation of conjugated linoleic acid. However, Ogawa *et al* (2001) identified HFA formation as final end products as well.

Different reaction media have been used for the formation of UFAs by LAB including skim milk, yeast extract, glucose, phosphate buffer, and bovine serum albumin , where bovine serum albumin was used as a surfactant for solubilizing fatty acid in the aqueous medium (Ogawa *et al*. 2001, Kishino *et al*. 2002, Kishimoto *et al*. 2003). Yields of hydroxylated products ranged from 0.4% (10,13-dihydroxystearic acid product originating from linoleic acid transformation) to 70% (10-hydroxystearic acid from oleic acid), after seven days of incubation period (Kishimoto *et al*. 2003, Wanikawa *et al*. 2001, 2002). While there have been many detailed reports on a wide variety of strains including *Pseudomonas aeruginosa* and *Bacillus* sp., there is substantially less information available for the optimization of hydroxy fatty acid production and yield from LAB.

In the present study, *P. aeruginosa* strains originating from environmental sources and LAB strains originating from different food fermentations were selected to evaluate their potential for transformation of UFAs. The strains were selected such that there was representation of the metabolic diversity of LAB. For example, *L. sakei* is the most prevalent type of LAB in various types of fermented sausages, *L. plantarum* is involved in vegetable fermentations, *L. sanfranciscensis* and *L. reuteri* are associated with sourdough fermentations, and *B. bifidum*, has been mentioned with regards to its probiotic effects (Hammes 1990). It was therefore, the aim of the study to analyze the formation of HFAs and calculate product yields under optimal bacterial growth conditions, characterize the pathway leading to this transformation, and develop methods to enhance the formation of hydroxy fatty acids of potential biological and synthetic applications.

2.2 Materials and Methods

2.2.1 Chemicals

Oleic (>95%), linoleic (95%), linolenic acid (95%), methyl heptadecanoic acid (98%), 12-hydroxystearic acid (>98%) were purchased from Sigma-Aldrich, (St. Louis, MO, USA). Reagent grade chloroform, ethyl acetate, methanol,

Tween 80, N, O-bis-(trimethyl silyl)-acetamide (BSA) were from Fisher scientific (Fair Lawn, NJ, USA).

2.2.2 Media and culture conditions

Bacterial strains used for this study are shown in Table 2.1. *P. aeruginosa* strains were maintained on Lauria Bertani (LB) agar plates at 30 °C. Overnight bacterial cultures were sub-cultured with a 1% inoculum and incubated in LB medium containing 1 g/L (4 mmol/L) individual oleic acid (LB-oleic) and with 1 g/L (4 mmol/L) linoleic acid (LB-Linoleic acid), unless otherwise mentioned.

Table 2.1. Microorganisms used for the transformation study of UFAs.

Microorganism	**Growth temperature**
P. aeruginosa MF 2	30 °C
P. aeruginosa MF 15	30 °C
P. aeruginosa MF 19	30 °C
P. aeruginosa MF 20	30 °C
P. aeruginosa MF29	30 °C
P. aeruginosa MF 30	30 °C
P. aeruginosa MF 18G	30 °C
P. aeruginosa MF NG2D	30 °C
L. plantarum TMW 1.460	28 °C
L. plantarum TMW 1.701	28 °C
L. sanfranciscensis DSM 20451	28 °C
L. reuteri LTH 2584	37 °C
L. sakei LTH 677	28 °C
B. bifidum BB12	28 °C

Lactic acid bacteria were maintained on modified MRS (mMRS) agar plates (Jänsch *et al*. 2007) at their optimal growth temperatures as shown in Table 2.1. Overnight bacterial cultures were sub-cultured with a 1% inoculum and

incubated in 15 mL Sarstedt culture tubes with 5 mL mMRS medium containing 1 g/L (4 mmol/L) individual oleic acid (MRS-oleic acid), 1 g/L (4 mmol/L) linoleic acid and 1 g/L (4 mmol/L) linolenic acid (MRS-linoleic acid) and (MRS-linolenic acid) respectively. The cultures were shaken in shaker incubators with a speed of 100 rpm at their respective optimum growth temperatures.

2.2.3 Transformation of fatty acids by *P. aeruginosa*

Eight different strains of *Pseudomonas aeruginosa* were grown in LB medium under aerobic conditions using 1% overnight culture as inoculum in 500 mL Erlenmeyer flasks, containing 250 mL of LB medium. After 36 h of incubation, bacterial cells were centrifuged at 10,000 rpm and growth medium was discarded. Cells were washed with 250 mL of 50 mM potassium phosphate buffer (pH 6) twice, and resuspended in the same buffer to OD_{600} of 2.0. Linoleic acid and oleic acid at a concentration of 1 g/L were individually tested as substrates and added to the washed cells. Sterile controls as well as strains incubated without added substrate served as controls.

Overnight bacterial cultures from 1% inoculum were exposed to oleic and linoleic acid in LB-oleic medium and LB-linoleic medium, to observe any changes in the product profile of growing cells versus washed cells.

2.2.4 Transformation of fatty acids by LAB

Bacterial fermentations were carried out in MRS-oleic acid, MRS-Linoleic acid, and MRS-Linolenic acid media for 4 days (15 mL Sarstedt culture tubes containing 5 mL medium). Sterile media in the presence of substrates as well as strains incubated without added substrate served as controls. At the end of fermentation, pH was adjusted to 2 with 1N HCl for complete extraction. Methyl heptadecanoic acid was added as internal standard and culture was extracted three times with equal amount (5 mL) of chloroform containing 15% methanol. Solvent extracts were dried under nitrogen and stored at -20°C for analysis by GC-FID and GC-MS. This method of extraction will be used for all the later experimentation mentioned below.

2.2.5 Kinetics of transformation of linoleic acid by LAB

Three strains of lactic acid bacteria, *L. plantarum* TMW 1.701, *L. plantarum* TMW 1.460, *L. sanfranciscensis* DSM 20451 were used for studying linoleic acid transformations daily for 4 days. Bacterial cells were grown in 20 mL MRS-linoleic medium. Optical density at 600 $_{nm}$ and pH were recorded at 0, 1, 2, 3, and 4 days for each culture. Aliquots were taken from the cultures at 24 h intervals and cell counts were determined by plate count methods.

2.2.6 Effect of oxygen on transformation of linoleic acid by LAB

Effect of oxygen on transformation efficiency of linoleic acid was studied using *L. plantarum* TMW 1.701 and *L. sanfranciscensis* DSM 20451. Under aerobic conditions MRS-linoleic medium was inoculated with bacterial culture in sterilized 125 mL Erlenmeyer flasks. Culture was incubated at 30 °C at 150 rpm for 4 days. For incubation under anaerobic conditions, all the culture handling and fatty acid transfers were carried out in Sarstedt culture tubes with screw cap lids in an Air Lock anaerobic chamber (Coy Laboratory Products Inc. Grass Lake, MI, USA). Fermentation was allowed to proceed for 4 days at 30 °C with shaker speed of 150 rpm. At the end of incubation period, reaction mixture was extracted as mentioned above.

2.2.7 Transformation of linoleic acid by bacterial crude cell wall preparation

Bacterial cells from *L. plantarum* TMW 1.701, *L. plantarum* TMW 1.460, *L. sanfranciscensis* DSM 20451 were grown in 900 mL mMRS medium. Cells were harvested by centrifugation at 10,000 *g* for 20 min at 0 °C and washed twice with 100 mM potassium phosphate buffer at pH 5.8. The cells were finally suspended in 100 mL of the same buffer and passed through M-110S Micro fluidizer (Biocompare, South San Francisco, CA) for disrupting the bacterial cell. Crude cell extract was centrifuged at 5000 *g* for 30 min to separate the cell wall and clear supernatant containing cytoplasmic proteins. Cell debris was resuspended in 20 mL of 100 mM potassium phosphate buffer pH 5.8 before the addition of 1 g/L linoleic acid for 24 h and 96 h incubation periods, at 30 °C in an incubator shaker with a shaking speed of 150 rpm. Linoleic acid in washing buffer under identical conditions was used as a control for this experiment. The

reaction was quenched by the addition of 1N HCl to bring the pH to 2 after 24 h and after 96 h before extraction according to the procedure mentioned above.

2.2.8 Effect of oxygen on transformation of linoleic acid by crude cell lysate of LAB

The effect of oxygen on transformation efficiency of linoleic acid by crude cell extract was studied using *L. plantarum* TMW1.701 and *L. sanfranciscensis* DSM 20451. Under aerobic conditions 20 mL of crude cell extract was poured in sterilized 125 mL Erlenmeyer flask, 1 g/L (4 mmol/L) linoleic acid was introduced into the reaction mixture and flasks were covered with aluminum foil on the bench. Reaction mixture was incubated at 30 °C at 150 rpm for 24 h. For reaction under anaerobic conditions, all the crude cell wall extract and fatty acid transfers were carried out in an anaerobic chamber and 20 mL of lysate was poured in 50 mL Sarstedt culture tubes with screw cap lids. Transformation was allowed to proceed for 24 h at 30 °C with shaker speed of 150 rpm. At the end of incubation period, reaction mixture was extracted according to the method mentioned above.

2.2.9 Effect of substrate concentration on transformation of linoleic acid by lysed bacterial cells

To develop an insight towards the mechanism of linoleic acid transformation and identify the primary product of reaction, a constant amount bacterial crude cell lysate containing 54 g/L proteins, was incubated with varying amounts of initial linoleic acid starting from 1, 4, 5, 10, 21, 40 mmol/L for 24 h. Reaction products from various concentrations were extracted and analyzed as mentioned above.

2.2.10 Derivatization and analysis by GC-MS

Concentrated bacterial extracts were methylated using diazomethane (DAM) for 30 min and silylated using N, O-bis-(trimethyl silyl) acetamide (BSA) as described by Nicolaides *et al*. (1983). Diazomethane was prepared in the lab according to Aldrich technical bulletin AL-180 using Diazald ® distillation kit purchased from Aldrich (St Louis, MO, USA). A hydroxy fatty acid standard, 12-hydroxystearic acid under similar derivatizing conditions was run with the

bacterial samples as an external standard for retention time and mass spectral analyses.

Gas chromatography–Mass Spectrometry (GC-MS)

In order to identify the fatty acids produced in response to bacterial transformation of oleic, linoleic and linolenic acid, total ion current chromatograms (TIC) and mass spectra of derivatized bacterial extracts were generated. For this purpose, GC-MS analyses were performed on a model 5975B EI/CI GC-MS, with a 7683B series injector and auto sampler (Agilent Technologies Inc., Santa Clara, CA). The inlet heater was set at 300 °C, pressure at 46.5 KPa, and detector at 250 °C. The total flow was set at 104 mL/min with a split ratio of 100:1. Analyses were carried out using a standard DB1 column (Agilent Technologies Inc., Santa Clara, CA) with column temperature held for 0.1 min at 90 °C and increased at the rate of 10 °C /min to 220 °C. Temperature was held at 220 °C for 8 min and then increased to 290 °C at the rate of 10 °C/min and held at 290 °C for 1.9 min. Total run time was 30 min. Electron impact ionization (EI potential 70 eV) was used and mass range between 40- 600 Dalton was scanned. Data were analyzed using Enhanced MSD ChemStation ™ D.03.00.611 soft ware (Agilent Technologies Inc., Santa Clara, CA). For GC-MS analysis under chemical ionization mode (CI-GC-MS), similar column and run programme was followed while using ammonia as ionizing gas. CI-GC-MS analyses were performed at the Department of Chemistry, University of Alberta.

2.2.11 Gas chromatography – Flame ionization detector (GC-FID)

Semi-quantification of fatty acids and products of fermentation were done by GC-FID. The derivatizing agents add extra C-H bonds to the product HFAs, therefore, the theoretical FID response was corrected and calculations were made relative to internal standard of known concentration based on moles of carbon. A Varian 3400 Gas chromatograph equipped with a Varian 8200 auto sampler (Varian Inc., Palo Alto, CA) coupled with an FID was operated at 240 °C. Operating head pressure was 25 psi. Initial injector temperature was 60 °C, and it ramped up to 230 °C at the rate of 150 °C/min and held for 28 min. Samples were injected on to a BP 20 column (column length was 30 m, 0.25 mm internal

diameter, and 0.25 μm film thickness) from Fisher scientific (Fair Lawn, NJ, USA).. Column programme started from 50 °C, held for 0.2 min and reached up to 170 °C at the rate of 20 °C/min, held for 5 min and ramped up to 230 °C at the rate of 10 °C/min and held for 13 min.

For the analysis of conjugated linoleic acid formation during the biotransformation studies, the procedure of Kramer *et al*. was followed with a SP-2560 column (Column dimensions: 100 m x 0.20 μm x 0.25 mm) (Kramer *et al*. 2008).

2.3 Results

2.3.1 Transformation of fatty acids by *P. aeruginosa*

Incubation of oleic acid and linoleic acid with bacterial culture in LB medium and with washed bacterial cells in phosphate buffer was studied to identify the potential for *P. aeruginosa* to transform these UFAs. GC-MS analysis of strains MF30 and 18G, showed the presence of 10-hydroxystearic acid (10-HSA) in response to oleic acid addition from washed cells of *P. aeruginosa* (Figure 2.1).

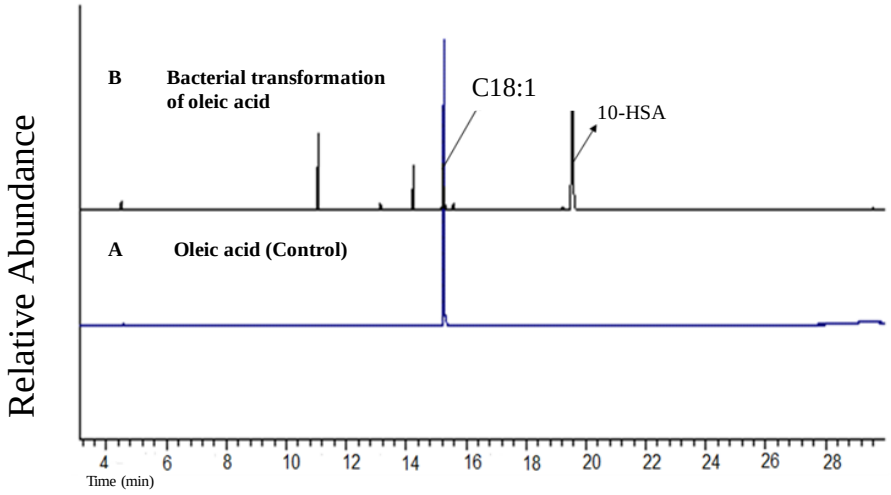
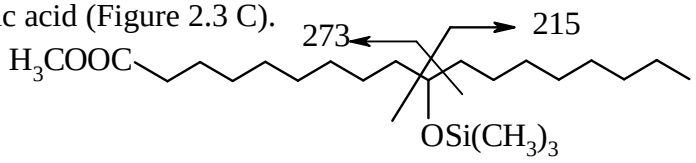


Figure 2.1. Total ion current chromatograms by GC-MS of diazomethane-BSA derivatised fatty acids and hydroxy fatty acids produced by washed cells of *P. aeruginosa* MF30 due to biotransformation of oleic acid (C18:1). Oleic acid in the absence of bacteria (control A), and washed bacterial cells in the presence of oleic acid (B). Peaks for oleic acid and 10-hydroxystearic acid (10-HSA) are indicated.

The mass spectra indicating 10-HSA formation in Figure 2.2 under identical derivatizing conditions have been reported previously (Kim *et al*. 2003, Wanikawa *et al*. 2000, Volkov *et al*. 2010). The expected molecular ion with *m/z* 386 was not observed. However, fragment ions with *m/z* 273 and *m/z* 215 showed cleavage at 10-hydroxyl position towards the ester and methyl end of the fatty acid respectively.

Analysis of culture supernatants from washed bacterial cells with linoleic acid did not show any novel hydroxylated products (Figure 2.3 A). Linoleic acid remained untransformed in both the live bacterial cultures and in washed cell extracts. Peaks arising from general bacterial metabolic activity were present in the control (Figure 2.3 B) and were identical to the bacterial washed cell preparation without linoleic acid (Figure 2.3 C).



TMS derivative of 10-hydroxystearic acid methyl ester

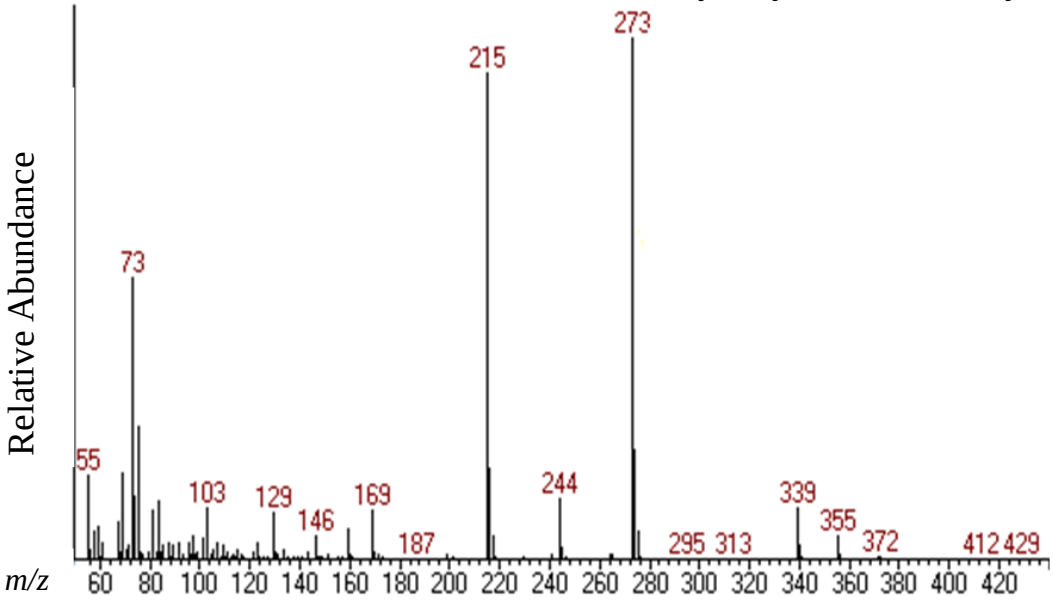


Figure 2.2. Mass spectrum of 10-hydroxystearic acid (10-HSA peak at 19 min retention time in Figure 2.1B). 10-HSA is formed due to the biotransformation of oleic acid by *P. aeruginosa* MF30. Figure is representative of *P. aeruginosa* MF30, 18G and all the LAB strains used in this study.

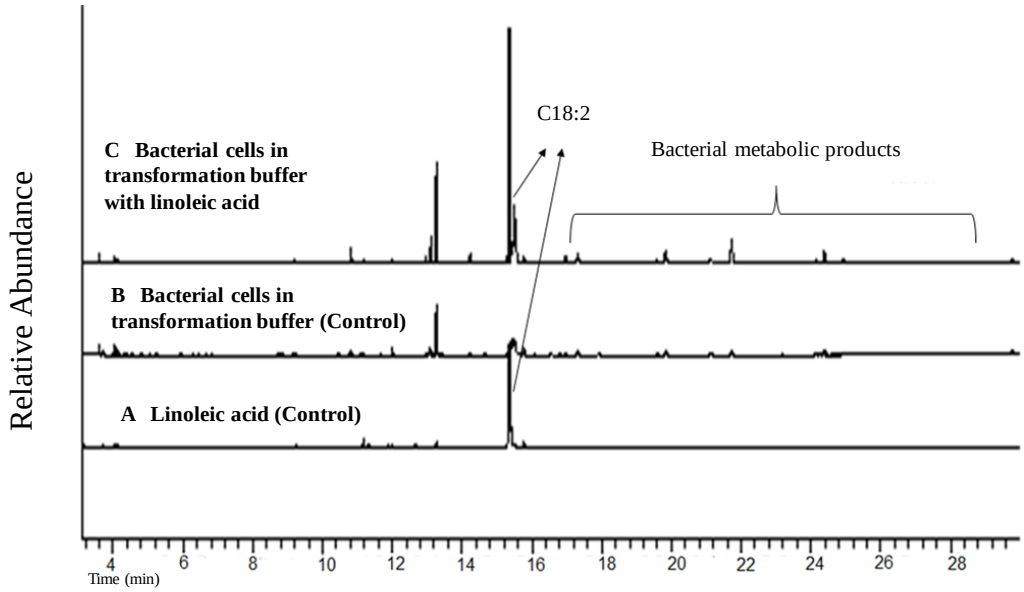


Figure 2.3. Total ion current chromatograms from GC-MS of diazomethane-BSA derivatised fatty acids and bacterial metabolic products produced by washed cells of *P. aeruginosa* MF 30 during incubation in the presence of linoleic acid. Figure shows linoleic acid in the absence of bacterial cells (control A), washed bacterial cells in the absence of linoleic acid (control B), and linoleic acid in the presence of washed bacterial cells (C). Peaks for linoleic acid, and for bacterial metabolites are labelled collectively. No specific hydroxy fatty acids were identified during this reaction.

It was concluded from the bio-transformation studies of *P. aeruginosa* strains that 10-HSA formation occurs in respose to oleic acid addition from two strains of *P. aeruginosa* (MF30 and 18G). However no hydroxy fatty acid was observed in response to linoleic acid addition.

2.3.2 Transformation of fatty acids by lactic acid bacteria

Transformation of unsaturated fatty acids to hydroxy fatty acids was observed by all the strains used in this study. Figure 2.4 and 2.5 illustrate the transformation of oleic, linoleic and linolenic acid (Peaks A, B and C) by *L.*

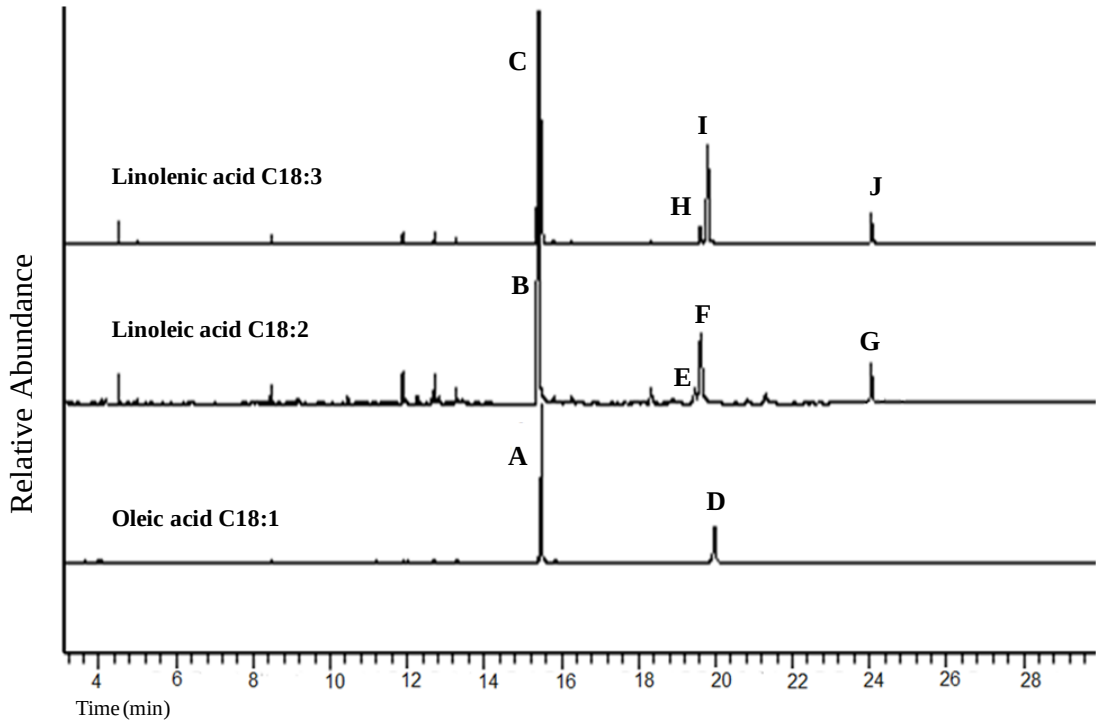


Figure 2.4. Total ion current chromatograms from GC-MS of diazomethane-BSA derivatised fatty acids and hydroxy fatty acids produced by *L. sanfranciscensis* DSM 20451 during growth in the presence of oleic (A), linoleic (B), and linolenic (C) acid. Peaks for 10-HSA (D), mono- and di-hydroxy products from linoleic and linolenic acid are indicated as E, F, G, H, I and J respectively.

sanfranciscensis DSM 20451 and *L. plantarum* TMW 1.701, respectively, as detected through total ion current chromatograms from GC-MS. Structural analysis of the fragmentation pattern of **peak D (**Figure 2.4 and Figure 2.5) shows it to be 10-HSA with an exact match to mass spectra cited in literature under similar derivatizing conditions (Wanikawa *et al* 2000). GC- MS (EI) *m/z* for the major product D were, 73 (38), 215 (90), 273 (100).

Reaction products labelled as E, F and G in Figure 2.4 arise due to transformation of linoleic acid by lactic acid bacteria. Mass spectra of the trimethyl silylated product derivatives did not show the expected molecular ions of *m/z* 384 (Figure 2.6). The fragment ion at *m/z* 73 was a base peak for **product**

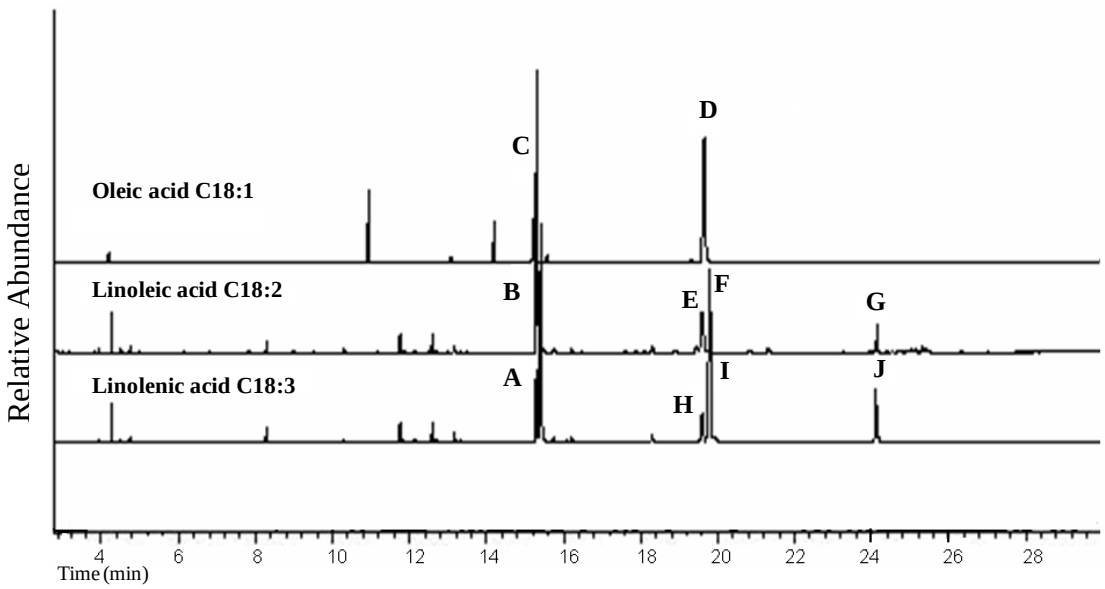


Figure 2.5. Overlay of total ion current chromatograms from GC-MS. Separation of diazomethane-BSA derivatised fatty acids and hydroxy fatty acids produced by *L. plantarum* TMW 1.701 during growth in the presence of oleic (A), linoleic (B), and linolenic (C) acid. Peaks for 10-HSA (D), mono- or di-hydroxy products from linoleic and linolenic acid are indicated as E,F, G, H, I and J respectively.

E while fragment ions with *m/z* 173 and *m/z* 313 indicated cleavage of derivatized hydroxyl moiety at position 13 towards methyl and ester end of the fatty acid respectively (Figure 2.6). The position of double bond could be any where from the 13-hydroxyl group towards the ester end. Hence based on the fragmentation pattern and previous literature **product E** can be referred as **13-hydroxy-9-octadecenoic acid**.

Product F shows a $[M]^+$ ion, *m/z* 384 (Figure 2.6). The fragment ion *m/z* 273 was a base peak and represented a cleavage alpha to 10-hydroxyl group while fragment ion *m/z* 73 accounts for the abstraction of $Si(CH_3)_3$ group. Hence based on the fragmentation pattern and previous literature (Volkov *et al*. 2010) **product F** was identified as **10-hydroxy-12-octadecenoic acid**.

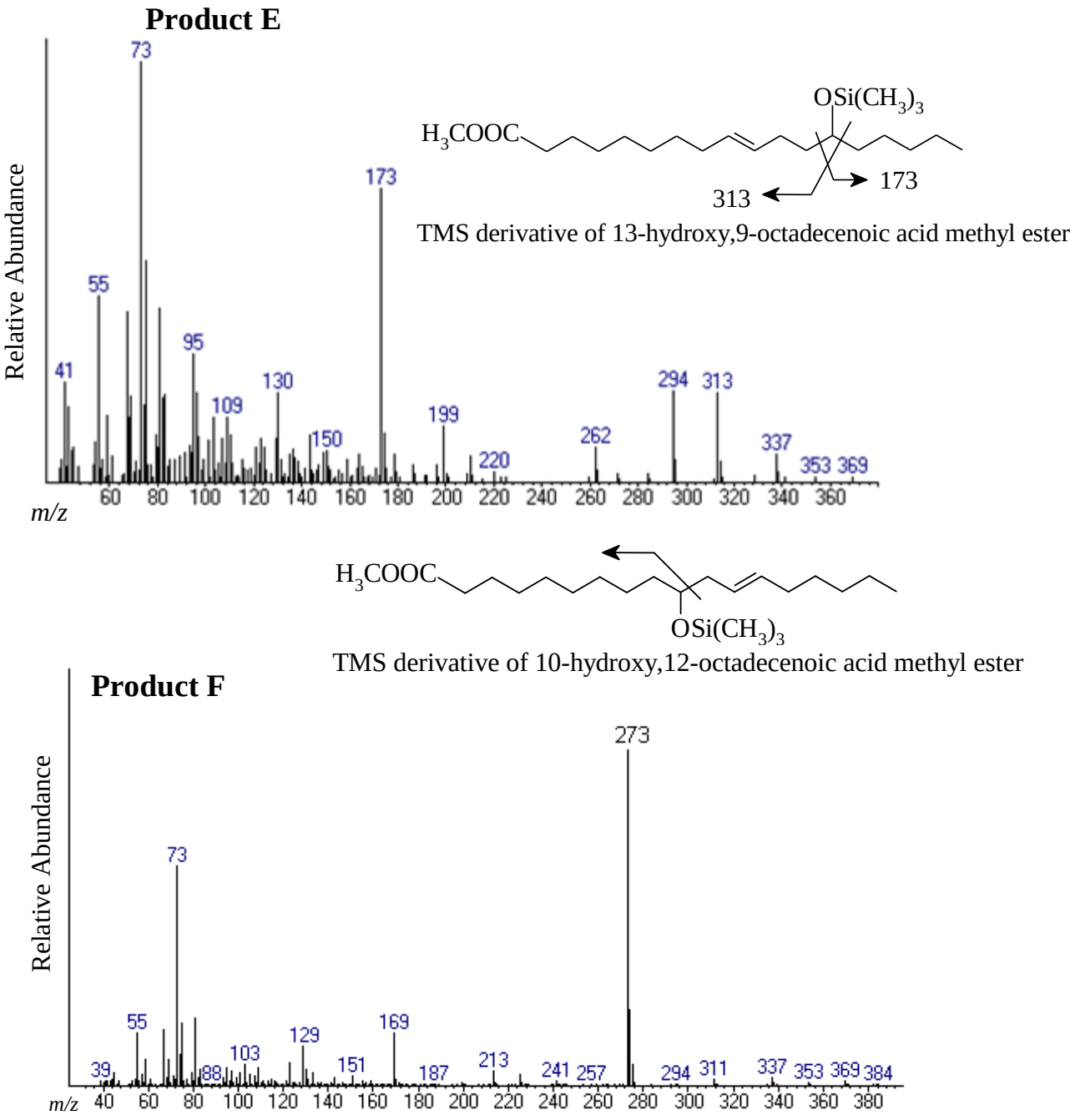


Figure 2.6. Analysis by GC-MS of diazomethane-BSA derivatised hydroxy fatty acids from a biotransformation reaction of *L. sanfranciscensis* DSM 20451 in the presence of linoleic acid. Mass spectra of the peaks at 19.3 min (product E) and 19.5 min (product F) with structural information are presented. Figure is representative of all the LAB strains used in this study.

Product G appears as single peak at 23.9 min. From the fragmentation pattern according to NISTO5a library source (2005) reference No. 182470, and previously cited literature, product G was identifird as 10,13-dihydroxy octadecenoic acid with molecular weight of 474 g/mol (Volkov *et al*. 2010). Analysis of Product G under chemical ionization mode of GC-MS with ammonia as ionizing gas gave a pseudo molecular ion of *m/z* 475 [M+H^+]. In light of the data generated by EI-GCMS, CI-GCMS and previous citations, product G was identified as **10,13-dihydroxystearic acid** (Volkov *et al*. 2010).

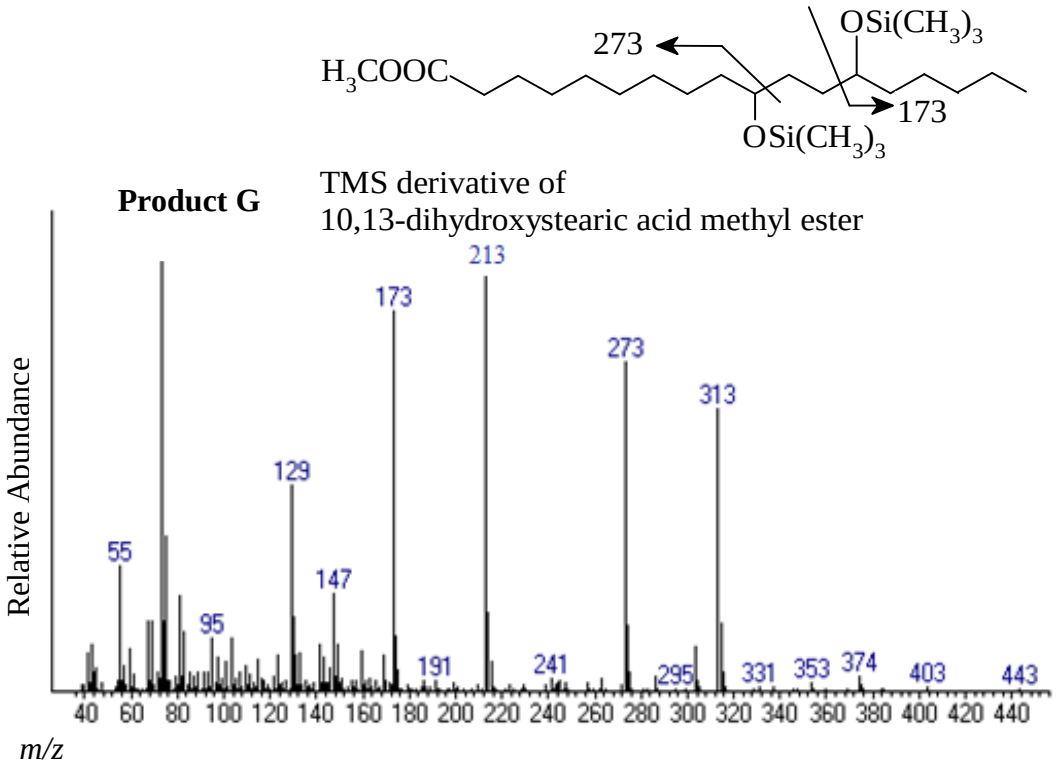


Figure 2.7. Analysis by GC-MS of diazomethane-BSA derivatised hydroxy fatty acids from a biotransformation reaction of *L. sanfranciscensis* DSM 20451 in the presence of linoleic acid. Mass spectra of the peak at 23.9 min (product G) with structural information are presented. Figure is representative of all the LAB strains used in this study.

Fermentation of linolenic acid resulted in the formation of reaction products H, I and J (Figure 2.4 and 2.5). Data generated by GC-MS exhibited mass spectra for all these products to be identical to the products formed during

fermentation with linoleic acid. Hence products H and I were identified as **13-hydroxy-9-octadecenoic acid** and **10-hydroxy-12-octadecenoic acid** respectively. Product J was identified as **10,13-dihydroxystearic acid**. GC-MS (EI) *m/z* for product E were, 73 (100), 173 (70), 294 (20), 313 (20), 337 (7). GC-MS (EI) *m/z* for product F were 73 (58), 129 (10), 169 (17), 273 (100) and GC-MS (EI) *m/z* for product G were 73 (100), 129 (41), 173 (74), 213 (92), 313 (64), 374 (4). Aanalysis of reaction products according to the method of Kramer et al (2008) did not indicate the formation of conjugated linoleic acid during this bio-transformation reaction.

2.3.3 Kinetic study of the transformation of linoleic acid

Growth of *L. sanfranciscensis* DSM 20451 and *L. plantarum* TMW 1.701 was followed for a period of four days in the presence of 4 mmol/L of linoleic acid under optimal growth conditions. Bacterial growth in the presence of the fatty acids followed the normal growth pattern and pH dropped from 6.5 to 3.4 (Figure 2.8 and 2.9). Transformation products started to appear during the stationary phase and showed an increasing trend in the amount of HFA formation by the 4^{th} day of fermentation. During the first 24 h 75% of the added substrate disappeared, however only 10% transformed into mono and di-hydroxy products. Twenty five percent of the initial substrate remained untransformed at the conclusion of the incubation.

2.3.4 Effect of oxygen on transformation of linoleic acid by LAB

In order to develop a relation between oxygen requirement and formation of oxygenated HFAs, bacterial transformation was performed by *L. plantarum* TMW 1.701 under aerobic and anaerobic conditions. From the chromatogram in Figure 2.10, it is likely that under anaerobic environment isomers of monohydroxy octadecenoic acid along with a dihydroxystearic acid were formed. Whereas, culture propagation under aerobic conditions generated auto-oxidation products of linoleic acid including two additional monohydroxy octadecenoic acid isomers. These isomers were also present in the control experiments, and correspondingly might have reduced the quantity of dihydroxystearic acid in aerobically treated samples (data not shown). Therefore it was concluded that the

presence of molecular oxygen for the the biotransformation reaction was not essential.

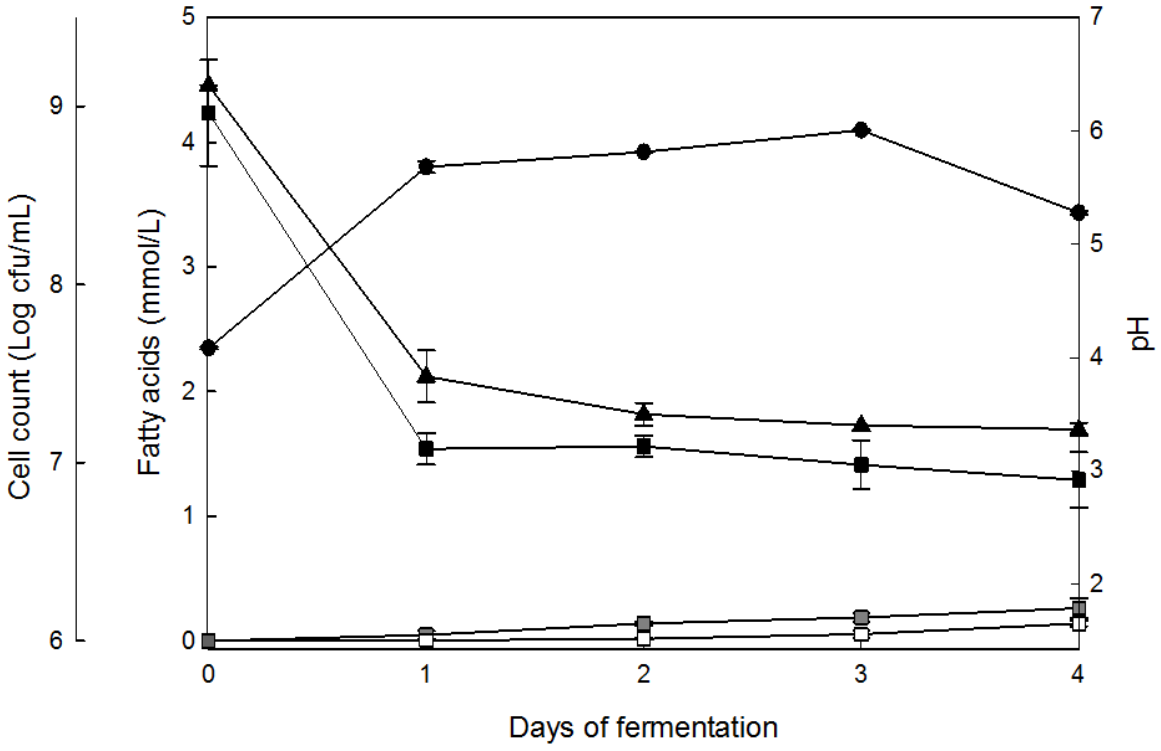


Figure 2.8. Kinetic study of fermentation of *L. sanfranciscensis* DSM 20451 in media containing 4 mmol/L linoleic acid. Shown are cell counts (●) and pH (▲) after 0, 1, 2, 3, and 4 days of fermentation as well as the concentration of C18:2 (■), mono-hydroxy fatty acids (▩) and di-hydroxy fatty acids (□).

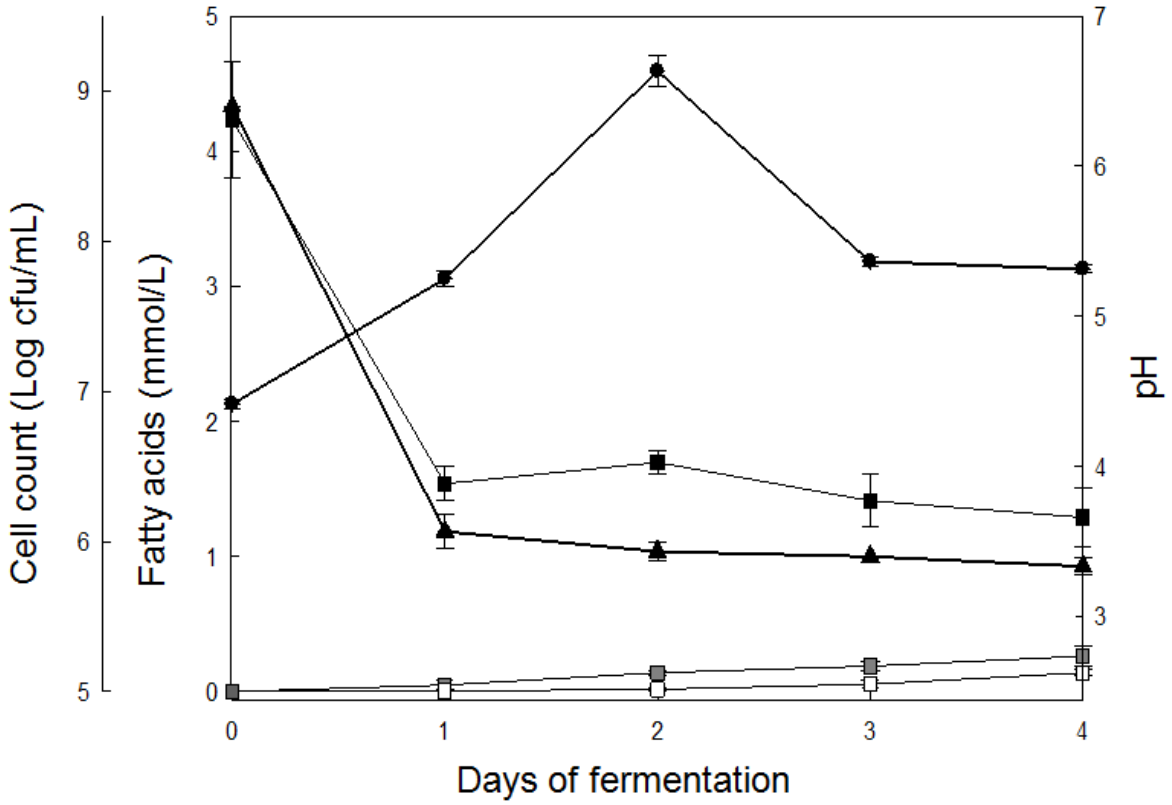


Figure 2.9. Kinetic study of fermentation of *L. plantarum* TMW 1.701 in media containing 4 mmol/L linoleic acid. Shown are cell counts (●) and pH (▲) after 0, 1, 2, 3, and 4 days of fermentation as well as the concentration of C18:2 (■), and the concentrations of mono-hydroxy fatty acids (▣) and di-hydroxy fatty acids.

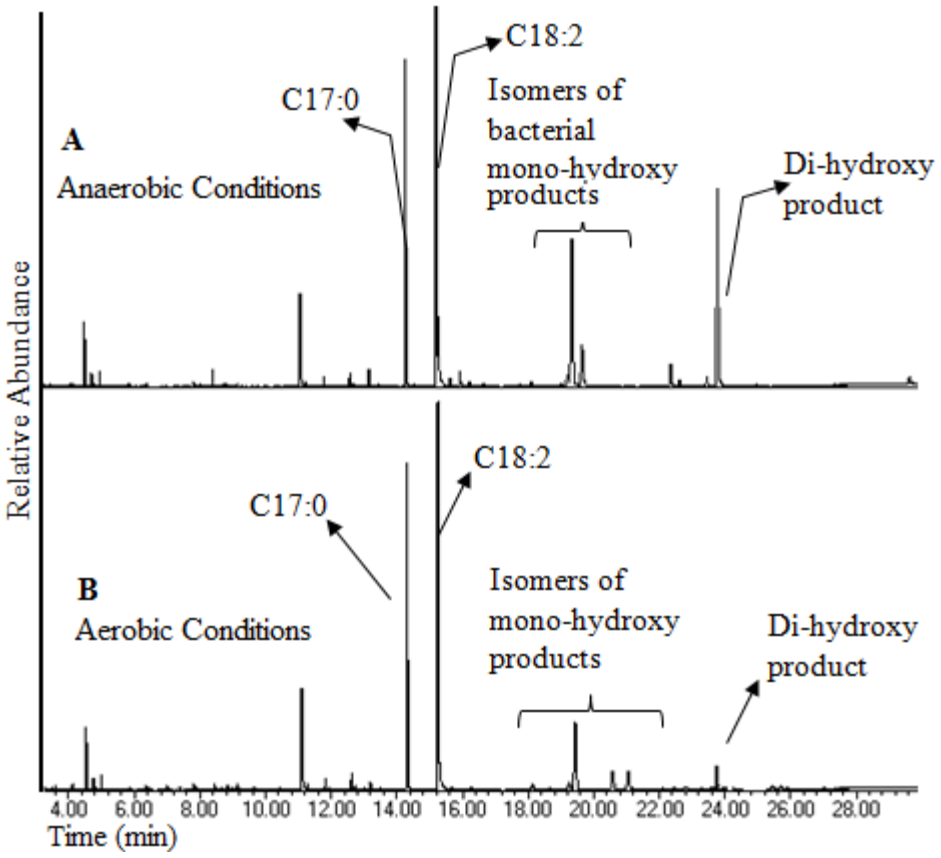


Figure 2.10. Total ion current chromatograms from GC-MS showing separation of diazomethane-BSA derivatised fatty acids and hydroxy fatty acids produced from linoleic acid by *L. plantarum* TMW 1.701 during growth under anaerobic conditions (Panel A) or under aerobic conditions (Panel B). Peaks for the internal standard (C17:0), linoleic acid (C18:2), and the mono- or di-hydroxy products are indicated.

2.3.5 Transformation of linoleic acid by disrupted bacteria

To facilitate increased transformation efficiency, bacterial cell wall extracts were generated through microfluidization of overnight cultures of *L. sanfranciscensis* DSM 20451 and *L. plantarum* TMW 1.701. The hydrolyzed cell

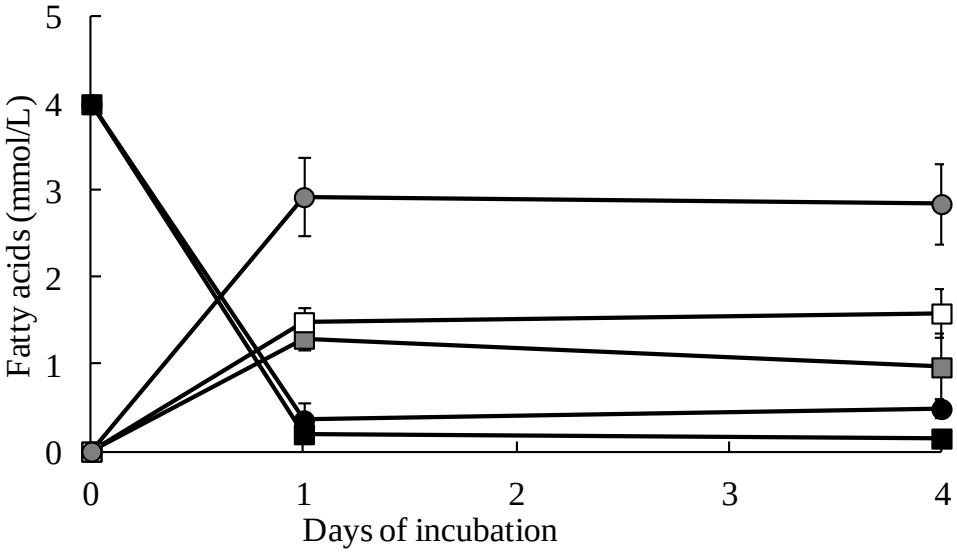


Figure 2.11. Conversion of fatty acids with bacterial crude cell lysate from *L. sanfranciscensis* DSM 20451 to monohydroxy fatty acids and dihydroxy fatty acids. Shown is the conversion of oleic acid (●) to 10-HSA (◍), and the conversion of linoleic acid (■) to mono-hydroxy fatty acids (▣) and di-hydroxy fatty acids (□). Results are shown as mean ± standard deviation of 3 independent experiments.

preparations were then incubated with 4 mmol/L of linoleic acid (Figure 2.11 and 2.12). It was found out that crude cell wall extracts noticeably enhanced the conversion of substrate fatty acid, as compared to whole cell preparations.

In 24 h, the use of disrupted cells resulted in a 5 fold increase in monohydroxy octadecenoic acid products and a 10 fold increase in dihydroxy product than observed for bacterial cultures (Figure 2.9 for *L. plantarum* TMW 1.701). The products generated did not undergo further transformation when incubation was continued for an additional 4 days, as shown in Figures 2.11 and 2.12. Semi-quantification using GC-FID revealed approximately 33% (1.3 mmol/L) mono-hydroxy octadecenoic acid isomers comprising of 13-hydroxy-9-octadecenoic acid (product E) and 10-hydroxy-12-octadecenoic acid (product F).

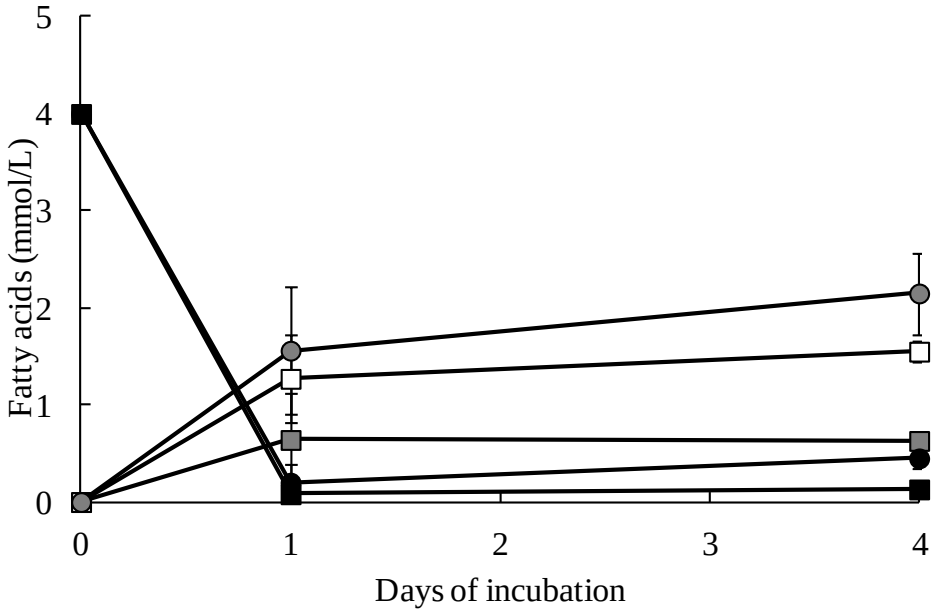


Figure 2.12. Conversion of fatty acids by bacterial crude cell lysate from *L. plantarum* TMW 1.701 to hydroxy fatty acids. Shown is the conversion of oleic acid (●) to 10-HSA (◍), and the conversion of linoleic acid (■) to mono-hydroxy fatty acids (◧) and di-hydroxy fatty acids (□). Results are shown as mean ± standard deviation of 3 independent experiments.

The major product at 38 % yield (1.5 mmol/L) was 10,13-dihydroxystearic (Figure 2.11). Untransformed linoleic acid was only 5 % while the rest of the fatty acid mass i.e. 27% remained unidentified. A comparable trend of linoleic acid conversion was observed for *L. plantarum* TMW 1.701 (Figure 2.12).

2.3.6 Effect of oxygen on transformation of linoleic acid by crude cell lysates of LAB

In order to find out the mechanism of this reaction, and to develop a relationship between the availability of molecular oxygen and hydroxylation efficiency of unsaturated substrates by LAB crude cell lysate from *L. plantarum* TMW 1.701, the reaction was carried out in the absence and presence of oxygen. From the results in Figure 2.13, it can be inferred that the reaction was not dependent upon oxygen and proceeded via hydration of double bonds, resulting in

10,13-dihydroxystearic acid as the major product during anaerobic incubation. A majority of products appeared as isomers of mono-hydroxy octadecenoic acid during aerobic incubation of substrate with a decreased amount of di-hydroxy compound. This can be explained by comparison with a control run under aerobic conditions without the biocatalyst (data not shown).

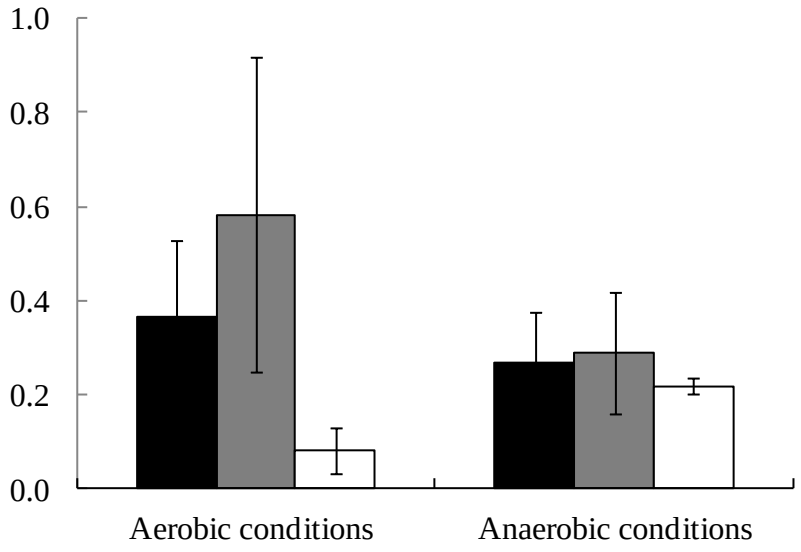


Figure 2.13. Incubation of 4 mmol/L linoleic acid with crude cell lysate of *L. plantarum* TMW 1.701 for 24 h under aerobic and anaerobic conditions. Products, mono-hydroxy fatty acids (▩), di-hydroxy fatty acids (□), and untransformed linoleic acid (■) are shown. Control contained fatty acids in lysis buffer in the absence of biocatalyst.

The difference in the amount of mono-hydroxy fatty acids under aerobic and anaerobic conditions was significantly different (*p* value 0.02). Hence the final yield of mono-hydroxy octadecenoic acid increased as compared to the di-hydroxy product during aerobic incubation. Under these conditions linoleic acid might have undergone auto-oxidation and therefore resulted in an overall higher amount of mono-hydroxy fatty acids. It was inferred from this experiment that LAB enzymes biotransform linoleic acid in the absence of molecular oxygen.

2.3.7 Effect of substrate concentration on transformation of linoleic acid by lysed bacterial cells

A further insight into the mode of action of the hydrating enzymes present in the crude cell lysate was provided by incubating linoleic acid with different concentrations of linoleic acid, starting from 1 mmol/L of linoleic acid (Figure 2.14).

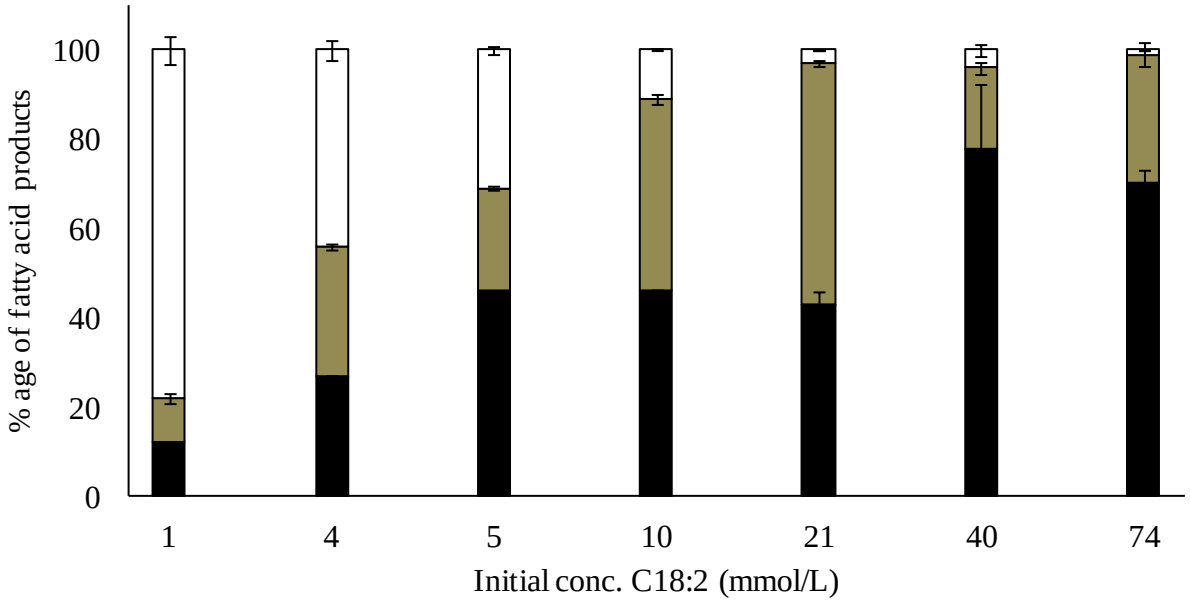


Figure 2.14. Conversion of linoleic acid by exposing *L. plantarum* TMW 1.701 crude cell lysate to increasing concentration of substrate C18:2. Figure represents the percentage of left over substrate C18:2 (■), and products; monohydroxy-products (▩), and dihydroxy-product (□). Results are shown as mean ± standard deviation of 3 independent experiments.

From the nature of monohydroxy octadecenoic acid products and their simultaneous presence it can be inferred that in the presence of sufficient amount of enzyme the most abundant product was 10,13-dihydroxystearic acid. When the amount of enzyme became limiting, with increasing concentrations of linoleic acid, monohydroxy octadecenoic acid products started to accumulate ultimately decreasing the amount of 10,13-dihydroxystearic acid. With 1 mmol/L of starting

linoleic acid concentration incubated with crude cell lysate from *L. plantarum* TMW 1.701 there was 78% dihydroxy product, 10% isomers of mono hydroxy and 12 % untransformed linoleic acid. 10,13-dihydroxystearic acid appeared as the most abundant product from this reaction. In Figure 2.14, with 40 mmol/L of starting linoleic acid concentration, there were 4% 10,13-dihydroxystearic acid, 18% isomers of mono hydroxy octadecenoic acid products and 78% untransformed linoleic acid.

2.4 Discussion

Transformation of oleic and linoleic acid to HFAs was studied from growing and washed cells of eight different strains of *P. aeruginosa*. Two strains converted oleic acid to 10-HSA during growth whereas, linoleic acid was not transformed. Formation of 10-hydroxy-octadecenoic acid products with various unsaturations has been mentioned in response to the addition of oleic, linoleic, and linolenic acid, under anaerobic conditions by Pseumonads (Wallen *et al*. 1962, 1971, Davis *et al*. 1969b). In contrast to the previous studies the strains utilized during this analysis hydrated oleic acid but had no effect on linoleic acid. Therefore it can be assumed that the 10-HSA formation occurred in response to the addition of oleic acid as a stress response, because palmitic, stearic and oleic acid are the main fatty acids in the cell membranes of *Pseudomonas* sp. (Kawai *et al*. 1988). The presence of oleic acid in the medium was considered as an induced stress and hydroxy fatty acids were produced in response to that stress (Murga *et al*. 2001).

Transformation of three unsaturated fatty acids to HFAs by different strains of lactic acid bacteria was analysed. In this study all the strains investigated produced various levels of 10-HSA when exposed to oleic acid in the culture. The products from linoleic acid and linolenic acid were identified as 10-hydroxy-12-octadecenoic acid, 13-hydroxy-9-octadecenoic acid and 10,13-dihydroxystearic acid. To our knowledge, this is the first report of 10,13-dihydroxystearic acid being produced from linolenic acid by LAB. Linolenic acid transformation by LAB, *Nocardia cholesterolicum* and *Flavobacterium* sp.

DS5 indicates the formation of 10-hydroxy-12,15-octadecadienoic acid (Kim *et al*. 2003, Hou 1995a, Sakata *et al*. 1986a, Koritala and Bagby 1992). Concomitant presence of three different hydroxy products, namely 10-hydroxy-12-octadecenoic acid, 13-hydroxy-9-octadecenoic acid and 10,13-dihydroxy-octadecanoic acid were observed during linoleic acid and linolenic acid transformation in contrast to the previous citations.

Conjugated linoleic acid was not formed by LAB. In lactic fermentations, HFAs arising from linoleic acid transformation have been reported to be the precursors of conjugated linoleic acid. 10-hydroxy-12-octadecenoic acid and 13-hydroxy-9-octadecenoic acid have been identified from linoleic acid transformation from strains of *Lactobacillus*, *Streptococcoccus,* and *Enterococcus*. In the present study, the yield of 10-HSA from the culture was in the range of 24% to 38% of 4 mmol/L initial substrate concentration, which substantially increased to 66% when LAB crude cell lysate was utilized for the biotransformation process after less than 24 hours. The yield of 10-HSA (10% - 70%) has been mentioned from washed cells of LAB in response to 4 mmol/L oleic acid addition after 20 hours of incubation by Wanikawa *et al*. (2000, 2002). Additionally, hydration of oleic acid to 10-HSA with very low turnover rates has also been mentioned for *Enterococcus faecalis* (Hudson *et al*. 1995).

Analysis of linoleic acid transformation demonstrated no correlation with bacterial growth. The transformation products form linoleic acid started to appear from 2nd day when culture reached the stationary phase and then gradually started to accumulate in the later stationary phase (day 3 and 4). These results are in agreement with the findings of Ogawa *et al*. (2001). However, Ogawa *et al.* (2001) identified isomers of hydroxy fatty acids en route to the formation of conjugated linoleic acid from washed cells of *L. acidophilous* under microaerobic conditions (oxygen concentration less than 1%), which gradually decreased in concentration due to the formation of conjugated linoleic acid (Ogawa *et al*. 2001). In previous studies, incubation of washed cells from *L. acidophilous* for seven days resulted in 65% 10-hydroxy octadecenoic acid and 4% of 10,13-dihydroxystearic acid yield, indicating the presence of biocatalyst in the

membranes of bacterial cells, which are released upon cell death and thus transform linoleic acid to predominantly monohydroxy fatty acids (Kistimoto *et al*. 2003).

Presence of 4-mmol/L (1 g/L) linoleic acid in the optimal growth medium was not found to be deleterious for the propagation of cells as evident from studying the cfu/mL in response to added linoleic acid, whereas Kim *et al*. (2003) observed certain LAB strains that were unable to propagate in the free fatty acid containing medium. From the current analysis it appears that the added fatty acids at 1 g/L concentration were used to increase the cellular mass. It is supported by the observation by Hofmann *et al*. (1957) that lactobacilli use exogenous fatty acids for the formation of lactobacillic acid present in their cell membranes (Hofmann *et al*. 1957). However, requirement of oleic acid for the growth of *L. reuteri* and *L. sanfranciscensis* has also been mentioned (Sugihara and Kline 1975, Gänzle *et al*. 2000). In our study, use of crude cell lysate indicated that transformation took place in less than 24 hours with a 5 fold increase in monohydroxy octadecenoic acid products and a 10 fold increase in 10,13-dihydroxystearic acid (10,13-dihydroxystearic acid being the major product) as compared to transformation occurring in whole cell culture. The biocatalyst for the transformation was found to be located in the fraction of the crude cell lysate containing cell debris. Incubation of bacterial crude cell lysate to increasing amounts linoleic acid revealed that 10,13-dihydroxystearic acid was the most abundant compound generated during the reaction, and isomers of monohydroxy-octadecenoic acid products appeared as the intermediates for the formation of 10,13-dihydroxystearic acid.

The nature of products analysed and quantified under anaerobic and aerobic conditions to evaluate the role of oxygen indicated that hydration of double bonds takes place in the absence of molecular oxygen. Presence of oxygen leads to auto-oxidation of the electron rich substrate. Auto-oxidation of linoleic acid generated monohydroxy-unsaturated products in addition to bacterial mono-hydroxy fatty acids, resulting in a relatively higher cumulative amount of monohydroxy-octadecenoid products under aerobic conditions of bacterial the

genusgrowth. Mass spectral analysis of LAB generated isomers of monohydroxy-octadecenoid compounds was markedly different from the auto-oxidation products of linoleic acid which were observed in the controls under aerobic conditions and from lipoxygenase generated products of linoleic acid (data shown in Chapter 3 Figure 3.3). Bacterial culture under anaerobic conditions specifically accumulated mono hydroxy products from bacterial metabolic activity, along with quantitatively higher amounts of 10, 13-dihydroxystearic acid.

These results are in agreement with the mechanism of hydration for transformation of unsaturated fatty acid by *Streptococcus pyogenes* (Volkov *et al*. 2010). A highly conserved protein family, called myosin cross-reactive antigen (MCRA) protein family was identified as an FAD enzyme which acted as hydratase on 9(*Z*) and 12(*Z*)- double bonds of C16 and C18 non-esterified fatty acids (Volkov *et al*. 2010). A BLAST search identified >75% sequence identity of *L. reuteri*, *L. plantarum*, and *L. sakei* for the MCRA protein from *S. pyogenes*, suggesting the presence of hydrating enzymes in the genus *Lactobacillus*. LAB transform UFAs to HFAs in their membranes in response to temperature stress (Murga *et al*. 2001). Therefore the presence of highly conserved MCRA in the genomes of LAB indicates that this hydratase is a house-keeping enzyme to maintain membrane homeostasis under stress conditions. House keeping enzymes with conserved sequences, are present among all the living organisms and are expressed constitutively to perform life functions (Pancholi and Chhatwal 2003).

In this work more emphasis has been placed on linoleic acid as the bacterial transformation in the presence of linoleic acid, results in products with more functionalities and the presence of these functional groups in turn allow for more chemical manipulation for a number of different applications. By controlling the amount of linoleic acid the method can be applied for the synthesis of predominantly monohydroxy octadecenoic acid or 10,13-dihydroxystearic acid. This will not only allow for the ease of downstream purification but also for the generation of specific hydroxy compounds for particular applications from technologically desirable strains of LAB.

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Chapter 3

Coriolic Acid Synthesis From Free and Immobilized Lipoxygenase

3.1 Introduction

Coriolic acid (13-hydroxy-9,11-octadecenoic acid), is a di-unsaturated, monohydroxy C18 fatty acid. Applications of coriolic acid as an industrial intermediate, and as a bioactive compound are described in Section 1.4.3. A highly purified preparation in bulk quantities is required for applications in biomedical processes and synthetic chemistry. Current methods for the synthesis of coriolic acid either use chemical processes or chemoenzymatic approaches including the use of soybean lipoxygenase (Rao *et al*. 1986, Jie *et al*. 1997, Babudri *et al*. 2000, Omar *et al*. 2003, Gardner 1996, Iacazio *et al*. 1990). In addition to the specificity of biocatalysis (Hassan *et al*. 2009), enzymatic reactions offer several other advantages. First, enzymatic synthesis occurs at lower physiological temperatures with decreased energy demand and by-product generation (Koeller and Wong 2001). Second, the environmental concerns favour the development of processes operating under milder reaction conditions, using less solvent, and minimal waste production. As a result, enzymatic conversions are desirable for research, development and industrial applications (Gunstone 2003). Another positive feature of biocatalysis is that enzymes are required at a concentration of 0.1-1% of the substrate. At the same time, recovery and reusability of enzyme through the process of immobilization on a suitable support gives the whole process economical viability and synthetically simplifies the process by one-step synthesis (Posorske 1984, Mateo *et al*. 2007, Koeller and Wong 2000). Therefore, an enzymatic process for one-step synthesis of coriolic acid was investigated during the current study.

Soybean lipoxygenase-1 (Lox) specifically generates 13-hydroperoxy-9,11-octadecenoic acid under alkaline conditions through the introduction of molecular oxygen to the substrate as shown in Figure 3.1 (Nanda and Yadav 2003). The catalytic products of the Lox reaction with linoleic acid are peroxides of unsaturated fatty acids which are highly reactive and can result in scission reactions leading to the formation of a variety of small chain aldehydes and

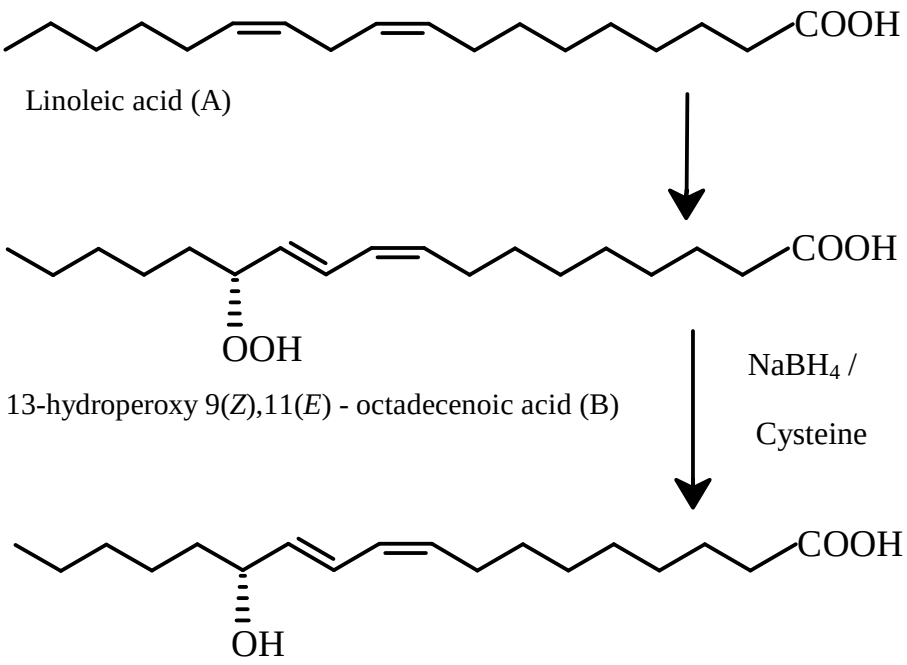

Figure 3.1. Reaction of lipoxygenase with linoleic acid (A) for the formation of 13-hydroxy-9,11- octadecenoic acid / coriolic caid (C) in the presence of reducing agents. Lox reaction medium in the absence of any added reducing agent converts linoleic acid (A) to 13-hydroperoxy-9,11-octadecenoic acid (B), which is reduced by $NaBH_4$ in a two step reaction to generate 13-hydroxy-9,11- octadecenoic acid (C). Cysteine when present in the Lox medium results in the formation of coriolic acid (C) in a single step. Reaction mechanism adapted with permission from Nanda and Yadav 2003.

carboxylic acids (Bentley 2001). The formation of coriolic acid, using free and particularly immobilized Lox systems, suggests a higher yield and purity of hydroperoxy compounds (Iacazio *et al*. 1990, Maguire *et al*. 1991, Chikere *et al*. 2000, 2001, Gardner *et al*. 1996). A standard protocol to generate coriolic acid from Lox reaction on linoleic acid initially generates linoleic acid hydroperoxides, which are extracted by organic solvents, and then reduced to coriolic acid.

The chemical approach for the reduction of peroxides utilizing sodium borohydride leads to the formation of optical and positional isomers of the products due to auto-oxidation of the substrate linoleic acid during the induction period. As a result, the yield and purity of final product decreases and down-stream purification is impacted (Bentley 2001). Lox converted linoleic acid to hydroperoxy octadecenoic acid with a 99% yield, however, coriolic acid yield was 70% after using sodium borohydride as reducing agent (Maguire *et al*. 1991, Kermasha *et al*. 2002). In addition to sodium borohydride, triphenylphosphine, strong alkali and horse radish peroxidase have been utilized for the purpose of reducing hydroperoxides to corresponding alcohols (Yadav *et al*. 2001, Gardner *et al*. 1996, Witting *et al*. 1997). For the synthesis of coriolic acid, all these procedures have been stated to occur either as two step processes, or with lower yields of coriolic acid as compared to the hydroperoxy product. Elshof *et al*. (1998) described a method for the formation of coriolic acid in a single step with cysteine as reducing agent using Lox enzyme from crude extract of defatted soybean flour. However, the formation of coriolic acid from purified or immobilized Lox in a single step process has not been described. It was therefore the aim of this study to produce coriolic acid in a single step process, using cysteine and commercial preparation of soybean enzyme, Lox-1 in free and immobilized form. It was also intended to analyse the stability of immobilized Lox during multiple reaction cycles.

In order to develop an efficient process for a pure isomeric preparation of coriolic acid the objective of the study was to establish the quantitative yield of coriolic acid by using different reducing agents *i.e.* cysteine and β–mercaptoethanol in a single step process as compared to sodium borohydride in a two step process. A second goal was to optimize the aqueous Lox reaction medium for solubilising maximum concentration of substrate (linoleic acid) during the single step process and, further replicate the method with immobilized enzyme system to develop a cost efficient approach for the formation of coriolic acid.

3.2 Materials and methods

Linoleic acid (98%), methyl heptadecanoic acid (98%), 12-hydroxy stearic acid (95%), cysteine-HCl (≥98.0%), sodium borohydride, trizma hydrochloride (> 99%), Lipoxidase from *Glycine max* (soybean) Type I-B (≥ 50,000 units / mg), Eupergit C (spheres of ~ 150 μm diameter), alginic acid (viscosity 20-40 cps), and chitosan were purchased from Sigma-Aldrich, (St. Louis, MO USA). Whatman flexible thin layer chromatography (TLC) plates (250 μm thickness), Davisil chromatographic silica gel 200-425 mesh, reagent grade chloroform, ethyl acetate, hexane, methanol, isopropanol, and Tween 20, N, O-bis-(trimethyl silyl)-acetamide (BSA) were purchased from Fisher Scientific (Fair Lawn, NJ USA).

3.2.1 Enzymatic conversion of linoleic acid

In order to study the quantitative effects of conducting Lox reaction in a single step or two step process, cysteine-HCl / β-mercaptoethanol and sodium borohydride were employed as reducing agents respectively. For process optimization, various molar ratios of cysteine and β-mercaptoethanol were tested for maximum yield during the process of single step coriolic acid synthesis. Three different types of buffers were prepared as shown in Table 3.1. Sodium borohydride was used as a reducing agent in a two step process with free enzyme according to Vega *et al*. (2005b).

In short, 2 mmol/L linoleic acid was dissolved in 0.1M Tris-Cl buffer (pH 9) containing 0.6% Tween 20. Linoleic acid was weighed each time to achieve a concentration of 2 mmol/L for an accurate mass balance analysis. Four different concentrations of cysteine, and three different concentrations of β-mercaptoethanol were added to the buffers in order to study the effect of different molar strengths of reducing agents on the final yield of coriolic acid (Table 3.1). The pH was adjusted to 9 after the addition of reducing agent through the use of 10 N NaOH. The selected molar ratios of cysteine : linoleic acid were 0.5:1, 1:1, 1:2 and 1:4, while β–mercaptoethanol : linoleic acid molar ratios were 1:1, 1:10, and 1:20. The percent yield of coriolic acid in Lox reaction was generally calculated as $[CA]_{Final}$ x 100/ $[LA]_{Initial.}$ Here CA and LA represent coriolic acid (mmol/L) and linoleic acid (mmol/L).

Table 3.1. Composition of Lox reaction medium for the formation of coriolic acid.

Reaction media	Composition	Reducing Agent (molar ratio to linoleic acid)
Two step process		
A	Tris-Cl 0.1M (pH 9) Tween 20 = 0.6% linoleic acid = 2 mmol/L (w/v)	Use $NaBH_4$ (25mM) for reducing hydroperoxides to hydroxy fatty acid
Single step process		
B1	Tris-Cl 0.1M (pH 9) Tween 20 = 0.6% linoleic acid = 2 mmol/L (w/v)	β-mercaptoethanol : C18:2 (0.5 : 1)
B2		(1:1)
B3		(5:1)
C1		Cysteine –HCl : C18:2 (0.5 : 1)
C2		(1 : 1)
C3		(2 : 1)
C4		(4 : 1)

Lox was added to a concentration of 0.08 g/L (91 U per 5 mL reaction volume) under a gentle stream of oxygen at room temperature for 5 min and the reaction vials were transferred to an incubator at 25 °C with shaking speed of 150 strokes per min. for 25 min in the absence of oxygen. Buffers containing linoleic acid treated with reducing agents and in the absence of enzyme were run in parallel as control in order to estimate the amount of auto-oxidation products generated under different sets of reducing conditions.

3.2.2 Extraction of hydroperoxides and coriolic acid

At the end of incubation period, pH of buffers with and without reducing agent, were adjusted to pH 2 with 1 N HCl to achieve complete extraction. Heptadecanoic acid was added as an internal standard and the aqueous solution was extracted three times with equal amount of chloroform containing 15% methanol. Solvent was removed from the extract through the use of a rotatory

vacuum evaporator. Solvent extracts from reaction mixtures containing β-mercaptoethanol and cysteine as reducing agents were stored at -20 °C until analysed, while extracts without reducing agents were dissolved in methanol followed by addition of a 5% solution of sodium borohydride in water (25 mM) to reduce the peroxides to coriolic acid. The reaction was performed on ice (0-4 °C) for 30 min with stirring followed by another 30 min at room temperature. At the end of the reaction, the pH was adjusted to 2 with 1N HCl and the solution was extracted with chloroform/methanol. All of the underivatized solvent extracts used in the study were applied on TLC plates, run in a solvent system of hexane/ethyl acetate 4:1 for initial identification of products. TLC plates were developed in an iodine vapour chamber. Following TLC analysis, samples were analysed by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID) after derivatization by diazomethane (DAM) and N, O-bis-(trimethyl silyl) acetamide (BSA) as described below.

3.2.3 Purification of coriolic acid

Column chromatography was utilized for purification of products. The column was packed using silica slurry made in hexane and poured into a glass column of 1.5 cm diameter up to a 22 cm column length. Hexane /ethyl acetate 4:1 was used as a mobile phase to purify coriolic acid from untransformed linoleic acid and from other unidentified impurities as described by Hou (1999). Purified extracts were analysed by GC-MS, Fourier transform infra-red spectroscopy (FTIR) and high performance liquid chromatography-tandem mass spectrometry (LC-MS-MS) as described below. FTIR analyses were performed at the Department of Chemistry, University of Alberta.

3.2.4 Selection of solvents for Lox reaction in a monophasic system

In order to incorporate higher concentrations of linoleic acid in soluble form, the compatibility of various water miscible solvents were analysed for the formation of coriolic acid in a single step with four times molar excess of cysteine as reducing agent. To generate a monophasic Lox reaction system, tween 20 was individually replaced by four different water miscible solvents; acetone 5% v/v,

methanol 5% v/v, isopropanol 10% v/v and glycerol 20 % v/v concentration during the reaction with 2 mmol/L linoleic acid. The reaction was carried out in 15 mL Sarstedt tubes with a 5 mL reaction volume. Cysteine was incorporated in the buffer to analyse the compatibility of cysteine in a solvent containing medium for the formation of coriolic acid in a single step process.

Buffers in the absence of solvents with 0.6% Tween 20, 2 mmol/L linoleic acid, and 8 mmol/L cysteine were used as control to assess any effect on the final yield of coriolic acid. Reactions were carried out in the presence of gentle stream of oxygen at room temperature by the addition of 0.08 g/L (91 U) of enzyme for 5 min, and transferred to incubator shaker at 25 °C for 25 min. At the end of incubation period, solvent extracts were prepared as mentioned above and stored at -20 °C until analysed by TLC plates, GC-MS and GC-FID.

3.2.5 Formation of coriolic acid in the presence of solvents and compatibility of system with cysteine and $NaBH_4$ as reducing agents

From the results of experiment mentioned in the above section, 5% isopropanol (v/v), 10% methanol (v/v), and 20% glycerol (v/v) in 0.1M Tris-Cl buffer, were found to be compatible with cysteine containing system for the formation of coriolic acid in a single step process and were therefore selected to be used with higher concentrations of linoleic acid. The solubility of linoleic acid in 5% isopropanol, 10% methanol, and 20% glycerol was assessed in the solvent mixtures containing Tris-Cl buffer by adding an excess amount linoleic acid. The mixtures were well shaken in a cold dark place overnight and samples were drawn from the lower aqueous layer for analysis on HPLC. HPLC was conducted using a Zorbax Eclipse XDB RP C18 column (4.6 x 150 mm, 5 μm) from Agilent Technologies (Santa Clara CA, USA) with a solvent system of 75% acetonitrile and 25% water in an isocratic elution. A diode array detector was used and wave length of 240 nm and 254 nm were used for quantification. The solubility of linoleic acid in 5% isopropanol (v/v), 10% methanol (v/v) and 20% glycerol (v/v), as recorded at 254 nm was 20 ± 3 mmol/L, 22 ± 4 mmol/L, and 38 ± 13 mmol/L respectively.

To test the efficiency of the solvent-assisted systems, the Lox reaction was conducted in a monophasic system by incorporating 20 mmol/L linoleic acid in buffers containing 5% isopropanol, 10% methanol, and 20% glycerol, individually, in 0.1 M Tris-Cl buffer pH 7.5 and 80 mmol/L cysteine as reducing agent. Addition of cysteine decreased the pH of the buffer which was adjusted to 7.5 by using 10 N NaOH. Identical buffer systems containing solvents in the absence of cysteine were used in parallel for the formation of hydroperoxides as control. These hydroperoxides were extracted and reduced with sodium borohydride in a two step process as mentioned above to compare the yield of coriolic acid formed in solvent containing media from cysteine and sodium borohydride as reducing agents. The reactions were carried out by the addition of 0.08 g/L enzyme under oxygen flow for 5 min, and for 25 min in an incubator shaker at 25 °C with shaking speed of 150 strokes per min. Solvent extracts from all the treatments were stored at -20 °C until analysed.

3.2.6 Use of borate buffer pH 9.5 with cysteine as reducing agent

The Lox reaction was carried out in an alkaline aqueous medium containing 0.1M sodium borate buffer pH 9.5 with 100 mmol/L of linoleic acid in a 3 mL reaction volume. The substrate was exposed to gentle stream of oxygen while being stirred at 0-4 °C. Lox was added to a total enzyme concentration was 3.3 g/L (2300 U per 3 mL reaction volume) in the reaction buffer. Four times molar excess of cysteine relative to the substrate concentration was introduced into the reaction system after 15 min as reducing agent and pH adjusted to 9.5 with 10 N NaOH. The reaction under oxygenated conditions was quenched after 2 h and after 4 h of introducing cysteine into the medium. After the completion of reaction pH was adjusted to 2 with 1N HCl for a thorough extraction of fatty acids and extracted three times with chloroform/methanol as mentioned above in Section 3.2.2..

3.2.7 Immobilization of Lox on different supports

Three different methods of immobilization were tested during the reusability study of lipoxygenase. Loading efficiency (percentage) was measured

by analysing the total unbound protein concentration in the washing buffer of the supports using standard Bio-Rad protein assay (Bradford 1976).

Each of the three immobilization/encapsulation methods utilized a unique support that was selected based on cost and performance. Procedures are given below for immobilization of Lox in Calcium alginate gel, polyvinyl coated chitosan beads and Eupergit C. The immobilization support showing the highest % loading efficiency after 1st cycle of reaction, and best retention efficiency in subsequent cycles was selected for further experimentation.

Sodium alginate beads were prepared by the method of Hsu *et al* (1997). In brief, 4% w/v solution of sodium alginate was prepared in 0.2 M sodium borate buffer pH 9. The sodium alginate solution was then mixed with an equal volume of Lox 5 mg/mL in 0.2 M borate buffer pH 9. The lipoxygenase and sodium alginate mixture was dispensed drop wise into a cold solution of 0.2 M calcium chloride to form beads. The beads were collected after 30 min and transferred to a flask containing enough hexane to cover the beads. Tetramethoxy orthosilicate was added to beads in 1:1 ratio v/v and left at room temperature overnight. Beads were then filtered and the filtrate was tested for unbound protein using Bio-Rad protein assay kit. Lipoxygenase activity was tested after vacuum drying the beads for six hours.

Polyvinyl coated chitosan beads were prepared by the procedure described by Dincer and Telefoncu (2007). Polyvinyl coated chitosan beads were modified with maleic anhydride. Subsequently lipoxygenase (5 mg protein/mL wet weight of beads) was immobilized on the beads using epichlorohydrin as a cross linking agent in a reaction at 4 °C for 6 hours while shaking. The beads were then washed 3 times with equal volume of 60 mM phosphate buffer pH 6.5. Wash buffers were stored and analyzed for free protein determination. Prepared immobilized enzyme preparations were stored in 0.1 M Tris-Cl buffer at 4 °C for activity measurement.

Eupergit C was used for the immobilization of Lox, and the protocol described by Chikere *et al*. (2001) was followed. Briefly, beads were left for soaking in 0.05M potassium phosphate buffer pH 7.5 for 1 hour at 4 °C. 20 mg

Lox per gram weight of beads (4500 U of enzyme activity) was used for immobilization for 72 hours at room temperature without shaking. After the immobilization process, beads were washed with 0.1 M potassium phosphate buffer and stored at 4 °C until used for enzyme reaction. Buffer from washes was stored for the estimation of any unbound protein using Bio-Rad protein assay kit.

3.2.8 Immobilization of Lox on Eupergit C and use of cysteine as reducing agent

From the comparison of the percent loading efficiency and retention efficiency of the three supports under examination as mentioned in Section 3.1.6 (Results Section 3.3.6), Eupergit C was selected as the support of choice for further experimentation. After each consecutive reaction cycle immobilized Eupergit C beads were washed three times with 0.05 M KPO_4 buffer pH 7.5. Two different buffers described in above sections were used with the immobilized Lox with the following descriptions.

(i) Reaction medium containing detergent, 0.6% Tween 20 with initial linoleic acid concentration of 2 mmol/L and four fold molar excess of cysteine as reducing agent.

(ii) Sodium borate buffer of pH 9.5 containing 100 mmol/L of linoleic acid and four fold molar excess of cysteine as reducing agent. Washing the support after the completion of reaction was done by including 10% methanol and 0.6% Tween 20 in the washing buffer. This was done to wash the microporous support which was completely covered by the product, coriolic acid and the substrate, linoleic acid.

Eupergit C beads (0.02 g), containing 0.4 mg Lox (91 U) were used in a 5 mL reaction volume for (i) reaction medium in 15 mL Sarstedt tubes. Whereas 0.5 g beads containing 10 mg Lox (2300 U) in 3 mL reaction volume was used for (ii) in 50 mL Sarstedt tubes.

3.2.9 Scale-up of free and immobilized Lox with cysteine as reducing agent for the production of coriolic acid

The Lox reaction with free enzyme performed in a Minifors 5 L fermenter vessel (Rose Scientific, Ltd., Mississauga, Ontario, Canada) to evaluate any

impact due to scale up of the reactants and products. The reaction buffer contained 1 L of 0.1 M Tris-Cl buffer at pH 9, 0.6% Tween 20, and 2 mmol/L linoleic acid. Cysteine was added to the buffer (8 mmol/L) and pH adjusted to 9 with 10 N NaOH. Oxygen was bubbled at the rate of 2 L/min and enzyme stock solution containing 0.1 g/L (20000 U) free Lox enzyme was introduced through the port.

For the reaction with immobilized Lox, 4 g Eupergit C beads containing 0.08 g/L (18000 U) enzyme were introduced into the buffer system. The fermenter agitation was set to 100 rpm at 25 °C with 1 L/min oxygen flow to prevent excessive foaming. Reactions were allowed to proceed for 1 h. At the end of the reaction time, 1 N HCl was added to bring the final pH to 2. The reaction solution was extracted twice with 500 mL chloroform / methanol (85%: 15%) and analysed by GC-MS and GC-FID as described below.

3.2.10 Derivatization and analysis by gas chromatography

The Lox reaction extracts were methylated after drying using diazomethane (DAM) for 30 min and silylated using N, O-bis-(trimethyl silyl) acetamide (BSA) as described by (Nicolaides *et al*. 1983). Diazomethane was prepared in the lab according to Aldrich technical bulletin AL-180 using Diazald® distillation kit purchased from Aldrich (St Louis, MO, USA). 12-Hydroxystearic acid derivatized under similar conditions was run as an external standard for retention time and mass spectral analyses. Derivatized extracts were analyzed by electron impact (EI) - GC-MS as described in the following section.

3.2.10.1 Gas chromatography–Mass Spectrometry (GC-MS)

Total ion chromatogram (TIC) and mass spectra of derivatized Lox reaction extracts were generated through GC-MS analyses performed on a model 5975B EI/CI GC-MS, with a 7683B series injector and auto sampler from Agilent Technologies Inc., Santa Clara, CA. The inlet heater was set at 300 °C. The constant column flow was set at 1 mL/min in a split injection model with a split ratio of 100:1. The detector temperature was set to 250 °C. Analyses were carried out using a standard DB1 column with column temperature starting with a

hold of 0.1 min at 90 °C and then increased at the rate of 10 °C / min until 220 °C. Temperature was held at 220 °C for 8 min and then increased to 290 °C at the rate of 10 °C/min and held at 290 °C for 1.9 min. The total run time was 30 min. Electron impact ionization (potential 70 eV) was used and mass ranges between 40- 450 Dalton were monitored. Data was analyzed using the Enhanced MSD ChemStation TM D.03.00.611 software (Agilent Technologies Inc., Santa Clara, CA). Chemical ionization (CI-GC-MS) was carried out under the similar column conditions using ammonia as ionizing gas.

3.2.10.2 Gas chromatography–Flame ionization detector (GC-FID)

Reaction extracts were analyzed by GC-FID to calculate the final amount of products formed as a result of fatty acid transformation by Lox, and to analyze the amount of substrate utilized. Calculations of fatty acids were made relative to internal standard of known concentration on a carbon molar basis. A Varian 3400 Gas chromatograph equipped with a Varian 8200 auto sampler (Varian Inc., Palo Alto, CA) coupled with an FID at 240 °C was used. Operating head pressure was set to 25 psi. Initial injector temperature was 60 °C and increased to 230 °C at the rate of 150 °C/min and held for 28 min. Samples were injected on to a BP 20 column (column length was 30 m, 0.25 mm internal diameter, and 0.25 μm thickness). The temperature program started at 50 °C, was held for 0.2 min and increased to 170 °C at the rate of 20 °C/min and held for 5 min. The temperature was then increased to 230 °C at the rate of 10 °C/min and held for 13 min. The total run time was 30 min.

3.2.10.3 HPLC-MS-MS

All samples were analyzed on an Agilent 1200 liquid chromatograph (Agilent Technologies; Palo Alto, CA, USA) coupled to a QStar Elite mass spectrometer (Applied Biosystems/MDS Sciex; Concord, ON, Canada) with a Turbospray ion source. Analyst QS 2.0 software was used for data acquisition and analysis. The mass range recorded was from 50 amu to 1000 a.m.u. Nitrogen was used for nebulizing gas, auxiliary gas and curtain gas. The electrospray ion source was used in negative ion mode with the following optimal conditions:

Curtain gas at 25, gas 1 (auxiliary) at 20, nebulizing gas 2 at 40, ion source temperature at 100 °C and ionspray voltage at -4500 V. The declustering potential (DP), focusing potential (FP), and DP2 were 50, 250, and 15V, respectively. An Ascentis TM RP C18 column (15 cm x 2.1 mm, 3 μm) from Supelco (Santa Clara CA, USA) was used for separation. The mobile phase composed of 90% acetonitrile in an isocratic elution at a flow rate of 200 μL/min. The injection volume was 5 μL. Mass accuracy as determined by LC-MS-Ms analysis was calculated by the formula given below.

$[M_{Measured} - M_{Theoretical} / M_{Theoretical}] \times 10^6$. Here M represents the molar mass of the compound.

Statistical analysis was carried out using Student's t-test in Microsoft Excel 2003.

3.3 Results

3.3.1 Qualitative and quantitative analysis of coriolic acid produced by using different reducing agents

In order to analyse the effect of different reducing agents on the yield of coriolic acid, three different reducing agents were compared. The Lox reaction was conducted in a single step for the generation of coriolic acid through the use of various molar concentrations of cysteine and β-mercaptoethanol, or by using sodium borohydride as a two step reaction. The Lox reaction with sodium borohydride as reducing agent resulted in the formation of 1.6 mmol/L of coriolic acid with 0.1 mmol/L of hydroxy product in an isomeric form. Untransformed linoleic acid after the reaction was not observed (Table 3.2). The control experiment carried out under identical reaction conditions in the absence of enzyme formed 0.33 mmol/L of hydroxy products. The qualitative effect of sodium borohydride as reducing agent showed the formation of positional isomers and scission products were generated during the reaction (Figure 3.2 IA). In Figure 3.2 IA a number of unidentified peaks due to the breakdown of long chain of fatty acid were observed, and the product was present in the form of positional

isomers visible between 17 and 18 min of the GC program, labelled collectively

Table 3.2. Comparison of different reducing agents for the formation of coriolic acid from 13-hydroperoxy octadecenoic acid in a single step and two* step reaction.

	Initial Linoleic acid	**Reducing Agent**	**Final Linoleic acid**	**Coriolic acid (Total hydroxy product isomers)**
	mmol/L	mmol/L	mmol/L	mmol/L
$NaBH_4$*	3±0.1	25	0±0.01	1.6±0.3 (1.7±0.4)
$NaBH_4$ & cys–HCl*	3.2±0.1	25 mmol/L $NaBH_4$ & 12 mmol/L cys	0±0.01	1.6±0.1 (1.8±0.01)
β-mercapto-ethanol	3.2±0.07	2	0.05±0	0.02± 0 (0.02±0)
	3.3±0.04	4	0.08±0	0.1± 0.14 (0.1±0.14)
	3.5±0.07	20	0.37±0	0.64± 0 (0.64±0)
cys-HCl	3.7±0.04	2	0.09±0	0.4±0.01 (0.4±0.01)
	3.6±0.04	5	0.12±0	1.2±0.03 (1.2±0.03)
	3±0.04	6	0.12±0	2.2±0.04 (2.2±0.04)
	2.9±0.1	12	0±0	2.3±0.2 (2.3±0.2)
Control with $NaBH_4$ (No Lox)	2.7±0.4	25	2.44±0.54	(0.33±0.046)
Control with cys (No Lox)	2.7±0.4	12	3.03±0.17	(0.045±0.008)

as isomers of hydroxy product.

A single pure product peak was observed by including cysteine in the Lox buffer for immediate reduction of hydroperoxides to hydroxides (Figure 3.2 IB). In order to confirm the scission of hydroperoxides for a considerably longer time under oxidizing conditions, a control experiment was performed by incubating

linoleic acid in the presence of $NaBH_4$ and cysteine. This control experiment (Figure 3.3 IIA and IIB) illustrates a similar trend of the formation of oxidation products of linoleic acid. The effect of including cysteine in the medium are shown in comparison with $NaBH_4$ treatment with higher yields of the product, coriolic acid in experimental and control reactions in Table 3.2 and Figure 3.2 IA and IB.

The effect of four different molar concentrations of cysteine on the formation of coriolic acid was studied. The results with cysteine, as compared to substrate, when used at concentrations, 0.5:1, 1:1, and in 2:1 ratio did not produce maximum amounts of coriolic acid. However, the presence of four-fold molar excess of cysteine as reducing agent resulted in the maximal yield of coriolic acid as a single pure compound with a yield of 2.3 mmol/L. The results also demonstrated that the use of β-mercaptoethanol resulted in a higher residual amount of linoleic acid, and very low yield of coriolic acid compared to cysteine and sodium borohydride treatments (Table 3.2). Therefore, among the three

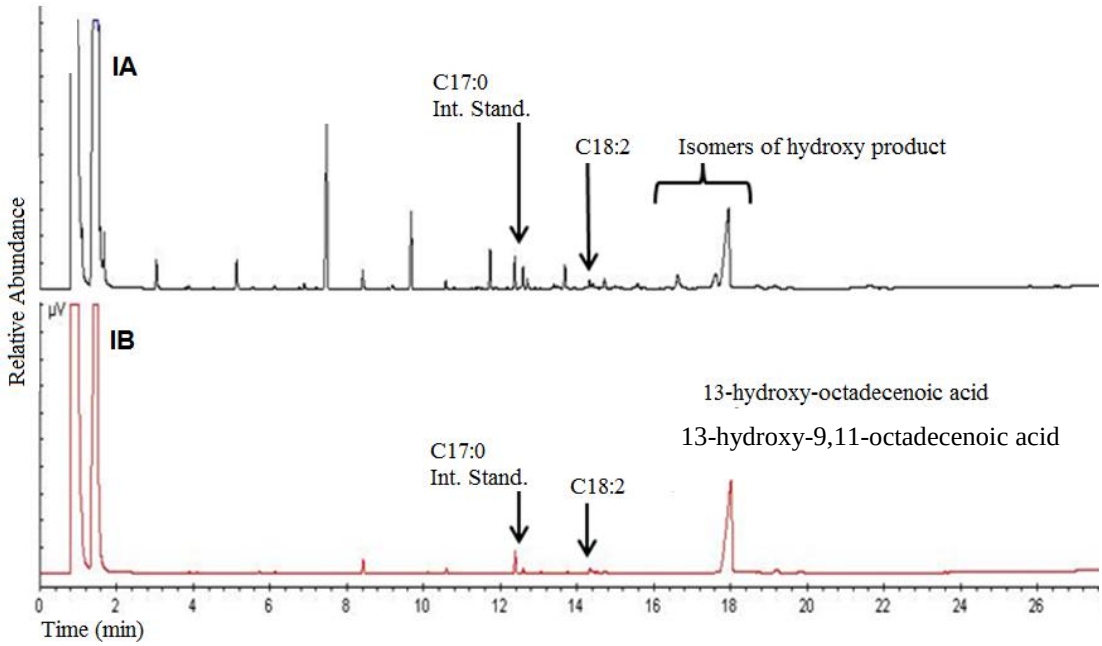

Figure 3.2. GC-MS chromatogram of derivatised extracts from Lox reaction for the formation of coriolic acid with sodium borhydride as reducing agent in a two step process (IA), and with cysteine as reducing agent in a single step process (IB). Peaks for internal standard C17:0, linoleic acid (C18:2), and hydroxy product 13-hydroxy-9,11-octadecenoic acid/coriolic acid are indicated.

reducing agents analysed during this study the maximum yield of coriolic acid was observed from the cysteine containing medium.

3.3.2 Purification of coriolic acid

To confirm the structural identity of coriolic acid, the product of Lox reaction purified from silica column was analysed by FTIR and HPLC-MS-MS in an underivatized form. After derivatization with DAM and BSA, the mass spectral studies were performed by EI-GC-MS and exhibited the following results.

EI-GC-MS: Mass spectra of peak labelled as 13-hydroxy-9,11-octadecenoic acid in Figure 3.2 IB, identified as trimethyl silyl derivative of methylated 13-hydroxy- 9,11-octadecenoic acid showed the fragment ions with charge to mass ratio **(***m/z*) of: 73 (98), 130 (49), 225 (25), 311 (29), and 382 (10). The structural explanation of these mass fragments is explained in Figure 3.4 where *m/z* 382 represents the molecular weight of the derivatized compound.

FTIR: From FTIR analysis, peak 3398 cm^{-1} (b) indicated the presence of hydroxyl group, 2929 cm^{-1} (b) and 2857 cm^{-1} (m) represented the presence of CH_2

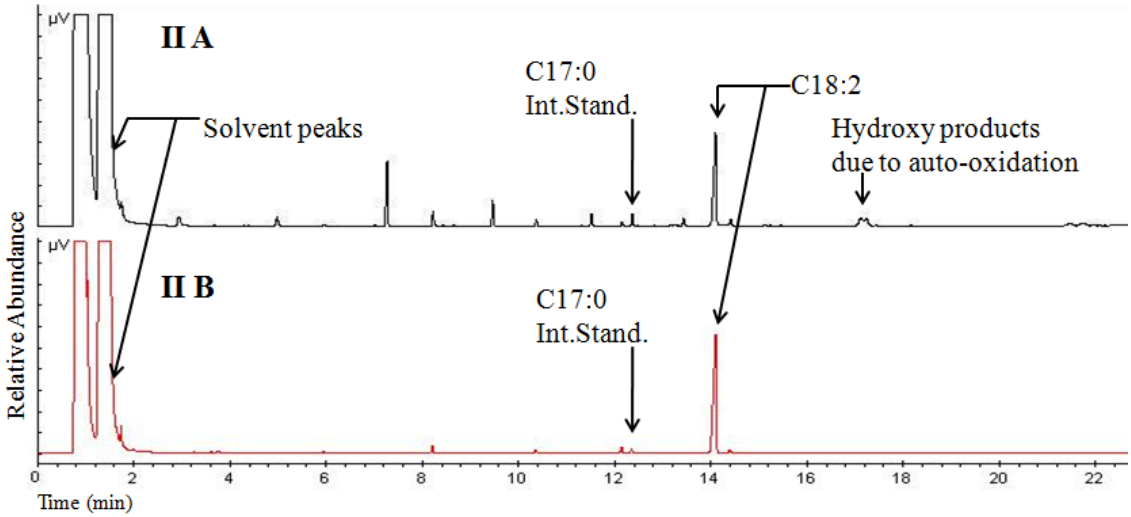

Figure 3.3. GC-MS chromatogram of derivatised extracts from linoleic acid subjected to reducing agents in control environment without the addition of Lox. Under control conditions linoleic acid, treated with sodium borohydride (IIA) and with cysteine (IIB). Peaks for internal standard C17:0, linoleic acid, and hydroxy products due to auto oxidation are labelled.

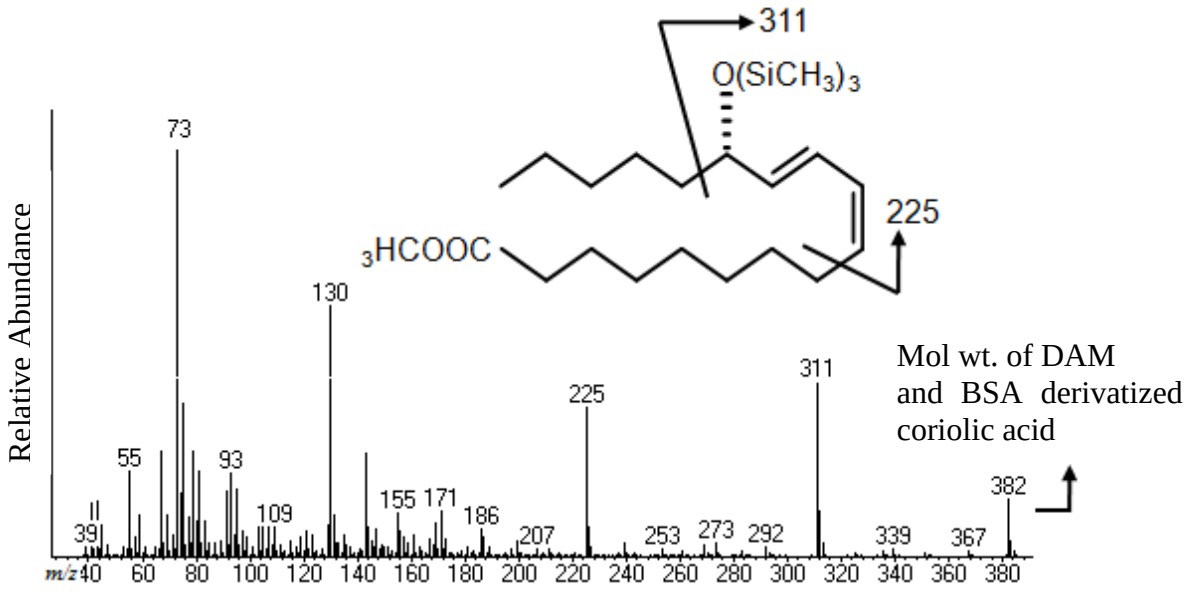

Figure 3.4. Mass spectrum of the total ion current chromatogram from GC-MS of DAM and BSA derivatized 13-hydroxy-9,11-octadecenoic acid (coriolic acid) with proposed structural information.

groups, and 1711 cm^{-1} (m) indicated the presence of carbonyl group, and 1109 cm^{-1} (s).

LC-MS-MS: Analysis of the partially purified coriolic acid from silica column, in an underivatized form showed the following mass spectra. Fragment ion with *m/z* 295.2291 indicates $[M-H^-]$. Fragment ion with *m/z* 277.2186 arises due to water loss from $[M-H^-]$, and fragment ion with *m/z* 195.1393 is the fragment corresponding to formula $C_{12}H_{20}O_2$. Mass measurements in Figure 3.5 are expected to be accurate within less than 2 mDa.

Therefore on the basis of these results, in all the further analyses during the course of this study, the peak labelled as 13-hydroxy-9,11-octadecenoic acid in Figure 3.2. IB will be referred to as coriolic acid.

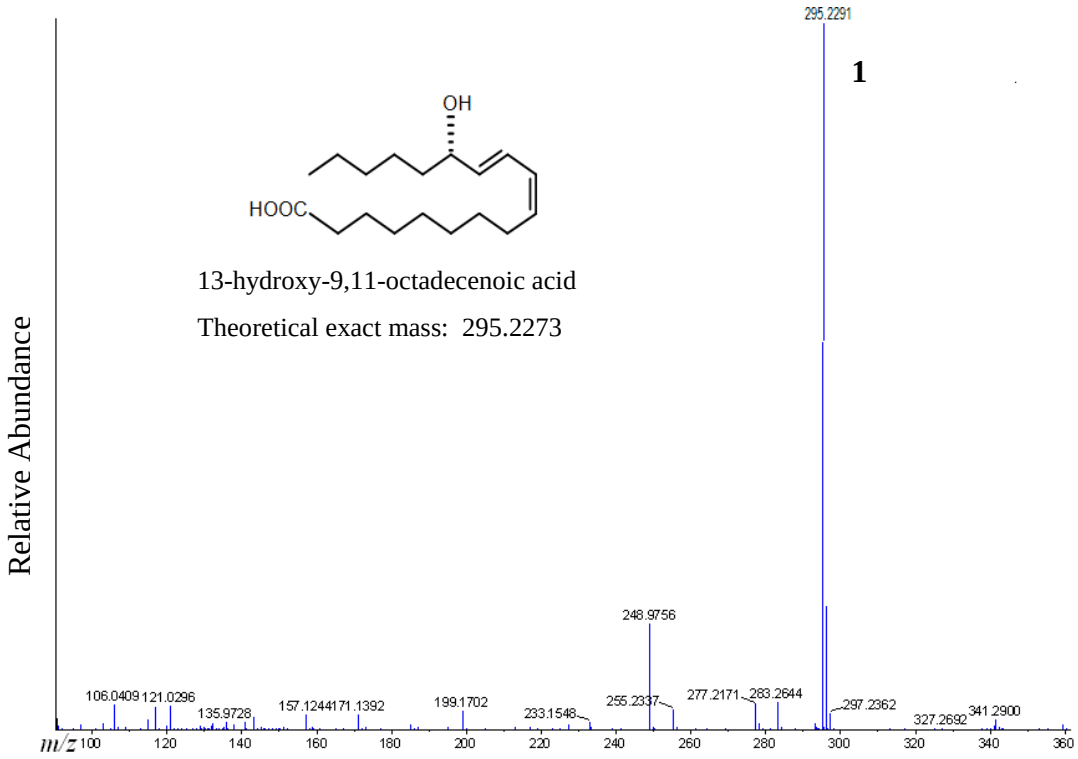

Figure 3.5. Mass spectrum and proposed structure of 2.9 min peak (labelled as 1) from HPLC-MS/MS analysis, showing Mol.wt. of 295.2291 $[M-H]^-$.

3.3.3 Compatibility of water miscible solvents with cysteine during Lox reaction

An experiment was designed to evaluate the effect of solvents added to Lox reaction system. Conversions in the presence of 2 mmol/L linoleic acid with 8 mmol/L cysteine during the reaction as a reducing agent demonstrated that acetone at 10% (v/v) concentration resulted in negligible product yield. However 10% methanol, 5% isopropanol, and 20 % glycerol (v/v) exhibited 85%, 75%, and 73% product yield, which was in the same range as that observed from the control medium in the absence of solvents (Figure 3.7). Therefore based on these results 10% methanol, 5% isopropanol, and 20 % glycerol were selected to incorporate higher concentrations of linoleic acid. Monophasic Lox reaction in the presence of these solvents with two reducing agents was compared to analyse the percent yield of coriolic acid.

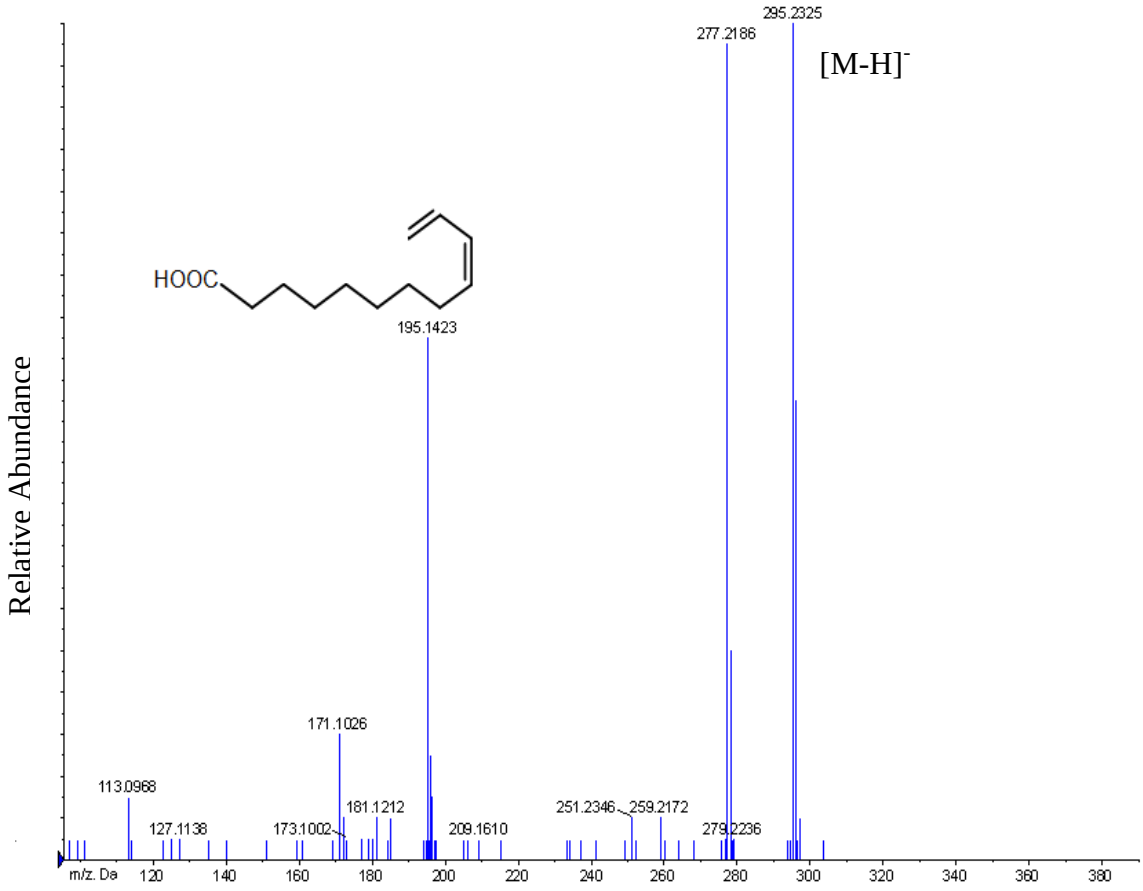

Figure 3.6. Proposed structural elucidation of underivatized coriolic acid and HPLC-MS/MS spectra for the fragment ion of *m/z* 295.2291 eluting at 2.9 min from a reverse phase C18 column.

3.3.4 The effect of solvents on the Lox reaction in the presence of cysteine and sodium borohydride

Coriolic acid yield from Lox reaction in the presence of near saturated solutions of linoleic acid in selected solvents was compared under cysteine and $NaBH_4$ as reducing agents. In the reactions with cysteine as reducing agent, the percent yield of coriolic acid from 20 mmol/L linoleic acid in isopropanol, methanol, and glycerol containing media was 33%, 37%, and 21% respectively (Figure 3.8). For this cysteine containing reaction medium, negligible residual linoleic acid was observed. Comparable reactions using $NaBH_4$ as the reducing

agent yielded 48%, 45%, and 55% coriolic acid, whereas, 20-30% linoleic acid was left untransformed under these conditions (Figure 3.9). The amount of leftover linoleic acid in a $NaBH_4$ treated system indicated the negative effect of hydroperoxides on the enzyme protein which appeared to affect its activity.

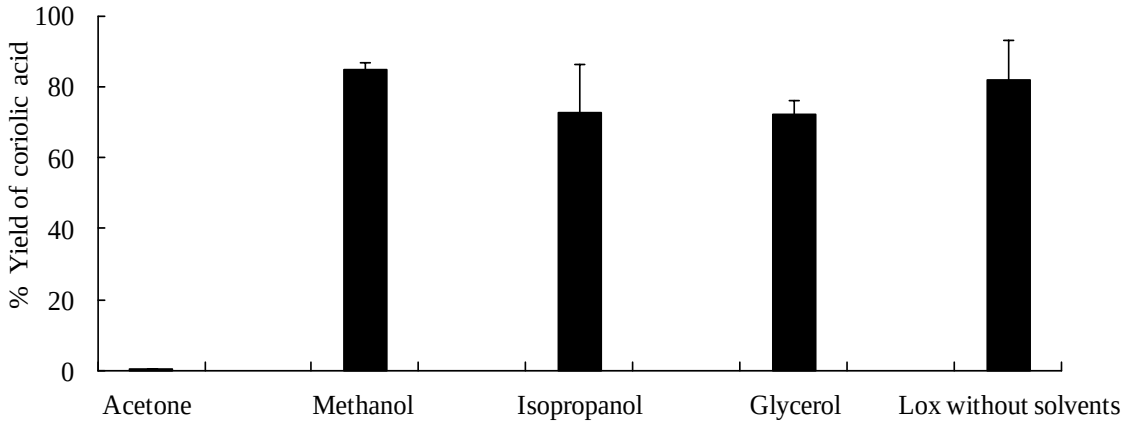

Figure 3.7. Lox reaction performed in a monophasic system to demonstrate the effect of solvents during the reaction with 2 mmol/L of initial linoleic acid and cysteine as reducing agent for a single step reaction. The detergent Tween 20 present during the reaction for solubility of linoleic acid was substituted by 20% acetone, 10% methanol, 5% isopropanol, and 20% glycerol (v/v) in Tris-Cl buffer in individual reaction systems. Figure shows the % yield (■) of coriolic acid (13-hydroxy-9,11-octadecenoic acid).

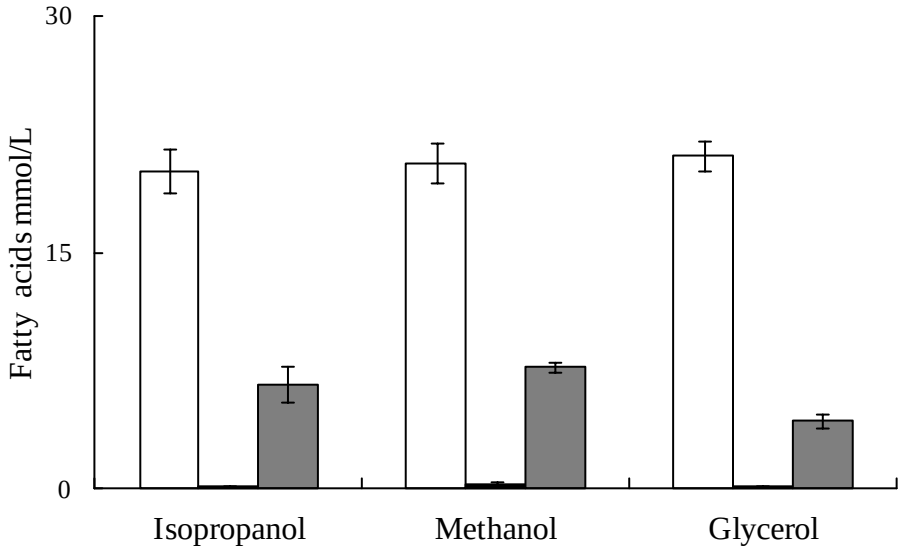

Figure 3.8. Lox reaction in the presence of solvents (5% isopropanol, 10% methanol, and 20% glycerol) in a medium to incorporate 20 mmol/L linoleic acid. Cysteine was used during the course of reaction as reducing agent. Changes in initial linoleic acid (□), final linoleic acid (■), and coriolic acid / 13-hydroxy-9,11-octadecenoic acid (▩) are shown.

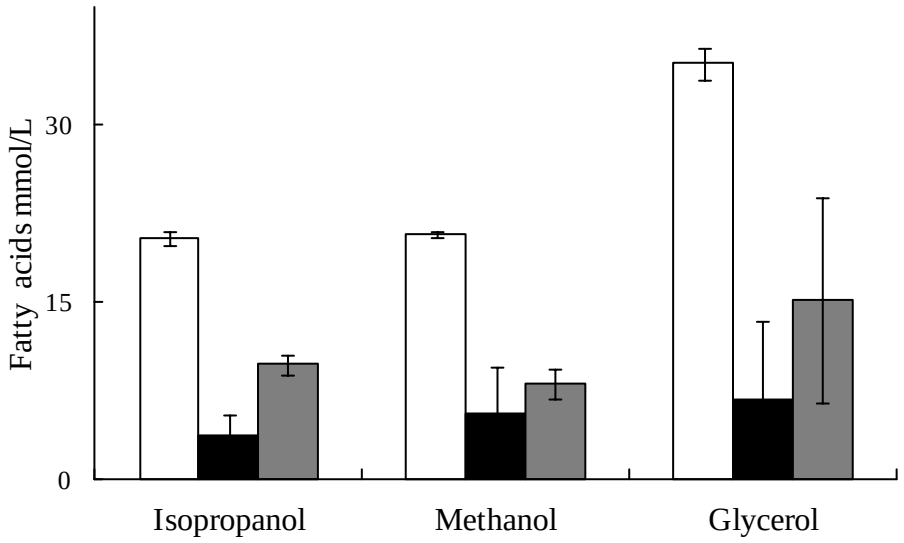

Figure 3.9. Lox reaction in the presence of solvents (5% isopropanol, 10% methanol, and 20% glycerol) in a medium to incorporate higher concentrations of linoleic acid. Sodium borohydride was used after the reaction as reducing agent. Changes in initial linoleic acid (□), final linoleic acid (■), and coriolic acid / 13-hydroxy-9,11-octadecenoic acid (▩) are shown.

3.3.5 Lox reaction with cysteine as reducing agent in aqueous alkaline medium

Lox reaction performed under alkaline conditions in the absence of any detergent or solvent with 103 mmol/L initial linoleic acid concentration resulted in 70 ±18% yield of coriolic acid after 4 h of incubation with cysteine as reducing agent, while leaving 26 ± 20% untransformed linoleic acid (Figure 3.10). The final coriolic acid concentration after 2 h and 4 h of reaction with cysteine were significantly different (p value of < 0.01). This implies that a high concentration of peroxides generated during the reaction required considerably longer time for reduction using cysteine *i.e*. the amount of coriolic acid generated was higher after 4 h of reduction time as compared to 2 h of reduction. However, the final leftover substrate concentration was 21 ± 5 mmol/L and 26 ± 15 mmol/L after 2 h and 4 h of reduction time, which was not significantly different under both time periods examined. It was concluded that 100 mmol/L of linoleic acid in a sodium borate buffer containing four fold molar excess of cysteine can result in 70% yield of coriolic acid in 4 h.

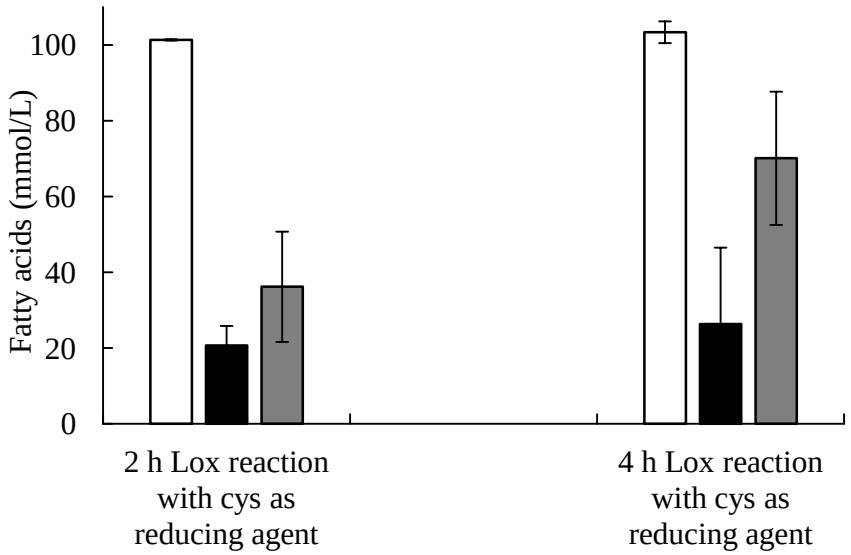

Figure 3.10. Lox reaction in 0.1 M borate buffer pH 9.5 with 100 mmol/L of initial linoleic acid. Lox reaction with cysteine as reducing agent in a single step reaction for the formation of 13-hydroxy-9,11-octadecenoic acid for 2 h and for 4 h of incubation. Changes in initial linoleic acid (□), final linoleic acid (■), and coriolic acid / 13-hydroxy-9,11-octadecenoic acid (▩) are shown.

3.3.6 Loading efficiency of Lox immobilized on different supports

Lox was immobilized on three supports to select a suitable immobilized biocatalyst. The amount of unbound protein was measured through the use of a Bio Rad protein assay kit (Bradford 1976). The percent loading efficiency of the supports was determined by subtracting the amount of measured unbound protein from the total amount of protein used for immobilization. Results from immobilization of lipoxygenase on calcium-alginate and modified chitosan supports showed that these supports encapsulate / bind less protein as compared to Eupergit C, due to the loss of protein during immobilization process.

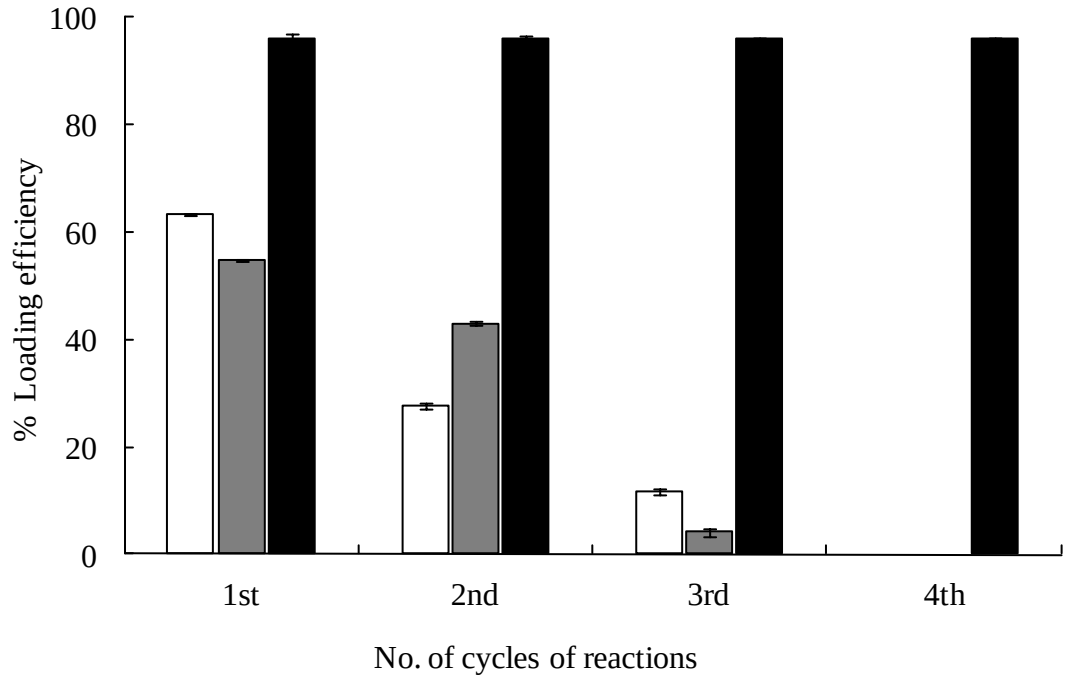

Figure 3.11. Percentage of loading efficiency of Lox immobilized by entrapment in calcium alginate gel (□), modified chitosan beads (▩) and through covalent linkage with commercially available support Eupergit C (■). Loading efficiency was measured after each cycle of reactions by analysing the amount of unbound protein in the washing buffer.

In the case of calcium-alginate 63% protein was entrapped, and only 55% was bound with modified chitosan. The loss of 37% and 45% protein from calcium-alginate and from modified chitosan supports, respectively, thus affected the product turnover. Additionally, in the consecutive reaction cycles followed by washing steps, a loss of protein due to leaching from these supports was observed. Eupergit C showed a percent loading efficiency, of 97 and 96% in the consecutive cycles of reactions (Figure 3.11). From the results of these experiments, Eupergit C was selected as the support of choice for conducting all the investigations.

3.3.7 Immobilization of Lox on Eupergit C and use of cysteine as reducing agent during reaction

The Lox enzyme immobilized on Eupergit C was used to produce coriolic acid in a one step process with cysteine as reducing agent. The immobilized enzyme was found to remain active and gave comparable percent yield of product in first 3 cycles of reaction (Figure 3.12). The initial concentration of linoleic acid utilized for this reaction was 2.2 mmol/L with 0.6% Tween 20 as detergent. After 13 cycles of reaction the product yield was found to drop to 34%. These experiments demonstrated that Lox can be successfully recycled for the generation of coriolic acid in a single step using cysteine as a reducing agent.

An immobilized Lox reaction system containing 100 mmol/L initial linoleic acid and cysteine as reducing agent resulted in coriolic acid yields of 41 mmol/L, 44 mmol/L, and 34 mmol/L from three consecutive cycles (Figure 3.12). The results from these experiments demonstrate that Lox can be recycled in both the systems under investigation, however, with lower linoleic acid concentrations (2 mmol/L), the enzyme retains its activity and can be recycled for 13 times before the final coriolic acid yield reaches 34%. In a similar system, with an initial linoleic acid concentration of 100 mmol/L, 34% coriolic acid yield was obtained after 3 reaction cycles.

3.3.8 Scale up studies of free and immobilized Lox reaction

When the Lox reaction was conducted in a fermentation vessel, with a 2 mmol/L starting concentration of linoleic acid and cysteine as reducing agent, a comparable percent yield of coriolic acid from free and immobilized Lox was observed. These results were in agreement with experiments in which the Lox reaction was performed in a 5 mL volume (Table 3.3).

It can be inferred that under the given conditions with lowered temperature, for enhanced availability of oxygen in the buffer, neither the greater volume nor immobilization affected the performance of Lox for the formation of coriolic acid with cysteine as reducing agent.

3.4 Discussion

For biotechnological applications, pure isomers of coriolic acid are required. However, by extracting reactive peroxides in the first step of reaction, and exposing them to $NaBH_4$ in the second step leads to the formation of volatile aldehydes and positional / optical isomers of the hydroxy product with low yield of coriolic acid (Bentley 2001, Spiteller *et al* 2001). This study evaluated simultaneous reduction of peroxides to hydroxides by introducing a reducing agent with in the Lox reaction medium to enable rapid conversion to coriolic acid. During the two step coriolic acid formation, the low coriolic acid yield was the result of deterioration of peroxides due to rise of temperature during pH adjustment or due to auto-oxidation of residual linoleic acid over considerably longer periods of time (Bentley 2001, Spiteller *et al* 2001). The oxidation of lipids is affected and promoted by elevated temperatures, light, or other external factors such as presence of oxygen in the environment, in addition to the geometry of double bonds (Tallman and Porter 2004, Tallman *et al*. 2004). Inclusion of cysteine -HCl as reducing agent invariably resulted in a qualitatively pure isomer of coriolic acid with quantitatively higher yield because of lesser scission products. It can be conluded that cysteine can be used for the generation of a pure product in a relatively less solvent, labour and time intensive manner.

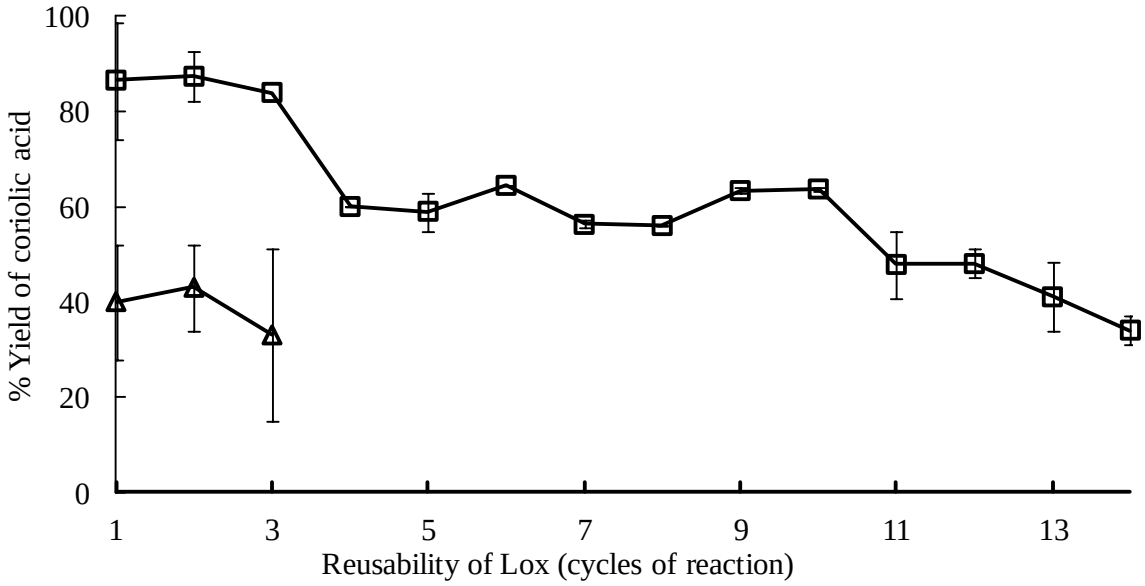

Figure 3.12. Reusability of Lox immobilized on Eupergit C to produce coriolic acid (13-hydroxy-9,11-octadecenoic acid) in a one step reaction by using cysteine as reducing agent. Percent yield of coriolic acid from initial linoleic acid concentration of 2 mmol/L (□) and from initial linoleic acid concentration of 100 mmol/L (Δ), during Lox reaction.

Table 3.3. Lox reaction in the presence of cysteine as reducing agent for the formation of 13-hydroxy-9,11-octadecenoic acid in a single step was extended to 1 L volume.

	Initial linoleic acid	**Final linoleic acid**	**Coriolic acid**	**% yield coriolic acid**	**Lox enzyme**
	mmol/L	mmol/L	mmol/L		g/L
Free Lox reaction in 5 mL volume	2.4±0.7	0±0.01	2.1±0.4	90±7	0.16 g/L
Free Lox reaction in 1 L fermenter	2±0.02	0.12±0.1	1.7±0.3	86±12	0.1 g/L
Immobilized Lox reaction in 1 L fermenter	2	0.005	1.62	81	0.08 g/L on 4 g Eupergit C beads

β-mercaptoethanol when included as a considerably cheaper alternate to thiols containing reducing agent in the Lox reaction, acted as an inhibitor for the enzyme activity as there was residual linoleic acid at the end of reaction (Table 3.2.1). Cysteine as a reducing agent, offers a comparatively simple approach for the preparation of coriolic acid. As only one isomer is generated during this process, the method offers an ease of purification as well. Therefore, cysteine, when present at four fold molar excess than linoleic acid during the course of reaction, offers a promising approach for single step synthesis of coriolic acid in an aqueous medium.

The confirmation of the coriolic acid structure was done by using GC-MS and HPLC tandem mass spectrometry. HPLC-ESI-MS-MS studies have been utilized in the past for the identification and analysis of positional specificity of hydroperoxides and hydroxides from Lox reaction (Schneider *et al*. 1997, Nunez *et al*. 2001, Martin-Arjol *et al*. 2010). The available data for this study indicates the occurrance of coriolic acid in the form of the most abundant peak from HPLC-MS-MS and GC-MS chromatograms showing the molecular ion of *m/z* 295 and *m/z* 382 in the respective systems. As the specificity of soybean Lox is impacted by the conditions of the medium including pH, temperature, and oxygen concentration (Hertmanni *et al*. 1992) giving rise to either 13-hydroperoxy-9,11-octadecenoic acid or 9-hydroperoxy-10,12-octadecenoic acid (Hertmanni *et al*. 1992, Pourplanche *et al*. 1994). The fragmentation pattern analysed from both gas and liquid chromatography coincided with the 13-hydroxy-9,11-octadecenoic acid. The data generated during the current study is in agreement with the fragment ions for coriolic acid under comparable conditions of HPLC-tandem mass spectrometery utilized for the identification of *Pseudomonas aeruginosa* 42A2 culture metabolites (Martin-Arjol *et al*. 2010). The fragment ion with *m/z* 195.1432 (Figure 3.6) is a decisive fragment for the identification of hydroxyl group at 13^{th} carbon atom on the alkyl chain in coriolic acid. Thus it can be inferred that coriolic acid was produced under all the experimental conditions utilized during this study.

The substrate and product for the Lox reaction are unsaturated fatty acids which are oils at room temperature and have low solubility in aqueous buffer media, which is required for optimal enzyme reaction (Vega *et al*. 2005, Kermasha *et al*. 2001). Therefore, efforts to increase the product yield from soybean lipoxygenase using various approaches aimed at different aspects of Lox reaction have been employed in the past. Previous efforts have included increasing the oxygen availability in reaction medium (Iacazio *et al*. 1990), enhancing the availability of soluble linoleic acid for a better enzyme substrate interaction through the use of surfactants (Srinivasulu and Rao 1995), water soluble cosolvents (Pourplanche *et al*. 1994), organic solvents (Emken and Dutton 1970), and by employing enzyme immobilization procedures (Piazza 1994, Chikere *et al*. 2000). In the present study, water miscible solvents in a monophasic system were used to improve substrate solubility in an aqueous system. Subsequently, the compatibility of these reaction media were analysed with cysteine and $NaBH_4$ as reducing agents. The Lox reaction produced comparable amounts of coriolic acid in the presence of isopropanol, methanol and glycerol, with cysteine as reducing agent and at a lower linoleic acid concentration of 2 mmol/L. However when a higher concentration of linoleic acid was introduced in the reaction medium, *i.e.* up to the solubility limits of these solvents, the production of coriolic acid was lower. A white precipitate was observed in the reaction vials and no residual unused linoleic acid was observed. Therefore it was concluded that Lox converted all the linoleic acid to the hydroperoxides, the lower yields of coriolic acid in solvent containing media with cysteine as reducing agents might be due to unavailability of cysteine in the reducing form. This unavailability of cysteine in solvent containing media was confirmed by including higher concentrations of cysteine in the Lox reaction medium (data not presented). Despite adding a 10 fold molar excess of cysteine as compared to linoleic acid, no improvement in coriolic acid yield was observed.

Identical conditions of the Lox reaction but with $NaBH_4$ as the reducing agent, showed substantial amounts of untransformed linoleic acid present at the end of the reaction, indicating the inhibitory action of the presence of peroxides

generated during Lox reaction on enzymatic activities. These results are in keeping with the previous findings which emphasize that, although oxygenated conditions are highly favourable for the regiospecificity of the product, presence of linoleic acid and hydroperoxy-product in an aerobic environment are deleterious for enzyme activity (Smith and Lands 1972, Berry *et al*. 1998). Water miscible solvents were used during this study to increase substrate concentrations for an enhanced product yield. Piazza *et al* (1994) used 6 mM concentration of linoleic acid in monophasic octane containing immobilized Lox reaction medium to exhibit 80% yield of hydroperoxides (Piazza *et al*. 1994). Whereas, mono- and bi-phasic organic solvent system comprising of chloroform, dichloromethane, hexane, iso-octane, octane and toluene have also been reported (Kermasha *et al*. 2001). A 2.6 fold increase of enzyme activity in monophasic iso-octane system has been reported as compared to the aqueous media (Kermasha *et al*. 2001). These monophasic systems, studied in the past, generated, hydroperoxides. Whereas, a method for producing Lox generated hydroxy compounds was desired for this study. Therefore, it can be inferred that Lox reaction can be conducted in the presence of methanol, isopropanol and glycerol containing medium with relatively higher initial concentrations of linoleic acid. However, this Lox medium cannot be used for the single step synthesis of coriolic acid due to its incompatibility with cysteine as reducing agent.

Linoleic acid exists in monomeric form for up to 167 μmol/L concentration at higher pH 10, and up to 10-20 μmol/L concentration at lower pH 8-9 (Verhagen *et al*. 1978). Beyond this concentration, in the respective pH zones, linoleic acid aggregates to form true transparent micelle solutions (Verhagen *et al*. 1978). Transparent miceller solutions of linoleic acid in aqueous buffer are formed due to complete ionization of the carboxyl groups at pH 9-10, due to ionic repulsion between adjacent polar groups of carboxylate anion (Kanicky and Shah 2003). In our study higher concentrations of linoleic acid were dissolved in buffer of pH 9.5 and product formation was measured after including cysteine in the reaction medium as reducing agent. A high coriolic acid yield was generated from this reaction medium and was the highest among all the

reaction media analysed during the course of this study. The results indicated that coriolic acid can be produced for biological or chemical applications in a single step by using cysteine as reducing agent during Lox reaction, using 100 mmol/L initial concentration of linoleic acid.

Lox was immobilized to increase the stability and to allow the recycling of the biocatalyst. Table 1.7 summarizes some of the salient features of the immobilization methods which included adsorption (Battu *et al*. 1994), entrapment (Hsu *et al*. 1997), cross linking by ionic and covalent bonding of support with the enzyme, (Kermasha *et al*. 2002, Chikere *et al*. 2001). For application in a bioreactor, with vigorous shaking and agitation, covalently bound supports are expected to be the preferred route as they allow the attachment of amino, thiol, and carboxylic groups of lysine, arginine and cysteine, residues on the enzyme molecule to react with the oxarine groups of support (Cabral and Kennedy 1991). In the current study, calcium alginate gel and polyvinyl coated chitosan beads allowed immobilization through entrapment of Lox, but, most of the protein leached out from the support during the washing step. This leaching of protein occurred due to weak enzyme-suport interaction and strong agitation. Only negligible amount of protein was left after two successive reaction cycles. However, commercially available support Eupergit C bound 96 % protein through covalent interaction with oxarine groups (Katchalski-Katzir and Kraemer 2000) and no loss of protein was observed in the successive reaction cycles through leaching. Eupergit C is preferred for enzyme immobilization because of its commercial availability, resistance to mechanical and chemical stresses and compatibility with all the processes that are carried out in the reactors (Chikere *et al*. 2001, Katchalski-Katzir and Kraemer 2000). Kermasha *et al*. (2002) reported the use of Eupergit C immobilized Lox for four times before a complete loss of activity, while Chikere *et al*. (2001) reported a 50% loss of activity after 3 cycles of reaction with 10 mmol/L initial concentration of substrate. Parradiaz *et al*. (1993) reported Lox immobilization from commercially available carbonyldi-imidazole activated support and its reusability after 7 reaction cycles in aqueous reaction medium, while in octane/buffer medium the activity of the immobilized

preparation decreased to 60% of its original activity after seven cycles. In the current study, two different reaction media with different initial linoleic acid concentrations were analysed with the immobilized Lox for the formation of coriolic acid as the final product with cysteine as the reducing agent. It was concluded that 2 mmol/L initial linoleic acid, in a cysteine containing system can be successfully applied with sixty percent yield for up to 7 reaction cycles. At higher substrate concentrations, the yield for immobilized Lox decreased and was observed to be less than as compared to the free enzyme in this case. It is suggested that the difference might be due to lack of oxygen availability in the reaction medium. The use of an immobilized support hindered the movement of stirrer in the reaction vial and might have affected the enzyme substrate interaction, leading to mass transfer restrictions and uneven oxygen supply. Use of immobilized enzyme requires extensive stirring as the support tends to settle down at the base of aqueous medium containing 100 mmol/L linoleic acid. Therefore a reactor design with vigorous and even stirring under oxygen rich medium will be required for an improved reproducibility and product yield.

Coriolic acid formation was scaled-up in a 5 L fermenter vessel containing 1 L aqueous buffer with a constant supply of oxygen using free and immobilized Lox. It was inferred that coriolic acid can be prepared at a 1 L scale from free and immobilized Lox starting with initial linoleic acid concentration of 2 mmol/L yielding 80% coriolic acid. Immobilized Lox under identical conditions of reaction, but, at a lower volume scale afforded ten consecutive cycles of reaction with at least 60% coriolic acid yield. Therefore, the scale up process investigated during this study offers an opportunity for attaining multiple reaction cycles from Lox immobilized on Eupergit C under the same conditions. Additionally, with higher linoleic acid concentration in an alkaline buffer containing cysteine as reducing agent, resulted in the single step formation of coriolic acid. This method when extended to an immobilized preparation of Lox resulted in reduction of coriolic acid yield to one half the yield obtained from the comparable free enzyme system. However the enzyme remained active even after two more reaction cycles. The conclusions drawn from the current study lead to a hypothesis for

future investigations that enzyme reaction employing Eupergit C as an immobilization support if conducted under physically agitated and oxygen pressurized vessel can lead to quantitative production of coriolic acid in a fed batch systems for numerous applications.

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Chapter 4

Overall discussion

The goal of this thesis was to develop a methodology for the transformation of pure unsaturated C-18 fatty acids to platform chemicals in the form of HFAs. It was intended to use microbes or enzymes for this purpose and improve the product yield to an industrial scale by optimizing the reaction parameters. Firstly, to achieve this goal, bacterial species were tested for their ability to biotransform oleic, linoleic and linolenic acid and effect of changes in the reaction media were studied to increase the amount of HFAs. Secondly, the use of commercially available enzyme soybean Lox was investigated for coriolic acid synthesis using linoleic acid as substrate. The objective was to develop a single step enzymatic process for coriolic acid synthesis by employing a higher concentration of linoleic acid in the reaction and use immobilized enzyme for process optimization.

Currently hydroxy fatty acids and lacquers are produced from petrochemicals i.e. natural gas and oil refinery streams (Sanchez-Riera *et al*. 2010). However, these can be generated from plant oils. Agri-based oils contain fatty acids with mono-, di- and poly-unsaturations, which serve as potential sites for chemical modifications. The development of processes using plant oils can be beneficial to invigorate rural economies by better use of agricultural resources, domestic supply of strategic raw materials, and decreased dependence on petroleum refinery streams for the generation of specialty chemicals (Tundo *et al*. 2000). In 2002, 320 million metric tonnes of oil crops were produced worldwide, valued at 60 billion US dollars (Dyer and Mullen 2005). The chemical industry used only 15% of this production to produce oleo-chemicals (Murphy 1999; Weselake 2005). Alberta offers ample opportunity to provide a consistent supply of feedstock to develop expertise in the field of transformation due to easy access to its livestock and plant oil refineries. This will result in sustainable economic solutions to problems of environmental deterioration currently faced by the chemical industry. Therefore, with growing awareness about depleting natural reserves, security challenges and environmental concerns, the green

biotechnology for industrial and biological applications is emerging (Li and Huang 2009, Tundo *et al*. 2000).

This dissertation investigates various methods of transforming UFAs which are produced as agricultural by-products from plant oil refineries comprising of 15% free UFAs into value added materials in the form of HFAs as bioactive compounds or industrial intermediates. Methods for biotransformation of UFAs have been under investigation since the 1960's. Despite exhibiting 90% conversion of added substrates by some of the bacterial strains (Table 1.4 and 1.5), development and industrial exploitation of microbial UFA biotransformation routes are still in infancy. The objectives for this study dealt with exploring biotransformation processes of pure C18 UFAs into HFAs of increased reactivity. Once established with pure fatty acids, the biotransformation methods could be extended to fatty acids derived from soap stock or deodorizer from plant refinery waste streams. The peculiar feature of microbial hydroxylation that makes it more attractive is the diversity of products generated by the same organism after being exposed to different substrates, in addition to the simple production methods, as opposed to the complex chemical synthetic routes for the identical product as mentioned in Section 1.6 of this dissertation. As a result, novel hydroxy products are formed, which have been documented to possess unique properties to serve as a potential source of intermediate compounds of multiple applications in various areas as described in Sections 1.4.1 – 1.4.3.

The second chapter of this thesis was focused on investigating the potential of different strains of *P. aeruginosa* and starter culture strains of LAB from *Lactobacillus* spp. and *Bifidobacterium* spp. for transformation of UFAs. Although extensive information is available about certain strains of *P.aeruginosa* for the formation of hydroxy products, there is inadequate literature about the optimization of hydroxy fatty acid formation from lactic acid bacteria. Therefore the study was designed to investigate the formation of HFAs from LAB with prospects of being utilized as bioactive compounds in food systems. In the first section, the aim of the study was to identify and develop methods for the formation of hydroxy fatty acids from microbial origin and analyse their

applicability for industrial purposes. Further objectives of the study were to analyse the mechanism of this transformation to enhance the yield of the products. To accomplish the objective of identifying the mechanism of transformation, two pronged approach was used. First, from the kinetic study of transformation it was analysed that hydroxylated fatty acids appear in the stationary phase. Secondly, the possible role of oxygen was ruled out during this process. Based on the findings, crude cell wall extract was found out to mediate the biotransformation reaction with the most optimal yield. The peculiar feature about this transformation was the effect of substrate concentration on the type of product generated. With oleic acid as substrate, transformation was a relatively straightforward process as the hydrating enzymes acted on the single unsaturation and resulted in 66% 10-HSA yield. However with linoleic acid containing two unsaturations, three different products were formed. 10-Hydroxy-octadecenoic acid appeared as the abundant mono-hydroxy isomer, while concomitantly; small quantities of 13-hydroxy-octadecenoic acid and most abundantly 10,13-dihydroxystearic acid were also observed. It was further noted that by altering the amount of crude enzyme preparation available for the linoleic acid as substrate, the process can be optimized for the formation of a selective product. The most abundant monohydroxy compound, 10-hydroxy octadecenoic acid, generated during this study has been reported as a biologically active compound present in human polymorphonuclear leukocytes (Yamada *et al*. 1996). This compound was found effective to decrease cardiac muscular tension in guinea pigs at 30, 100 and 300 μM concentrations (Yamada *et al*. 1996). The current study demonstrated that this bioactive compound was the most abundant product being generated by crude LAB cell extract containing 54 g/L total cellular proteins and 40 mmol/L of linoleic acid (Figure 2.14). LAB strains used during this study produced 10,13-dihydroxystearic acid in response to linolenic acid exposure in contrast to the previously cited 10-hydroxy-12,15-octadecadienoic acid (Kim *et al*. 2003, Hou 1995a, Sakata *et al*. 1986a, Koritala and Bagby 1992). In conclusion, it can be stated that all LAB strains utilized during this analysis produced potentially

bioactive hydroxy fatty acids. However the hypothesis about increasing the yield of these hydroxy fatty acids to an industrial level was disproved.

The third chapter of this thesis was aimed at optimizing coriolic acid synthesis in a single step process by modulating the reducing agent and composition of the Lox reaction medium for the synthesis of coriolic acid from immobilized Lox. The conclusion of this investigation indicated the incorporation of 100 mmol/L of linoleic acid into a system with continuous oxygen supply and conversion of linoleic acid to coriolic acid with 70% yield in the presence of cysteine as reducing agent. Therefore the first hypothesis about performing the reaction in a step process with high concentration of linoleic acid was proved. The same reaction conditions were employed with immobilized Lox, and the yield of coriolic acid in the first cycle was reduced to almost one half compared to the free enzyme reaction yield under identical conditions. In the previous studies Chikere *et al*. (2000) used 100 mM linoleic acid in a supercritical carbondioxide medium to generate hydroperoxy octadecenoic acid from immobilized Lox. Although, they reported a high yield of the product, but that product was in the form of reactive hydroperoxides which needed further reduction to coriolic acid. This study represents the novel method for the formation of coriolic acid in a single step from immobilized Lox using cysteine in the medium. Although the yield was reduced to half as that obtained from free enzyme system, it was reusable. It was a speculation that during these experimental conditions, Lox reaction medium was being agitated by a magnetic stir bar while, Lox immobilized on Eupergit C created hindrance to the movement of the whole system. Consequently there was an uneven enzyme-substrate interaction and insufficient supply of oxygen, which resulted in a lower yield of coriolic acid in even the first cycle of Lox reaction.

A comparison of the process of microbially generated hydroxy products, and the products generated by Lox reveal that the microorganisms require carbon source in the form of sugars and other nutrients in mMRS medium for growth and thus for the production of hydrating enzyme. Whereas, purified Lox reaction medium merely consists of buffer without any special chemicals. However the

use of commercially available purified Lox enzyme makes the process of coriolic acid formation equally expensive. Although bacterial system offers a diverse nature of products from linoleic and linolenic acid and is equally effective on oleic acid, the final yields of hydroxy products were much lower as compared to the ones observed from purified enzyme system. In addition to higher yields, the enzyme system offers an ease of purification if cysteine is used as reducing agent by lowering the chances of isomer formation. The hydroxy products from bacterial transformation offer a potential end use as bioactive compounds as these are effective in milli or micro molar quantities. However, the process for enzymatic conversion can be extended to a higher scale in a bioreactor for increased product turnover. Provided with sufficient oxygen supply in a well agitated system, immobilized Lox can be incorporated and Lox can be recycled for the formation of coriolic acid, which serves as a precursor for a number of bioactive compounds.

As bacterial systems enable a diversity of products, the formation of product is dependent upon the type of substrate, bacterial species, and the reaction conditions, while a particular type of Lox can be used for the formation of a specific type of product. During the current analysis Lox reaction were performed to ensure the synthesis 13-hydroxy 9,11-octadecenoic acid, however by changing the pH of the medium the same enzyme has been used to prepare 9 hydroperoxy octadecenoic acid (Spiteller *et al*. 2001). Furthermore, Lox extracts from rice, corn, potato, and tomato offers the potential of forming the 9-hydroxy 10(*E*), 12(*Z*)-octadecenoic acid product (Kimura and Yokota 2004, Zamora *et al*. 1987). Therefore, Lox generated product specificity can be modulated and is dependent not only upon the source of enzyme but on the reaction conditions as well. Additionally genetically engineered Lox created by site-directed or random mutagenesis can have a profound effect on the type of catalytic mechanism and the nature of products generated. The advantage of using Lox systems is that it does not require a cofactor for being active. Except for the presence of abundant supply of oxygen there is no other reaction demand and therefore Lox can be an

enzyme of choice, where multiple reaction cycles from immobilization process adds to the practicability of Lox at a larger scale synthetic operation.

4.1 New contributions to knowledge

During this research the following key conclusions were drawn.

1) All the LAB strains utilized for bio-transformation process (Table 2.1) have the potential to produce hydroxy fatty acids from oleic, linoleic and linolenic acid. Five different hydroxy fatty acids identified during this study are summarized in Table 4.1.
2) Quantitatively higher amount of hydroxy products were generated by using crude bacterial cell extract as opposed to the fermentation of bacteria in the presence of fatty acids.
3) Biotransformation of UFAs take place independent of the presence of oxygen. The use of crude cell lysate indicated the presence of hydrating enzymes in the bacterial cell membrane.

Table 4.1. Biotransformation of UFAs to HFAs.

Substrate	Product	Source of HFAs
Oleic acid	10-hydroxystearic acid	LAB strains *P.aeruginosa* MF30 *P.aeruginosa* 18G
Linoleic acid	10-hydroxy-12-octadecenoic acid	LAB strains
	13-hydroxy-9-octadecenoic acid	LAB strains
	13-hydroxy-9,11-octadecenoic acid	Lox enzyme
	10,13-dihydroxystearic acid	LAB strains

4) For the bio-synthesis of coriolic acid, among all the Lox reaction medias investigated during this research, the most optimal coriolic acid yield was obtained in a single step process starting from 100 mmol/L linoleic acid, in

borate buffer of pH 9.5 containing four fold molar excess of cysteine as reducing agent.

5) Immobilization of Lox on Eupergit C offers a more suitable method for the recycling of enzyme, as no attrition of protein was observed during consecutive reaction cycles.
6) Scale-up studies of aqueous Lox reaction medium for the synthesis of coriolic acid in 1 L fermenter resulted in more than eighty percent yield from free and immobilized Lox. The reaction was performed with 2 mmol/L starting linoleic acid concentration with cysteine as reducing agent. This method offers a novel approach with multiple reaction cycles for the synthesis of coriolic acid from immobilized Lox in a single step process.
7) The research presented in this thesis also presents a new method for the synthesis of coriolic acid from immobilized Lox preparation when used with 100 mmol/L initial linoleic acid concentration. This however, resulted in forty percent coriolic acid yield, as opposed to seventy percent yield from the free enzyme under identical conditions.

4.2 Suggestions for future research

During the current research, a very low yield of hydroxy products was observed from the biotransformation of oleic, linoleic and linolenic acid using LAB crude cell lysate. This low yield makes the process unsuitable for industrial manipulation. Future studies realted to LAB can be performed at two levels.

1. In order to increase the hydroxy product turnover, specific primers, as mentioned by Bevers *et al*. (2009) can be designed for the heterologous expression of these hydrating enzymes. For this purpose, the nucleotide sequence of hydratase from the genomes of *L. plantarum, L. reuteri Enterococcus*, and *Streptococcus* will be screened for any conserved motifs to design primers for amplification of the hydratase gene in the selected bacterial strains described in Chapter 2 of this document.

2. These hydroxy products from LAB starins can be isolated and used in food systems or their biological effects can be characterized in different physiological conditions as mentioned by Yamada *et al*. (1996) and, Gautam and Jachak (2009).

The formation of coriolic acid using Lox enzyme in a single step process produced a 70% yield of this product when free enzyme was used. However with immobilized enzyme preparation the percentage of coriolic acid yield dropped to about 40 percent. This lower coriolic caid yield can be improved by making changes in the reactor design. Further research suggestions for coriolic acid formation from Lox immobilized on Eopergit C are given below.

1. The process can be optimized and procedure can be developed for a tubular rotationg fed-batch bioreactor with constant oxygen supply. This kind of system will reduce the mass transfer problems as were encountered during the current research using Lox immobilized on Eupergit C.

2. The immobilized support will be retaind in the reactor by the use of a sieve (< 100 μm diameter). This will minimize the probable loss of immobilized support during the isolation of products and during the successive washing steps after the 1st cysle of Lox reaction.

3. After the completion of 1st reaction cycle the immobilized support will be washed three times with 10% methanol in 9.5 M sodium borate buffer. This will help to remove the fatty substrate and product from the microporous support and enable a better enzyme-substrate interaction.

4.3 References

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