August 2012
PhD Dissertation

Purification, Biochemical Characterization and Corroborated Applications of Protease, Lipase and Xylanase from Korean Soil Bacteria

Graduate School of Chosun University

College of Pharmacy

Poonam Mander

Purification, Biochemical Characterization and Corroborated Applications of Protease, Lipase and Xylanase from Korean Soil Bacteria

August 24, 2012

Graduate School of Chosun University

College of Pharmacy

Poonam Mander

Purification, Biochemical Characterization and Corroborated Applications of Protease, Lipase and Xylanase from Korean Soil Bacteria

Advisor: Prof. Jin Cheol Yoo

A dissertation submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

April 2012

Graduate School of Chosun University

College of Pharmacy

Poonam Mander

The submitted doctoral dissertation of
Poonam Mander is accepted

Committee Chair	Prof. Chosun University	Prof. Kim, Sung Jun
Committee Member	Prof. Chosun University	Prof. Hong, JoonHee
Committee Member	Prof. Chosun University	Prof. Woo, EunRhan
Committee Member	Prof. Chosun University	Prof. Choi, HongSeok
Committee Member	Prof. Chosun University	Prof. Jin, Cheol Yoo

June 2012

Graduate School of Chosun University

ABSTRACT

Purification, biochemical characterization and corroborated applications of protease, lipase and xylanase from Korean soil bacteria

Poonam Mander
Advisor: Prof. Jin Cheol Yoo
Department of Pharmacy
Graduate School of Chosun University

Biologically active enzymes may be extracted from any living organisms, including animals, plants and microorganisms. Among them microbial sources are preferred for the production of enzymes since they provide diversity of catalytic activities and can be produced more economically.

In this dissertation, we report the extraction, purification, and comprehensive biochemical characterization of three different EC[1] class 3 enzymes from the different strains of Korean soil bacteria that belongs to *Streptomyces* genus. In

[1] The Enzyme Commission number (EC number) is a numerical classification scheme for enzymes, based on the chemical reactions they catalyze. EC number has up to three subclasses. Details on http://www.chem.qmul.ac.uk/iubmb/enzyme/

addition, their suitability in various industrial applications is corroborated, albeit with lab scale experimentations.

Firstly, a protease with fibrinolytic activity was purified from *Streptomyces* sp. CS624 and its potential application in the fibrinolysis was experimentally confirmed. The results demonstrated that the purified protease is capable to degrade fibrin clot by direct fibrinolysis. Biochemical characterization of the protease revealed some interesting properties. Sodium dodecyl sufate-polyacrylamide gel electrophoresis (SDS-PAGE) of the protease showed a single polypeptide chain with molecular mass of 18 kDa, which is the lowest among the so far reported *Streptomyces* fibrinolytic enzymes. Its activity was optimum and highly stable at pH 7.0, suggesting that it is a neutral enzyme. Furthermore, the activity was maximum at 60 °C and stable at or below 50 °C. In fibrin plate assay, the protease showed stronger fibrinolytic activity than that of plasmin. It hydrolyzed *A*α-, *B*β- and γ-chains of fibrinogen within 5, 10 and 150 min, respectively. The protease showed higher specificity towards *N*-succinyl-Ala-Ala-Pro-Phe-pNA, a substrate for chymotrypsin. Its activity was inhibited by serine protease inhibitor pefabloc as well as metalloprotease inhibitors EDTA and EGTA. In addition, metal ions showed varied effects on its activity. Altogether, these results suggest that the purified protease is a chymotrypsin-like serine metalloprotease. K_m and V_{max} for the substrate *N*-succinyl-Ala-Ala-Pro-Phe-pNA

were 0.218 mM and 84.03 mM $min^{-1}mg^{-1}$, respectively. The first fifteen amino acid residues of the N-terminal sequence were APNVDAIYLPQYRLS, which are significantly dissimilar from the sequences of previously reported fibrinolytic enzymes.

Secondly, a lipase that can be used as an effective biocatalyst for the production of biodiesel was purified from *Streptomyces* sp. CS133. It has been well understood that the enzymatic route for biodiesel production has been noted to be cost ineffective due to the high cost of biocatalysts. Reusing the biocatalyst for successive transesterification cycles is a potential solution to address such cost inefficiency. However, when organic solvent like methanol is used as acyl-acceptor in the reaction, the biocatalyst (lipase) gets severely inactivated due to the inhibitory effect of undissolved methanol in the reaction medium. Thus, organic solvent-tolerant lipase is highly desirable for enzymatic transesterification. The lipase that we purified form *Streptomyces* sp. CS133 naturally possesses such organic solvent tolerance. The catalytic involvement of the purified lipase in biodiesel production process was confirmed by performing enzymatic transesterification of vegetable oils with methanol. Various biochemical assessments were made to biochemically characterize the purified lipase. SDS-PAGE of the purified lipase showed its molecular mass to be 39.8 kDa. The purified lipase was found to be stable in pH range 5.0-9.0 and at temperature lower

than 50°C, while its optimum lipolytic activity was achieved at pH 7.5 and 40°C. It showed the highest hydrolytic activity towards long chain *p*-nitrophenyl palmitate with K_m and V_{max} values of 0.152 mM and 270.2 mmol min^{-1} mg $^{-1}$, respectively. It showed non-position specificity for triolein hydrolysis. The first fifteen amino acid residues of its N-terminal sequence, AIPLRQTLNFQAXYQ, were noted to have partial similarity with some of the previously reported microbial lipases.

Finally, from the same strain *Streptomyces* sp. CS624 that was used in the production of the protease with fibrinolytic activity, an extracellular and cellulase-free xylanase was produced very economically using agricultural wastes and residues as the substrates. The purified xylanase was noted to be a potential candidate for being used in enzymatic hydrolysis of cellulosic agricultural waste for the production of value-added products (for example xylooligosaccharides) as it was able to hydrolyze various xylans yielding xylose and xylobiose as the major hydrolytic end products. SDS-PAGE and the zymogram analysis of the purified xylanase indicated molecular mass of 40 kDa. Biochemical characterization of the purified EX624 xylanase revealed its highest activity at a temperature of 60°C and pH 6.0. The xylanase was adequately stable in the pH range 4.5~10.0 and at temperatures ≤ 50°C. The purified xylanase displayed enhanced activity in the presence of several metal ions including Fe^{2+}, Co^{2+} and Ca^{2+}.

국문 초록

한국 토양세균이 분비하는 단백분해효소, 지질분해효소, 자일란분해효소의 정제, 생화학적 특성분석 및 산업적 응용 연구

맨더 푸남
지도교수 : 유 진 철
조선대학교 대학원 약학과

동식물과 미생물을 포함한 다양한 생물들은 생리활성을 나타내는 효소들을 생산하는데 그 중에서도 미생물 자원은 그의 생산성이나 경제성의 이유로 가장 많이 연구되고 분야이다.

본 논문은 토양미생물인 방선균속에 속하는 몇몇 국내 토양 미생물들에서 분리한 서로 다른 EC class 의 세가지 효소에 대한 분리, 정제 및 생화학적 활성조사와 이를 이용한 산업화, 상품화로서의 적합성도에 관해 서술하였다.

첫번째, 섬유소 용해작용이 있는 단백분해효소를 생산하는 CS624 균주는 *Streptomyces* sp. 에서 분리하였고, 혈전증 치료에 대한 잠재적인 의학적 적용가능성이 있음을 실험적으로 증명하였다. CS624 로부터 분리, 정제한

단백분해효소는 피브린의 덩어리를 직접적으로 용해시키며, 이 단백분해효소는 흥미로운 생화학적 특성을 띄고 있음 확인하였다. Sodium dodecyl sufate-polyacrylamide gel electrophoresis (SDS-PAGE)를 통한 전기영동을 통해 이 단백분해효소의 크기가 18kDa 크기, 이는 지금까지 발견된 방선균 소재 피브린 용해효소들 가운데 가장 작은 크기의 단백질임을 확인하였다. 이 효소는 pH 7.0 과 50 °C 이하에서 안정적이고 최대 60 °C 에서도 활성을 나타내었다. Fibrin plate assay 에서 이 단백분해효소는 플라스민 보다 강한 섬유소용해활성을 가지며, 이 효소는 피브리노겐의 *A*α-, *B*β-, γ-chains 을 각각 5, 10, 150 분에 가수분해 하였다. 그리고 chymotrypsin 의 기질 중 *N*-succinyl-Ala-Ala-Pro-Phe-pNA 에 높은 특이성을 보였다. 이 단백분해효소는 metalloprotease inhibitors 인 EDTA 나 EGTA 뿐만 아니라 serine protease inhibitor 인 pefabloc 에 의해서도 그 활성이 저해 되었으며, 따라서 본 효소는 금속 원소들에 의해 영향을 받는 것을 확인하였다. 이런 결과들로 미루어 보아, CS624 에서 분리 정제된 단백분해효소는 chymotrypsin 과 유사한 serine metalloprotease 이며, *N*-succinyl-Ala-Ala-Pro-Phe-pNA 기질에 대한 K_m and V_{max} 는 각각 0.218 mM, 84.03 mM $min^{-1}mg^{-1}$ 이다. N-말단 아미노산 염기서열분석결과 아미노산 서열은 APNVDAIYLPQYRLS 으로

확인되었으며 이는 현재까지 보고된 섬유소 용해 효소들과는 다른 서열을 나타내었다.

두 번째로, *Streptomyces* sp. CS133 에서 분리 정제한 지질분해효소는 바이오디젤 생산과정에서 효과적인 생물학적 촉매제로 사용될 수 있음을 확인하였다. 기존의 효소를 이용한 바이오디젤 생산은 비용적인 측면에서 비효율적인 방법이라고 생각되어져 왔으며 이에 대한 해법으로서, transesterification cycles 에서의 지질분해효소의 재사용이 생각되어 왔다. *Streptomyces* sp. CS133 에서 분리된 지질분해효소가 자연적인 유기용매내성을 가지며, 정제된 지질분해효소의 바이오디젤 생산에 있어서의 연관성은 메탄올과 식물유의 효소의 에스테르결합전이반응 을 통하여 밝혀졌다. 분리 정제된 CS133 지질분해효소는 SDS-PAGE 를 통해 분자량을 확인한 결과 39.8 kDa 정도임이 밝혀졌다. 그리고 pH 5.0~9.0, 50°C 이하에서 안정적인 활성을 보였다. pH 7.5, 40°C 에서 가장 높은 지방분해 효소 활성을 나타내었다. 이 효소는 *p*-nitrophenyl palmitate 를 가수분해 잘 시키는데, K_m 과 V_{max} 값은 각각 0.152 mM, 270.2 mmol min^{-1} mg^{-1} 이다. N-말단 아미노산 염기서열분석결과 AIPLRQTLNFQAXYQ 이며, 이는 현재까지 보고된 미생물 유래 지질분해효소들과 부분적인 유사성이 있지만 100% 상동성을 나타내지는 않았다.

마지막으로, 첫 번째와 같은 미생물인 *Streptomyces* sp. CS624는 cellulase-free xylanase도 생산해내는데 이는 기질로서 농산물 쓰레기를 사용할 수 있는 부분에서 매우 경제적이다. 정제된 자일란 분해효소 는 셀룰로오스로 이루어진 농산물쓰레기의 효소적 가수분해를 일으켜서 가치있는 산물(e.g. xylooligosaccharides)을 생산해내는 후보물질로서 주목받고 있다. 이는 다양한 자일란들을 가수분해해서 자일로오스와 자일로바이오스 등을 생성해낸다. SDS-PAGE와 zymogram 분석에서 정제된 자일란분해효소는 40 kDa정도 크기이다. EX624 자일란 분해효소에 대한 생화학적 활성은 60°C, pH 6.0에서 가장 좋은 생리활성을 띈다. 이 효소는 pH 4.5~10.0에서, 50°C 이하에서 안정하며 정제된 자일란 분해효소는 Fe^{2+}, Co^{2+}, Ca^{2+} 같은 금속이온을 첨가하였을 때 더 좋은 생리활성을 나타내었다.

Abbreviations

BLAST	Basic Local Alignment Search Tool
β-Met	β-Mercaptoethanol
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate)
CMC	Carboxy Methyl Cellulose
CVD	Cardiovascular Diseases
DMSO	Dimethyl Sulfoxide
DNS	3,5-dinitrosalicyclic
EDTA	Ethylene Diamine Tetraacetic Acid
EGTA	Ethylene Glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′, *N*′-Tetraacetic Acid
MUF	Methylumbelliferyl
NCBI	National Centre for Biotechnology Information
PMSF	phenyl-methylsulfonyl fluoride
pNA	Para-Nitroaniline
p-NP	Para-Nitrophenol
p-NPP	*p*-NP-Palmitate
PVDF	Polyvinylidene Difluoride
rDNA	Ribosomal DNA

SDS-PAGE	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SEM	Scanning Electron Microscopy
TCA	Trichloroacetic Acid
TLC	Thin Layer Chromatography
t-PA	Tissue Plasminogen Activator
WHO	World Health Organization

Table of Contents

List of Tables

List of Figures

1. Introduction

Enzymes are sustainable and environment-friendly alternative to the use of harsh chemical catalysts used in many chemical transformation processes in industrial applications. It has been well understood that majority of such industrial processes have several inherent shortcomings not only from commercial point of view but also from environmental point of view. For instance, high temperature and/or high pressure required to conduct reactions lead to high energy costs and may require large cooling water downstream. In addition, unwanted by-products and may prove difficult or costly to dispose off. High chemicals and energy consumption as well as harmful by-products have a negative impact on the environment. In a number of cases, some or all of these drawbacks can be eliminated by using enzymes.

It was not until 1926 that the first enzyme (i.e. urease) was obtained in pure form. Because of improved understanding of production biochemistry, purification strategies and methodologies, fermentation process engineering, and recovery methods, today, enzymes can be produced and purified in affordable price from the natural sources. Over the several decades, huge effort has been made in discovering a variety of new and more active enzymes by exploiting the diverse terrestrial and aquatic natural sources including plants, animals and microorganisms. Among them microbial sources are preferred for the production of enzymes since they provide diversity of catalytic activities and can be produced more economically. In

addition, the resulting enzyme from microbial sources can possibly be maximized using the recombinant strains of the microorganisms or by simply optimizing or modifying the production process (Uusitalo et al. 1991).

In this dissertation work, we aim to produce different enzymes from the various strains of Korean soil bacteria, which are identified to be belonging to the *Streptomyces* genus. Note that *Streptomyces* is the largest genus of Actinobacteria with over 500 known species, belonging to the family *Streptomycetaceae*. They are gram positive, spore-forming bacteria found mostly in soil. They are characterized by their tough, leathery, frequently pigmented colonies and their filamentous growths. When first discovered, these organisms were thought to be fungi, but closer examination revealed a lack of a nuclear membrane and the presence of peptidoglycan, demonstrating their prokaryotic origin (Mayfield et al. 1972).

1.1 Enzymes and their applications

All enzymes are proteins (or in some cases RNA) with highly specific catalytic functions. Enzymes, like other proteins, have molecular weights ranging from 12,000 to over one million Dalton. The common feature of proteins is that they are polypeptides. That is, their structure is made up of a linear sequence of α-amino acid building blocks joined together by amide linkages (Bugg 2004).

Enzymatic catalysis happens at the *active site* of the enzyme. The active site of enzyme typically consists of 3-12 amino acid residues, organized into a precise three-dimensional arrangement in a pocket or crevice in the protein, that directly participate in the making and breaking of the bonds. This site has a strong affinity for the substrate because the chemical nature of these amino acid residues and their three dimensional arrangement form a region that complements certain groupings on the substrate molecule. Such enzyme-substrate binding is generally described using the simple *Lock-and-key* model or the more realistic *Induced-fit* model that extends the former model by taking into consideration the changing of the conformation of the active site to fit the substrate after binding (Fig. 1.1).

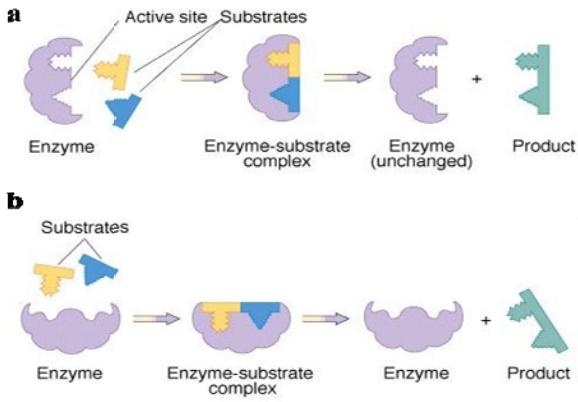

Fig. 1.1: Enzyme substrate binding: (a) Lock-and Key model, (b) Induced-fit model (Adopted from the web material of Brooklyn College Learning Center)

Once the enzyme binds to the substrate forming the enzyme-substrate complex, there is conversion of the substrate into end product, whilst enzyme remains

unchanged. Many enzymes catalyze reactions in their stand-alone presence, but some require an additional non-protein component known as a co-factor. Co-factors may be inorganic metal ions or consist of organic or metalloorganic molecules known as co-enzymes.

Enzymes can be categorized into different classes according to the substrates they act upon. In other words, they are classified into different classes based on the type of reaction that they catalyze. Table 1.1 presents the six major classes of enzymes. The scope of the enzymes considered in this dissertation confined within the EC 3 hydrolases, particularly proteases (Rao et al. 1998), lipases (Aripgny and Jaeger 1999) and xylanases (Subramaniyan and Prema 2002). Proteases are enzymes that break the peptide bond that joins amino acids together in proteins. Lipases are enzymes classified as hydrolases (glycerol ester hydrolases, EC 3.1.1.3) and act on ester bonds of several compounds, with acylglycerols being the most proper substrates, catalyzing reactions of hydrolysis, synthesis and trans- and interesterification. Xylanases are enzymes which randomly cleave the beta 1,4 backbone of the complex plant cell wall polysaccharide xylan.

The field involving the application of enzymes is expanding every day. Enzymes have been used in food, dairy, brewery, nutraceutical, pharmaceutical, agriculture, pulp and paper, leather, textile, detergent, and biofuel industries, among many others. Table 1.2 highlights the representative industrial application of enzymes.

Table 1.1: Classes of enzymes

EC	**Class of Enzyme**	**Reaction Profile**	**Industrial Enzymes**
EC 1	Oxidoreductases	Catalyse oxidation-reactions that involve the transfer of electrons from one molecule to another.	Catalases, Laccases
EC 2	Transferases	Catalyze the transfer of groups of atoms from one molecule to another.	Fructosyltransferases Glusyltransferases
EC 3	Hydrolases	Catalyze hydrolysis reaction, the cleavage of substrates by water.	Amylases, Cellulases, Phytases, **Lipases, Proteases, Xylanases** etc.
EC 4	Lyases	Catalyze the addition of a group across a double bond	Pectate lyases
EC 5	Isomerases	Catalyze the transfer of groups from one position to another in the same molecule	Glucose isomerases
EC 6	Ligases	Catalyze bond formation between two compounds	No used at present

Table 1.2: Some of the representative industrial applications of enzymes

Application	**Enzymes**	**Remarks**
Laundary detergent	Proteinase (91%)	Used in pre-soaks to remove protein based strains
	Lipase (6%)	Now commonly included to digest oils and fats
	Amalase(2%)	Removes resistant starch residues
	Cellulase(1%)	Digest the cotton 'fuzz' which accumulates with excessive washing
Starch industry	Amylases, amyloglucosidases, glucoamylases	Converts starch to glucose and other sugar syrups
	Glucose isomerase	Converts glucose syrups into fructose syrups
Dairy industry	Rennin	Manufacture of cheese
	Lipases	Enhances ripening of blue-mold cheeses
	Lactases	Break down lactose to glucose and galactose
Textiles industry	Amylase	Removing starch from woven fabrics
Brewing industry	Amylases, glucanases, proteinases	Splits polysaccharides and proteins in the malt
	Proteinases	Reduces clouding of beers
	Amyloglucosidase	Low calorie beer production
	β-glucanase	Improves filteration characteristics
Baking industry	α-amaylase	Catalyzes the breakdown of starch
		Improves the characteristics and rising of breads
	Proteinases	Used in biscuits manufacture
Leather industry	Proteinase	
Pulp and paper industry	β-xylanase	Emerging technology for enhancing pulp-bleaching
	Lipases	Reduces 'pitch' which causes paper to stick to rollers and tear
Medical	Protease	Antitumor therapeutic agent or digestive aid
	Peptidase	Anti-inflammatory
Biofuel industry	Lipases	Catalyze transesterification reactions

1.2 Motivation of the dissertation

The motivation of this dissertation is to produce, purify and biochemically characterize different EC 3 enzymes, namely protease, lipase and xylanase, from gram positive Korean soil bacteria that could be useful in the following three application domains:

1.2.1 Enzymatic fibrinolysis for the treatment of thrombosis

Two major aspects of the cardiovascular diseases (CVDs) are atherosclerosis and thrombosis. Atherosclerosis refers to the progressive thickening and hardening of the walls of medium-sized and large arteries caused by fat deposits on their inner lining. On the other hand, thrombosis is the formation or presence of a blood clot in a blood vessel. These diseases are the leading cause of death throughout the world. According to a fact-sheet released by World Health Organization (WHO), about 17.5 million people died from CVDs in 2005 representing 29% of all global deaths. WHO has recently predicted that the situation will be further worse with passage of time; by 2030, about 23.6 million people will die from CVDs every year (Joshi et 2008).

Treatment for CVDs due to thrombosis is primarily based on a process of hydrolyzing the accumulated fibrin using thrombolytic enzymes. Based on different mechanisms that the thrombolytic agents act upon, they are divided into two types: plasminogen activators and plasmin-like proteins (Peng et al. 2005). The

former type of thrombolytic agents that includes tissue type plasminogen activator (t-PA), urokinase and streptokinase activates the endogeneous fibrinolytic system to generate plasmin which eventually hydrolyzes fibrin. They are popular in clinical practice for the treatment of intravascular thrombosis. On the other hand, the later type of thrombolytic agents that includes nattokinase and lumbrokinase directly hydrolyzes fibrin. Despite their widespread clinical use, they induce hemorrhagic side effects, have short half-life in the body, and are also relatively expensive (Uesugi et al. 2011). Therefore, the search for safer and more economical thrombolytic agents from various natural sources has been receiving huge attention. Thus, in this dissertation, we are motivated to extract, produce, and purify the fibrinolytic enzyme from the Korean soil bacteria that can be used as an inexpensive, but effective, thrombolytic agent.

1.2.2 Enzymatic transesterification for the production of biodiesel

Biodiesel is receiving growing attention worldwide as a foremost substitute for conventional petroleum-derived diesel because it is simple to use, biodegradable, non-toxic, and essentially free of sulfur and aromatics (Demibras 2009). Biodiesel comprises of mono-alkyl esters of long chain fatty acids which are derived from renewable feedstock, such as vegetable oils and animal fats, using a method known as transesterification (alcoholysis) (Meher 2006). Such method exchanges the alcohol functional groups of the feedstock triglycerides with that of a short chain

alcohol (for example methanol, ethanol etc), preferably in the presence of suitably selected catalyst. By far alkali catalysts such as metal alkoxides and hydroxides have become default catalysts (Huber et al. 2006) for large scale biodiesel production processes since they are cheap, and produce high yields at short reaction time in mild temperature and pressure settings (Robles-medina et al. 2009). Despite the excellent conversion capability of such alkali catalyzed transesterification process, it is often criticized for some of its inherent drawbacks. Firstly, it mandates feedstock triglycerides to be in the highest purified form. Thus non-virgin sources, such as waste cooking oils and rendered animal fats which are obviously the cheap feedstock (Georgogianni et al. 2009; Canakci 2007) , may not be useful in such process. Even a small fraction of free fatty acids in such feedstock interfere with the transesterification process by reacting with the alkali catalysts to create soaps. Additional downstream processing is required to separate such soaps. Secondly, the produced biodiesel using an alkali catalyst has a high pH value and need subsequent washing. The generated alkali effluent may pose some environmental risks. Finally, the recovery of the process by-product, glycerol, in its pure form is difficult which thus shrinks the value-added application of this process.

To overcome aforesaid demerits of alkali catalyzed transesterification, different route for biodiesel production has been continuously sought. Enzymatic transesterification is one of them which can address all of the aforesaid drawbacks

in alkali catalyzed transesterification. Different lipases from various sources, (for example *Photobacterium Lipolyticum* (Yang et al. 2009), *Pseudomonas aeruginosa* (Ji et al. 2010), *Ralstonia* sp. CS274 (Yoo et al. 2011) etc) have previously been reported that can well catalyze transesterification reactions. However, due to the nature of lipases being relatively costly than the alkaline catalysts there exists a bottleneck (high production cost) which has limited this technique from being commercialized. It has been suggested that such bottleneck can be reduced by reusing enzymes for successive cycles of production (Nielsen et al. 2008) by exploiting the non-altering property of lipases in the reaction medium. However, when organic solvent like methanol is used as acyl-acceptor, the used lipases might severely get inactivated due to inhibitory effect of undissolved methanol present in the reaction medium. In order to combat such instability of the enzymes in organic solvents, several approaches, such as immobilization and chemical/physical modification of enzymes, have been suggested (Doukyu and Ogino 2010). Such biotechnological approaches for biodiesel production using bio-catalyzed transesterification have recently been reviewed by Parawira (Pariwara 2009). Alternatively, it would be much desirable to screen for naturally evolved lipases that have self-possessed organic solvent tolerance property. In response to such desirability, in this dissertation we aim to produce an organic solvent-tolerant lipase from the Korean soil bacteria.

1.2.3 Enzymatic hydrolysis of cellulosic agricultural waste

In recent years, there has been an increasing interest towards more efficient utilization of agro-industrial residues which are generally considered to be a waste and may cause environment pollution. With the advent of various innovative bioprocess engineering approaches, the huge amount of such residues, for example various plant biomass, can potentially be converted into different value added products including biofuels, food additives, nutraceuticals, and so on. Hydrolysis of xylan, one of the main components of hemicelluloses in plant cell walls and second most plentiful polysaccharide after cellulose, is an important step towards proper utilization of such an abundantly available agro-industrial lignocellulosic residues (Yan et al. 2009; Ninawe et al. 2008).

Synergistic actions from various enzymes are necessary for the complete hydrolysis of xylan, among which xylanases (β-1,4-endoxylanases EC 3.2.1.8) plays a key role. The xylanases are glycosyl hydrolases that catalyze a random hydrolysis of the β-1,4-glycosidic bonds in a xylan via a double displacement mechanism (Bae et al. 2007). The other accessory enzymes which could be helpful for the complete hydrolysis include ß-xylosidase, α-arabinofuranosidase, acetyl esterase, and α-glucuronidase (Qiu et al. 2010). Variety of microorganisms, including bacteria, yeast, actinomycetes and filamentous fungi, have been reported to produce different types of xylanases (Wejse et al. 2003; Bastawde et al. 1994;

Taneja et al. 2002). In particular, xylanases produced from mesophilic actinomycetes including several species of *Streptomyces* such as *S. flavogresieus*, *S. lividans*, and *S. cyaneus* are of endotype (β-1,4 xylan xylanohydrolase). Such enzymes are generally produced when microorganisms are grown in culture media supplemented with xylan or xylan hydrolysates as carbon source (Nascimento et al. 2002). However, the commercially available xylan is not cost effective for being used in industrial-scale fermentation since it significantly increases the cost of xylanase production. Alternatively, agro-industrial wastes and residues that are rich in cellulose, hemicelluloses and ligin, could serve as inexpensive substrates for economical xylanase production (Chapla et al. 2010). In this dissertation, we thus aim to produce xylanase from Korean soil bacteria, using very cheap agricultural waste as the substrate, which can be potentially used in enzymatic hydrolysis of cellulosic biomass for the production of xylooligosaccharides that are one of the most preferred natural food additives and nutraceuticals.

2. Chymotrypsin-like Fibrinolytic Enzyme

With the aim of producing an enzyme suitable for the enzymatic fibrinolysis for the treatment of thrombosis, we have produced and purified a metalloprotease, referred to as FES624, from the *Streptomyces* sp. CS624.

To date, many thrombolytic agents have been identified and characterized from different sources including microbes (Peng et al. 2005), animals (Zhang et al. 1995; Nakajina et al. 1993; Hahn et al. 2001), plants (Chung et al. 2010) and fermented foods (Choi et al. 2009; Sumi et al. 1995). Among the aforesaid sources, microbes are implicitly understood to be one of the most preferable sources due to their broad biochemical diversity, feasibility of mass culture and ease in genetic manipulation. Hence, over time, numerous fibrinolytic enzymes from various microbes including bacteria, actinomyces, fungi, and algae have been discovered (Peng et al. 2005, Table 1). Among them, only a few have been reported from the microbes belonging to *Streptomyces,* for e.g. from *Streptomyces omiyaensis* (Uesegi et al. 2011), *Streptomyces* sp. CS684 (Simkhada et al. 2010), *Streptomyces megaspores* SD5 (Chitte and Dey 2000), *Streptomyces* sp. Y405 (Wang et al. 1999), etc.

In what follows, we report the methods that we followed for the purification and biochemical characterization of FES624 and discuss the results highlighting its peculiar characteristics.

2.1 Materials and methods

2.1. 1 Materials

Human fibrinogen, human thrombin, human plasmin, azocasein, trichloroacetic acid (TCA), Folin Ciocalteu's phenol reagent and tyrosine were obtained from Sigma-Aldrich (Missouri, USA). Sepharose CL-6B and phenyl Sepharose CL-4B gel were obtained from Pharmacia (Uppasala, Sweden) and Applied Biosystems (California, USA), respectively. Various protease inhibitors including phenyl-methylsulfonyl fluoride (PMSF), pefabloc SC, ethylenediamine tetraacetic acid (EDTA) and ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′, *N*′-tetraacetic acid (EGTA) were obtained from Roche Applied Science (Mannheim, Germany). Chromogenic substrates including S-2222, S-2238, S-2251, S-2288, S-2444, S-2765 and S-7388 were obtained from Chromogenix (Milano, Italy). Other reagents and chemicals used were of analytical grade and were commercially available.

2.1.2 Strain and cultivation

Strain CS624 was isolated along with other several strains from the soil samples collected from Cheonnam province, Korea. The strain was identified as a strain related to *Streptomyces* based on morpholigical and 16S rDNA sequences (Yoo et al. 2007) (16S rDNA showed 100 % homology with *Streptomyces griseofuscus* (Accession no AB184206), *Streptomyces murinus* (Accession no AB184155) and *Streptomyces costaricanus* (Accession no. AY999910). It was grown for a week at

28 °C on OSYM agar plate containing (in g/l): oatmeal, 20; soybean meal, 10; yeast extract, 10; mannitol, 10; and agar, 15. A loopful of spores were scraped from the plate and inoculated into seed medium containing (in g/l): beef extract 4; yeast extract, 1; peptone, 4; glucose, 10 and NaCl, 2.5. It was cultivated for 24 h in shaking incubator (180 rpm) at 28 °C. This preculture, at 4% (v/v), was transferred to protease producing culture medium which consisted of (in g/l): glucose, 15; oatmeal, 15; K_2HPO_4, 3 and NaH_2PO_4, 3, and further cultivated in a shaking incubator (180 rpm) at 28 °C for 72h.

2.1.3 Protein estimation

Protein concentration was determined by the method of Bradford (Braford 1996) using bovine serum albumin as standard. The concentration of protein was determined by measuring the absorbance at 595 nm.

2.1.4. Purification of fibrinolytic enzyme

The culture broth of *Streptomyces* sp. CS624 was centrifuged at 6000 × *g* for 1 h at 4 °C and $(NH_4)_2SO_4$ at 80% saturation was added to the supernatant to precipitate the proteins. Precipitated proteins were recovered by centrifugation at 6000 × g for 1 h at 4 °C. The precipitate, suspended in 10 mM Tris-HCl buffer (pH 7.0), was dialyzed against the same buffer and then concentrated by ultrafiltration using YM 10 membrane (Milipore Corp., USA). The resulting concentrated sample was purified using gel filtration with Sepharose CL-6B column (116 × 2.2 cm)

previously equilibrated with 10 mM Tris-HCl buffer (pH 7.0). Proteins were eluted in the same buffer at 20 ml/h. The active protease fractions with high specific activities were pooled, concentrated, and then purified using hydrophobic interaction with phenyl Sepharose CL-4B column (20 × 1.2 cm) that was pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing 0.5 M $(NH_4)_2SO_4$. The bound proteins were eluted by gradient of 10 mM Tris-HCl buffer (pH 7.0) containing 0.5 to 0 M $(NH_4)_2SO_4$ at elution speed of 30 ml/h. After monitoring the enzyme purity by gel electrophoresis, various biochemical properties of the enzyme were determined.

2.1.5 Determination of molecular weight

The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (Laemmli 1970) using a 5% (w/v) stacking and 12 % (w/v) polyarcylamide resolving gels. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue (CBB) R-250 and destained with a solution containing methanol: glacial acetic acid: distilled water (1:1:8 by volume). A low-range protein marker kit (Fermentas, USA) was used for the calibration.

2.1.6 Analysis of N-terminal amino acid sequence

The N-terminal amino acid sequence of the purified enzyme was determined by the automated Edman degradation method. For sequencing, the Procise 491 amino acid sequencer (Applied Biosystems, USA) was used.

2.1.7 Protease assay

Protease activity was assessed using the Folin-Ciocalteu method of Oda and Murao (Oda and Murao 1974) with slight modifications. Reaction mixture was prepared by mixing 0.1 ml enzyme sample with 0.05 ml 1% (w/v) azocasein in 10 mM Tris-HCl buffer (pH 7.0). Following incubation at 60 °C for 20 min, 0.06 ml ice-cold 10% (w/v) TCA was added and then immersed in ice water for 10 min. After centrifugation at 10,000 × g for 20 min, the supernatant was mixed with 0.3 ml Folin-Ciocalteus phenol reagent (0.33 M) and 0.45 ml Na_2CO_3 (10%, w/v) solution. The mixture was incubated at 30 °C for 30 min and the optical density of the produced color was measured at 660 nm using a UV-1601 spectrophotometer (Shimadzu Corporation, Japan). To assess the enzyme activity, a standard curve was generated using tyrosine solutions with different concentrations, ranging from 1-15 mg/l. Protease activity was quantified in unit (U), where 1U is defined to be the amount of enzyme required to release 1 µg tyrosine per min under the assay condition.

2.1.8. Enzyme assay

2.1.8.1. Fibrinolytic activity assay

Quantitative analyses of fibrinolytic activity were conducted using fibrin plates, following the method described by Astrup and Mullertz (Astrup and Mullertz 1952) with slight modifications. Fibrin plates were prepared by pouring the solution composed of 4.5 mg/ml fibrinogen in 10 mM Tris-HCl buffer (pH 7.0), 1.2% agarose and 0.45 U/ml thrombin into the petri dish. The solution in the plate was left for half an hour at room temperature to form fibrin clot. Ten µl of the purified enzyme with various concentrations (1 to 5 µg/well) was carefully loaded onto each circular well (3.5mm in diameter) on the plate and then the plate was incubated at 37 °C for 18 h. Fibrinolytic activity was estimated by measuring the diameter of the lytic circle around the well. The activity was expressed in the unit of lysed area per µg of the protein ($mm^2/\mu g$) (Giron et al. 2008). Plasmin from the human plasma was used as the positive control.

2.1.8.2 Fibrinogenolytic activity assay

Quantitative analyses of fibrinogenolytic activity were conducted following the reported method (Kim et al. 2008) with slight modifications. 150 µl fibrinogen (1 µg/µl) in 10 mM Tris-HCl buffer (pH 7.0) consisting 0.15 M NaCl was incubated at 37 °C with 45 µl purified enzyme (1 µg/µl) for various time durations. A fraction of the incubated sample (20 µl) was withdrawn at each considered time interval,

boiled for 5 min to terminate the reaction, and then cooled in ice-water. The resulting degradation products were analyzed by SDS-PAGE according to the specified method (Rao et al. 1998). Plasmin from human plasma was used as the positive control.

2.1.9. Effect of pH on enzyme activity and stability

To determine the optimal pH, enzyme assay was carried out with azocasein at various pH values (pH 4.0-12.0), under the standard assay protocol described earlier. Standard pH buffers (100 mM) were: citric acid-sodium phosphate buffer (pH 4.0-6.0), Tris-HCl buffer (pH 7.0-9.0), sodium bicarbonate-sodium hydroxide buffer (pH 10.0-11.0), and potassium chloride-sodium hydroxide buffer (pH 12.0). Likewise, stable pH range was determined by measuring the residual activities after incubating the enzyme for 24 h at 4 °C in the respective pH buffer (100 mM). The residual enzyme activities were calculated with reference to initial activity (prior to incubation in the buffers) considering 100%.

2.1.10 Effect of temperature on enzyme activity and stability

To determine the optimal temperature, enzyme assay was carried out at pH 7.0 and various temperatures ranging from 4 °C to 80 °C. Likewise, the thermal stability was evaluated by measuring the residual activities after incubating the enzyme sample at pH 7.0 and various temperatures for 15-90 min. The initial enzyme activity (prior to exposing to the various temperatures) was considered to be 100%.

2.1. 11 Effects of enzyme inhibitors and metal ions

In order to characterize the nature of the purified enzyme, effect of following protease inhibitors were examined: PMSF, pefabloc SC, EDTA, and EGTA. The purified enzyme was pre-incubated with various inhibitors for 1 h at room temperature before measuring the residual activity. The effect of various metal ions were investigated by adding 5 mM divalent metal ions such as $CaCl_2$, $CoCl_2$, $MgSO_4$, $ZnSO_4$, $CuSO_4$ and $FeSO_4$ to the reaction mixture. Enzyme activity measured in the absence of metal ions or inhibitors was considered 100%.

2.1. 12 Amidolytic activity of the enzyme

Amidolytic activity was measured spectrophotometrically using the following synthetic substrates: S-7388 (*N*-succynyl-Ala-Ala-Pro-phe-pNA for chymotrypsin), S-2765 (Z-D-Arg-Gly-Arg-pNA-2HCl for Xa), S-222 (Bz-Ile-(OR)-Gly-Arg-pNA for Xa), S-2251(H-D-Val-Leu-Lys-pNA for plasmin and streptokinase activated plasminogen), S-2238 (H-d-Phe-pip-Arg-pNA for thrombin), S-2288(H-D-Ile-Pro-Arg-pNA for t-PA) and S-2444(Pyro glu-gly-Arg-pNA for urokinase). After incubating the reaction mixture (total 0.5 ml containing 5µg/200µl purified enzyme and 0.5 mM substrate) in 10 mM Tris-HCl buffer (pH 7.0) for 5 min at 37 °C, the amount of liberated p-nitroaniline (pNA) was measured at 405 nm.

2.1.13 Determination of kinetic constants

The kinetic constants, Michaelis constant (K_m) and the maximum reaction rate (V_{max}), of the purified enzyme were determined by fitting the observed enzyme activities at different concentrations (0.05 to 1 mM) of *N*-succynyl-Ala-Ala-Pro-Phe-pNa to the standard Lineweaver-Burk double reciprocal plot (Lineweaver and Burk 1934). The reactions were performed in 10 mM Tris-HCl buffer (pH 7.0) at 60 °C for 5 min.

2.2 Results and discussion

2.2.1. Enzyme purification and molecular weight

Purification of FES624 was carried in the three sequential steps which are summarized in Table 2.1.

Table 2.1: Summary of purification of protease from *Streptomyces* sp. CS624

Purification steps	Total protein (mg)	Total activity[a] (U)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Crude extract	63.6	1770	27.84	100	1
Ammonium sulfate	34.5	1316	38.14	74	1.37
Sepharose CL- 6B	17.7	1002.9	56.66	56	2.03
Phenyl Sepharose CL-4B	0.086	91.19	1060	5	38

[a] Protease activity was assessed by Folin-Ciocalteu method using azocasein as the substrate.

The crude extract contained 63.6 mg protein; maximal activity was precipitated with ammonium sulfate at 80% saturation. Gel filtration of the ammonium sulfate fraction yielded a single major proteolytic peak (Fig.2.1a).

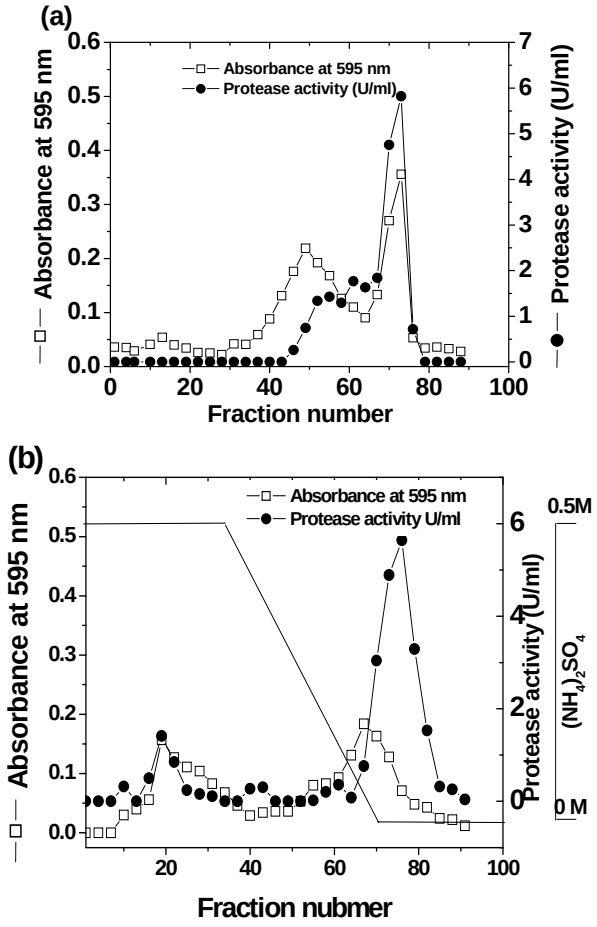

Fig. 2.1: Elution profile of FE624 from *Streptomyces* sp. CS624. **(a)** Gel filtration chromatography with Sepharose CL-6B column (116 × 2.2 cm). The proteins were eluted at a flow rate of 20ml/h. **(b)** Hydrophobic interaction chromatography with Phenyl Sepharose CL-6B column (20 × 1.2cm). The proteins were eluted at a flow rate of 30 ml/h with a linear gradient of 0.5-0 M $(NH_4)_2SO_4$.

Protein concentration (□) and the enzyme activity (●) in both cases were measured at 595 and 660 nm, respectively.

The active fractions were pooled, concentrated and purified using Phenyl Sepharose CL-4B which eluted two subsequent proteolytic peaks (Fig. 2.1b). The second and major peak retained the pure protein. Overall, 38-fold purification and 5% activity recovery (yield) was obtained after completion of the purification steps. SDS-PAGE of the purified enzyme was performed in order to verify the enzyme purity and to determine the molecular weight. The protein migrated as a single band and the molecular weight was estimated to be 18 kDa (Fig. 2.2). Molecular weight of the reported microbial fibrinolytic enzymes was in the range of 22.7 to 63.3 kDa (Peng et al. 2005; Lu et al. 2010). Thus, FES624 was one of the low molecular weight microbial fibrinolytic enzymes (Table 2.2) and the value is close to the recently reported 14 kDa serine protease from mushroom (*Pleurotous eryngii*) (Cha et al. 2010). The molecular weight of FES624 was exactly same as the fibrinolytic enzyme from *Rhizopuz chinensis* 12 (Liu et al. 2005). It is noteworthy to mention that the molecular weight of FES624 is the lowest among so far reported *Streptomyces* fibrinolytic enzymes as illustrated in Table 2.2.

Table 2.2: Comparison of different molecular and biochemical characteristics of FES624 with other fibrinolytic proteases/enzymes

Source	Enzyme	M.W.	Optimal		Stability		Specific Activity	Fibrinogenolysis	References
		(kDa)	pH	T (°C)	pH	T(°C)	(U/mg)	[Degradation Pattern]	
Streptomyces sp. CS624	Chymotrypsin like SMP	18	7	60	5.0-8.0	<50	1060	[Aα(5m),Bβ(10m),γ (2.5h)]	A
Streptomyces sp	Chymotrypsin like SMP	33	7.8	35-50	NA	NA	NA	NA	B
Streptomyces megasporus SD5	Chymotrypsin like SP	35	8	55	6.0-9.0	37-60	4200	NA	C
Streptomyces sp. CS684	SMP	35	7.5	45	6.0-9.0	<40	19	[Bβ(15m)]	D
Streptomyces corcohrussi	Plasminogen activator	34	6	50	5.0-8.0	40-50	1295	NA	E
Streptomyces sp Y405	Serine MP	30	8		4-9	4-37	2952.3	NA	F
Paenibacillus polymyxa EJS-3	Chymotrypsin like SP	63.3	7.5	37	6.0-8.0	30-40	2096	[Aα(15m),Bβ(30m),γ (4h)]	G
Korean *Cordyceps militaris*	Chymotrypsin like SMP	34	7	40	5.0-9.0	20-40	499	[Aα(60m),Bβ(2h),γ (4h)]	H
Armillaria mellea	Chymotrypsin like MP	21	6	33	NA	NA	1097	[Aα(1h), β(12h),γ(12h)]	I
Perenniporia fraxinea	Chymotrypsin like MP	42	6	35-40	5.0-6.0	<45	900	[Aα(5m),Bβ(30),γ(1h)]	J
Polychaete Neanthes japonica	SP	28-32	9	60	6.0-11.0	40-80	11232	[Aα(1m),Bβ(10m),γ (1h)]	K
Bacillus subtilis A26	SP	28	9	60	7.0-12.0	<50	3211	NA	L
Rhizopuz chinensis 12	MP	18	10.5	45	6.8-88	<37	2143.4	[Aα, Bβ, γ]	M
Bacillus subtilis TP-6	Subtilisin like P	27.5	7	50	6.0-6.5	ND	1197	[Aα(5m)]	N
Bacillus subtilis DC33	Subtilisin like SP	30	8	55	5-12.0	<60	15,494	[Bβ,Aα,γ]	O
Pleurotus eryngii	Plasmin like SP	14	5	40	4.0-6.0	<40	52.8	[Aα(5m),Bβ(10m),γ (6h)]	P

A: Current work; B: Bono et al. 1996; C: Chitte and Dey 2000; D: Simkhada et al. 2010; E: Kim et al. 2010; F: Wang et al. 1999; G: Lu et al. 2010; H: Choi et al. 2010; I: Lee et al. 2005; J: Kim et al. 2008; K: Deng et al. 2010; L: Agrebi et al. 2009; M: Liu et al. 2005; N: Kim et al. 2006; O: Wang et al. 2006; P: Cha et al. 2010

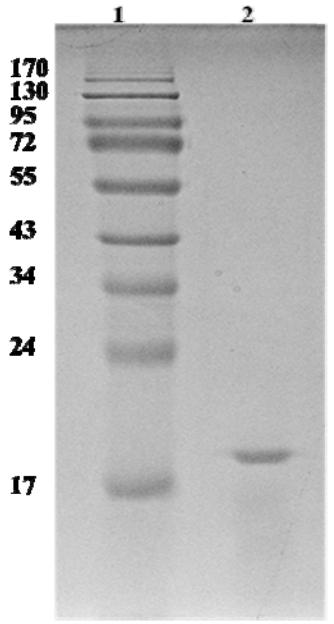

Fig. 2.2: SDS-PAGE of FES624; lane 1 corresponds to the standard protein marker with the corresponding value in kDa on the left, and lane 2 corresponds to the purified FES624. Electrophoretic analysis was carried out in 12% (w/v) gel. Gels were stained with CBB R-250 and destained with methanol: glacial acetic acid: distilled water (1:1:8 by volume).

2.2.2 Fibrinolytic and fibrinogenolytic activities

Fibrinolytic activity of FES624 was assayed using the fibrin plate method. After incubation at 37 °C, FES624 was noted to form relatively bigger clear zone around the well than that by equal amount of plasmin, as illustrated in the photograph

taken at 9 h (Fig. 2.3a). Since area of the clear zone is directly proportional to the activity, it can be said that the FES624 offers stronger fibrinolytic activity than that

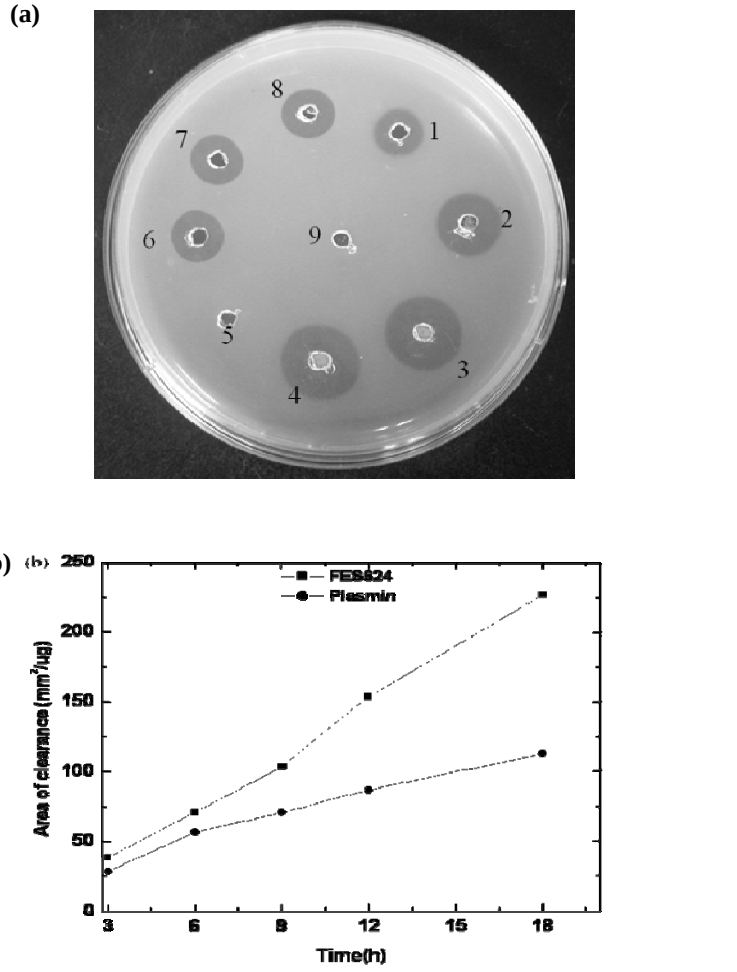

Fig. 2.3 Fibrino(geno)lytic activity of FES624. (**a**) Fibrinolytic activity on the fibrin plate. Numerically marked lytic circles were: 1-3, purified enzyme (1 μg, 3 μg and 5 μg, respectively); 4, plasminogen (10 mU) plus purified enzyme (5 μg); 5, plasminogen (10 mU); 6-8, plasmin (1 μg, 3 μg and 5 μg, respectively; and (9) negative control (buffer). (**b**) Comparison of fibrinolytic activities between the purified enzyme (1 μg) and plasmin (1 μg) after 3, 6, 9, 12 and 18 h. (**c**) SDS-PAGE of fibrinogen hydrolysis catalyzed by FES624. Lane 1 represents protein marker, lane 2 represents fibrinogen (20 μg) before incubation, and lane 3 to 9 represent hydrolyzed aliquots after 5, 10, 30, 60, 90, 120 and 150 min, respectively.

of plasmin. As can be illustrated in Fig. 2.3 b, the area of clear zone (at 18h) formed by FES624 was observed to be approximately 227 $mm^2/\mu g$ which was approximately 2-fold bigger than that of plasmin (113 $mm^2/\mu g$) (photograph at this time was not shown, since some lytic circles were large and overlapped). Furthermore, the fibrinolytic activity of FES624 was compared in the presence (10 mU) and the absence of plasminogen. Additional plasminogen did not contribute in the enhancement of the clear zone (Fig. 2.3a) implies that FES624 is a plasmin-like protease which directly degrades fibrin. It was thus different from the urokinase, streptokinase and t-PA which activate plasminogen. Fibrinogenolytic activity of FES624 was analyzed by SDS-PAGE (Fig. 2.3c). FES624 completely hydrolyzed the *A*α- and *B*β-chains within 5 and 10 min, respectively. The complete hydrolysis of γ-chain, however, took relatively longer time of 150 min. Thus, fibrinogen degradation pattern by FES624 is *A*α>*B*β>γ which is similar to other fibrinolytic

proteases (Kim et al. 2008; Lu et al. 2010; Cha et al. 2010; Deng et al. 2010; Choi et al. 2010; Lee et al. 2005). Even though the pattern is similar, FES624 completely degraded all the chains in comparatively less time than the other proteases do, except (Kim et al. 2008) and (Deng et al. 2008). Fibrinogen degradation pattern of FES624, however, is different to the pattern noted for fibrinolytic serine protease from *Streptomyces* sp. CS684 (Simkhada et al. 2010) and serine protease from *Bacillus subtilis* DC 33 (Wang et al. 2006), where *B*β–chain gets hydrolyzed first.

2.2.3 Effect of pH and temperature

Comprehensive comparison of pH and temperature profiles of FES624 with related enzymes is presented in Table 2.2. The optimal pH for FES624 was found to be 7.0 (Fig. 2.4a). The enzyme was completely stable at pH 7.0 and <90% activity remained between pH 6.5 and 7.5 (Fig. 2.4b). The activity vigorously tends to decrease in acidic and alkaline range; therefore, it is a neutral enzyme. FES624 showed maximum activity at 60 °C and remained stable at or below 50 °C (Fig. 2.5). The pH optimum of FES624 coincides with that of subtilisin-like protease from *Bacillus subtillis* (Kim et al. 2006) and chymotripsin-like serine metalloprotease from *Cordyceps militaris* (Choi et al. 2010), while its pH stability is observed to be comparatively wider than *B. subtillis* (Kim et al. 2006). Likewise, the optimal temperature for FES624 coincides with that for serine proteases from *Polychaete neanthes japonica* (Deng et al. 2010)and *B. subtillis* A26 (Agrebi et al.

2009). Thermal stability range for FES624 is observed to be comparatively wider than that of chymotrypsin-like serine protease from *Paenibacillus polymyxa* EJS-3 (Lu et al. 2010) and chymotripsin like serine metalloprotease from *Cordyceps militaris* (Choi et al. 2010).

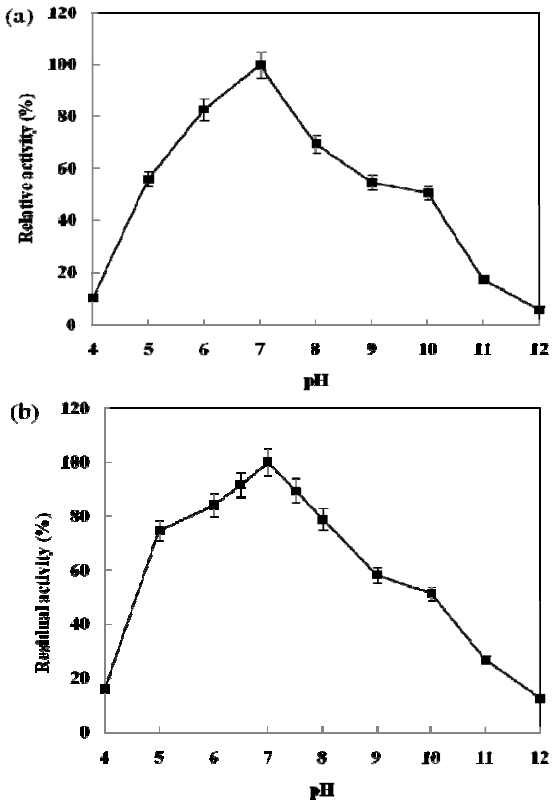

Fig. 2.4: Effect of pH on enzyme activity of FES624. **(a)** Optimal pH was determined by assessing the enzyme activity in the pH range 4.0-12.0. **(b)** Stable pH range was determined by measuring the residual enzyme activities after incubating the enzyme at various pHs for 24 h.

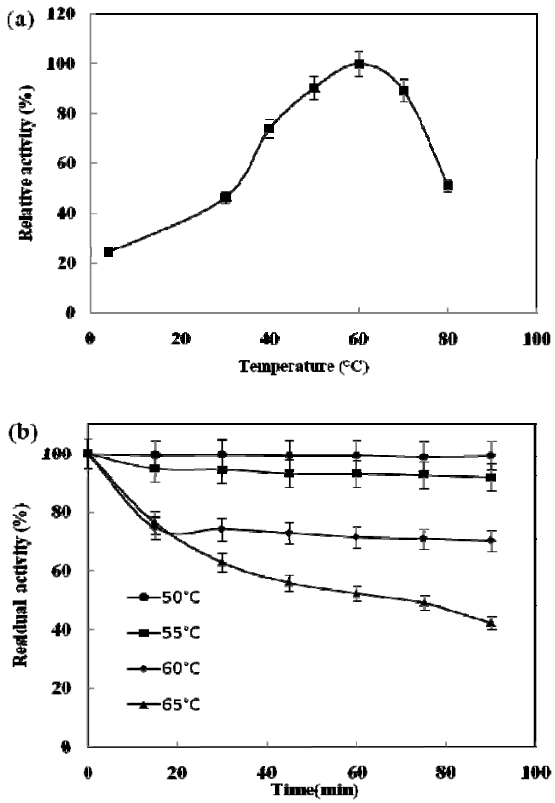

Fig. 2.5: Effect of temperature on the enzyme activity of FES624. **(a)** Optimal temperature was determined by performing the enzyme assay in different temperature ranging from 4-80 °C. **(b)** Thermostability of the enzyme was evaluated by measuring the residual enzyme activities after incubating the enzyme in various temperatures for 15-90 min at pH 7.0.

2.2.4 Effect of protease inhibitors and metal ions

The effects of various inhibitors and metal ions are summarized in Table 2.3. The activity was resistant to serine protease inhibitor PMSF while the activity was

noticeably inhibited by other serine protease inhibitor Pefabloc SC. It is consistent with the previous reports of *Streptomyces* serine proteases (Simkhada et al. 2010; Bono et al. 1996). It suggests that FES624 may belong to serine protease family which is known to have involvement of serine in the catalytic activity. The activity was also significantly inhibited by metalloprotease inhibitors EDTA and EGTA, suggesting that FES624 also belongs to metalloprotease family. Furthermore, metal ions showed varied effect on the FES624 activity. The activity was completely inhibited by Fe^{2+}, significantly inhibited by Cu^{2+}, Zn^{2+} and Co^{2+} and slightly enhanced by Ca^{2+} and Mg^{2+} (Table 2.3). Considering these all, FES624 can be categorized as a serine metalloprotease as our previous report (Simkhada et al. 2010).

Table 2.3: Effect of metal ions and inhibitors on FES624

Metal ions or inhibitors	Concentration (mM)	Relative activity[a] (%)
None	—	100
$CaCl_2$	5	129± 3
$CoCl_2$	5	10 ± 0.6
$MgCl_2$	5	116 ± 1.8
$ZnSO_4$	5	8 ± 0.5
$CuSO_4$	5	8 ± 0.3
$FeSO_4$	5	0
Pefabloc SC	2	81 ± 3.28
	4	61± 5.14
PMSF	4	99± 3.46
EDTA	1	12 ± 2.49
EGTA	1	23 ± 1.64

2.2.5 Amidolytic activity

The amidolytic activity of FES624 was assessed with several synthetic chromogenic substrates (Table 2.4). FES624 exhibited highest activity against N-succinyl-Ala-Ala-Pro-Phe-pNa, a substrate for chymotrypsin, suggesting that it is a chymotrypsin-like protease. This type of fibrinolytic enzymes has also been reported from *Cordyceps militaris* (Choi et al. 2010), *Perennipiria fraxinea* (Kim et al. 2008), *Armillaria mella* (Lee et al. 2005) and *Fomitella fraxinea* (Lee et al. 2006). Amidolytic study incorporating with the protease inhibitors study (Table 2.3) suggests that FES624 is a chymotrypsin-like serine metalloprotease.

Table 2.4: Substrate specificity of FES624

Chromogenic substrate	Amino acid sequence	Characteristics	Specific activity (mM min^{-1} mg^{-1})
S-7388	N-succinyl-Ala-Ala-Pro-Phe-pNA	For chymotrypsin	67.17
S-2765	Z-D-Arg-Gly-Arg-pNA-2HCl	For factor Xa	5.76
S-2222	Bz-Ile-(OR)-Gly-Arg-pNA	For factor Xa	5.99
S-2251	H-D-Val-Leu-Lys-pNA	For plasmin and SAP*	15.56
S-2238	H-d-Phe-pip-Arg-pNA	For thrombin	9.7
S-2288	H-D-Ile-Pro-Arg-pNA	For t-PA	18.42
S-2444	Pyro glu-gly-Arg-pNA	For urokinase	7.07

*Streptokinase activated plasminogen

2.2.6 Kinetic parameters

The Michaelis constant (K_m) and the maximum reaction velocity (V_{max}) of FES624 for the substrate N-succynyl-Ala-Ala-Pro-Phe-pNa were determined to be 0.218 mM and 84.03 mM min^{-1} mg^{-1}, respectively. The K_m value of FES624 for the mentioned substrate was found to be comparable with some of the previously reported fibrinolytic enzymes: 0.21 mM, 0.214 mM and 0.226 mM for *Cordyceps militaris* (Choi et al. 2010), *Fomitella fraxinea* (Lee et al. 2006), and *Rhizopus chinensis* 12 (Liu et al. 2005), respectively.

2.2.7 Amino acid sequence analysis

The first fifteen N-terminal sequence of FES624 was APNVDAIYLPQYRLS. The sequence was compared with the other sequences available in the protein database maintained by National Centre for Biotechnology Information (NCBI) using basic local alignment search tool (BLAST) (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The BLAST search did not suggest perfect homology with the other protease sequences but it showed a high degree of homology with the sequences of serine proteases from *Pacifastacus leniusculus* and *Penaeus monodon*, and low homology with the sequences of a serine protein kinase from *Micromonas* sp. RCC299. The sequences homology between FES624 and that of fibrinolytic serine proteases from *Streptomyces* (*S. fradiae* and *S. griseus*) is very low (Table 2.5), suggesting that FES624 may be a new enzyme of its kind.

Table 2.5: Alignment of N-terminal sequences of FES624 and closely related serine proteases or a fibrinolytic enzyme from different sources

S. No	Enzyme	N-terminal amino acid[a]
1	FES624	APNVDAIYLPQYRLS
2	Serine protease from *Pacifastacus leniusculus*	APNVDTLCLPNQDQN
3	Serine protease from *Penaeus monodon*	APNVDTVCLPQANQK
4	Serine protease from *Streptomyces fradiae*	IAGGEAIYAAGGGRC
5	Serine protein kinase from *Micromonas sp.* RCC299	SAAMAEIYLPNYRMG
6	Fibrinolytic enzyme from *Streptomyces griseus*	RAAOGEFPFMMVRLS

[a]The identical amino acid residues between FES624 and the other compared enzymes are shaded.

2.3 Conclusion

A chymotrypsin-like serine metalloprotease with fibrinolytic activity was purified from *Streptomyces* sp. CS624. Its molecular weight was determined to be 18 kDa which is the lowest among the so far reported *Streptomyces* fibrinolytic enzymes. It showed a very low N-terminal amino acid sequences homology with the previously reported fibrinolytic enzymes. The enzyme of this study, which is highly active and stable in neutral pH and moderate temperature range and is capable to degrade fibrin clot by direct fibrinolysis, more effectively than plasmin, can potentially be utilized for the treatment of thrombosis.

3. Organic-Solvent Tolerant Lipolytic Enzyme

With the aim of producing an enzyme suitable for the enzymatic transesterification of vegetable oils for the production of biodiesel, we have produced and purified, an organic solvent tolerant lipase, referred to as LS133, from the *Streptomyces* sp. CS133. In what follows, we report the methods that we followed for the purification and biochemical characterization of LS133 and discuss the results highlighting its peculiar characteristics.

3.1 Materials and Methods

3.1.1 Materials

Para–nitrophenol (*p*-NP) esters (except *p*-NP stearate) and triolein were purchased from Sigma (St. Louis, MO, USA). *p*-NP stearate was purchased from MP Biomedicals (Solon, OH, USA). Sepharose CL-6B and Phenyl Sepharose CL-4B gel were obtained from Pharmacia (Uppasala, Sweden) and Applied Biosystems (CA, USA), respectively. PMSF and EDTA were obtained from Roche Applied Science (Mannheim, Germany). Thin layer chromatography (TLC) silica gel 60 plates and organic solvents were purchased from E-Merck (Darmstadt, Germany).

3.1.2 Screening and identification of microbial strain

Among the several hundreds of microbial strains stocked in our laboratory, a strain CS133 was found to possess lipolytic activity and was selected for this study. For

the identification, morphological characteristics and 16S rDNA sequences of the strain were analyzed as described by Yoo (Yoo et al. 2007). The analysis revealed maximum sequence homology (99.8 %) with *Streptomyces xanthocidicus.* The 16S rDNA sequence (accession number: JN177516) was deposited in the GenBank® (http://www.ncbi.nlm.nih.gov/genbank/). The strain was grown for a week at 28 °C on OSYM agar plate containing (in g/l): oat meal, 20; soybean meal, 10; yeast extract, 10; mannitol, 10; and agar, 15. A loopful of spores were scraped from the plate and inoculated into the seed medium containing (in g/l): beef extract, 4; yeast extract, 1; peptone, 4; glucose, 10, and NaCl, 2.5. It was cultivated for 24 h in a shaking incubator (180 rpm) at 28 °C. Four percentages of this preculture was transferred to lipase producing culture medium containing (g /l): lactose, 10; soybean, 10; and KCl, 1 and was further cultivated for 96 h in a shaking incubator (180 rpm) at 28 °C.

3.1.3 Purification of lipase

The culture broth of strain CS133 was centrifuged at 6000 × *g* for 1 h at 4 °C. In order to precipitate proteins, solid $(NH_4)_2SO_4$ was added to the supernatant to the final concentration of 80 % saturation. Precipitate was collected by centrifugation at 6000 × *g* for 1 h at 4 °C, dissolved in 10 mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer for 24 h. The dialysate was then loaded on Sepharose CL-6B column (1.1 × 90 cm) equilibrated with 10 mM Tris-HCl buffer

(pH 8.0). All fractions were assayed for protein content and lipase activity. Fractions showing lipase activity were pooled, concentrated using 10 kDa centricon (Amicon, USA), and then loaded on phenyl Sepharose CL-4B column (1.2 × 20 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.5 M $(NH_4)_2SO_4$. The bound proteins were eluted by gradient of 10 mM Tris-HCl buffer (pH 8.0) containing 0.5 to 0 M $(NH_4)_2SO_4$ at flow rate of 30 ml/h. Finally, fractions containing high lipase activity were concentrated using 10 kDa centricon.

3.1.4 Protein determination and lipase activity assay

Protein concentration was determined spectrophotometrically (at 595 nm) following the method of Bradford (Bradford 1996) using bovine serum albumin as the standard. Lipase activity was assayed using *p*-NP-palmitate *(p*-NPP) as a substrate. This assay was performed according to Winkler and Stuckman (Winkler and Stuckman 1979) with some modifications. Firstly, the substrate *p*-NPP was dissolved in acetonitrile (10 mM). Then the substrate (25 µl) was mixed with 350 µl Tris-HCl buffer (100 mM, pH 7.5) containing 0.5 % triton X-100 and 0.15 M NaCl. The reaction was performed at 40 ºC by adding 25 µl of suitably diluted enzyme solution to the mixture. The reaction was terminated after 30 min by immersing the reaction mixture into ice water. The aliquots were centrifuged at 6000 rpm for 10 min. The absorbance of the supernatant containing released *p*-NP was measured at 405 nm using a Multiskan EX microplate reader (Thermo

Scientific, USA). Appropriate blanks were incorporated to subtract possible unwanted absorbance due to factors other than specific hydrolysis of *p*-NPP. One unit of enzyme activity was defined as the amount of enzyme needed to liberate 1 μM min^{-1} of *p*-NP under the standard assay conditions.

3.1.5 Determination of molecular mass

3.1.5.1 SDS-PAGE

The molecular mass of the purified enzyme was determined by SDS-PAGE as described in Chapter 2.

3.1.5.2 Zymography

Zymograms were developed using methylumbelliferyl (MUF) heptanoate as the substrate as described by De Sousa (De Sousa et al. 2010) with minor modifications. After electrophoresis, the gel was washed in 2.5 % Triton X-100 (30 min.). The gel was then transferred to 10 mM Tris– HCl buffer (pH 7.5) for protein renaturation. Next, the gel was incubated in 4-MUF heptanoate solution (prepared with 3.2 mg MUF- heptanoate in 1ml toluene). After 10 min, the fluorescence signal was detected and captured using a UV transilluminator.

3.1.6 Analysis of N terminal amino acid sequence

For N-terminal amino acid sequencing, the purified lipase (15 μg) after SDS-PAGE was electrophoretically transferred to a polyvinylidene difluoride (PVDF)

membrane using Tris-HCl buffer (10mM, pH 7.5). Sequencing was carried out in the Procise 491 amino acid sequencer (Applied Biosystems, USA) according to the automated Edman degradation method.

3.1.7 Effect of pH on lipase activity and stability

To determine the effect of pH on lipase activity and stability, various pH buffer systems were used: citric acid-sodium phosphate buffer (pH 4.0-6.0), Tris-HCl buffer (pH7.0-9.0), and sodium bicarbonate-sodium hydroxide buffer (pH 10.0-11.0). To determine the optimum pH, lipase activities were measured at different pH values (pH 5.0-10.0) using different buffers (100 mM) following the standard assay protocol. Similarly, the stability of lipase was determined by measuring the residual activities upon incubating it for 1 h at 40 °C in different pH buffers (pH 4.0-11.0). The lipase activity (prior to the incubation in the buffer) was considered to be 100 %.

3.1.8 Effect of temperature on lipase activity and stability

To determine the effect of temperature on lipase activity the reaction was performed at various temperatures ranging from 10 °C to 50 °C. Similarly the thermal stability of enzyme was evaluated by measuring the residual activities at different time intervals (0 to 120 min) after incubating the enzyme solution (pH 8.0) at various temperatures (50 °C to 80 °C). The residual activity was measured

according to the *p*-NPP method as described earlier. The initial lipase activity (prior exposing to the various temperatures) was considered to be 100 %.

3.1.9 Effect of chemicals on lipase stability

The effect of various metal ions (Ca^{2+}, Co^{2+}, Na^{+}, Mn^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}), enzyme inhibitors (EDTA and PMSF), reducing agent (mercaptoethanol), and oxidizing agents (hydrogen peroxide and sodium perborate) on the lipase activity was investigated at the final concentration of 1 mM, 5mM and 10 mM. The enzyme was pre-incubated with any of the selected chemicals for 1 h at 40 °C. The residual lipase activity was measured against control (100 %, no chemical added).

3.1.10 Effect of detergents on lipase activity

The effect of various detergents (0.5% v/v), including Triton X-100, Tween-20, Tween-80, Deoxycholic acid, N-Laurylsarcosine, SDS and 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) (CHAPS), on the lipase activity was investigated following the *p*-NPP method as described earlier. Lipase activity measured in the absence of detergents was considered to be 100 %. As to be reveled in next section, Triton X-100 significantly enhanced lipase activity than the other considered detergents. Hence, to further examine the effect of Triton X-100 on enzyme, activities were assayed in the broad range of concentrations, 0.0625 % to 1 % (v/v).

3.1.11 Substrate specificity and determination of kinetic parameters

To determine the substrate specificity, the following *p*-NP esters (0.625 mM) of various chain length were used to measure enzyme activity under standard assay condisions: *p*-NP acetate (C_2), *p*-NP butyrate (C_4), *p*-NP decanoate (C_{10}), *p*-NP myristate (C_{14}), *p*-NP palmitate (C_{16}) and *p*-NP stearate (C_{18}). The maximum initial rate of consumption of substrates (V_{max}) and Michaelis-Menten constant (K_m) of the purified lipase were determined by fitting the observed lipase activities at different concentrations (0.097 mM to 3.125 mM) of *p*-NP palmitate to the standard Lineweaver-Burk double reciprocal plot (Lineweaver and Burk 1934). The reactions were performed with 1µg of purified lipase in 10 mM Tris-HCl buffer (pH 8.0) at 40 °C for 30 min.

3.1.12 Effect of organic solvents on lipase stability

To study the effect of organic solvents on enzyme stability, suitably diluted enzyme in 10 mM Tris-HCl (pH 8.0) was mixed with different organic solvents to yield a final concentration of (25 %, v/v) and then the mixture was incubated on a shaking incubator (180 rpm) at 30 °C for 48 h. The residual lipase activity was measured using *p*-NPP method. The used organic solvents were decane, octane, heptane, hexane, xylene, toluene, benzene, dichloromethane, diethylether, ethylacetate, isopraponal, acetone, ethanol, acetonitrile, methanol and dimethyl sulfoxide (DMSO). The initial lipase activity (without containing organic solvents)

was considered to be 100 %. Among the evaluated organic solvents, methanol has popularly been used as an organic media in enzymatic transesterification of vegetable oils and animal fats. Thus, to examine the stability properties of the purified lipase in such media, residual activities were examined after 24 h of incubation at various ranges of methanol concentration ranging from 5 % to 50 % (v/v).

3.1.13 Positional specificity

Positional specificity was determined by analyzing hydrolyzed products of triolein on TLC plate (silica gel 60) as described by Demir and Tukel (Demir and Tukel 2010) with some modifications. Reaction mixture (150 µl) was prepared by mixing purified enzyme (50 µl) and triolein (30 µl) in 10mM Tris–HCl buffer (70 µl). The reaction mixture was incubated in a shaking incubator (200 rpm) at 30 °C for 24 h. Then the mixture was extracted with 60 µl of chloroform, centrifuged and the upper supernatant layer was applied to silica gel plates. The plates were then developed in a chamber containing solvent mixture of chloroform, acetone and acetic acid (95:4:1) and the spots of the hydrolyzed products were visualized using saturated iodine vapor.

3.1.14 Application of the purified lipase in biodiesel production

Motivated by the organic solvent tolerance property of the purified lipase, its catalytic ability in biodiesel production was accessed. Enzymatic transesterification

of commercial soybean and olive oil in methanol was performed according to the method described in (Yang et al. 2009) with some modifications. First of all, a mixture of soybean oil or olive oil (3.94 ml) and methanol (0.49 ml) were mixed. To this mixture, enzyme solution (250 U by *p*-NPP spectrophotometric assay) prepared in 10mM Tris-HCl buffer (pH 8.0) was added. Then the reaction mixture was put in screw capped glass vials (20 ml) and the transesterification reaction was conducted up to 48 h at 30 °C with rotary shaking (200 rpm). Aliquots (100 µl) were withdrawn at different predetermined time interval and mixed with (200 µl) of hexane. Upon performing centrifugal separation of the mixture, 5 µl of the upper layer was applied to TLC plate. The plate was developed in the chamber containing a solvent mixture of hexane, acetone, and acetic acid (95:4:1) and was air dried. The plate was then immersed in potassium permanganate solution (0.5 % w/v) and the biodiesel spot were visualized. The visualized spots were compared with a reference biodiesel, fatty acid methyl ester.

3.2 Results and discussion

3.2.1 Purification of lipase and determination of molecular mass

Three sequential purification steps were carried to increase the specific activity of LS133 while retaining as much as possible of the activity. The results are summarized in Table 3.1.

Table 3.1: Summary of purification of lipase from *Streptomyces* sp. CS133

Purification steps	**Total protein**	**Total activity**	**Specific activity**	**Yield**	**Fold**
	(mg)	(U)	(U/mg)	(%)	
Ammonium sulfate precipitation (80 %)	41.50	7100	171.0	100	1.0
Sepharose CL-6B	9.60	2964	308.7	41.7	1.80
Phenyl Sepharose CL-4B	1.74	1240	712.6	17.4	4.17

After the first purification step that employed 80 % ammonium sulfate precipitation, 41.5 mg of protein was obtained with specific activity of 171 U/mg. The subsequent step of gel permeation chromatography using Sepharose CL-6B yielded 1.8-fold purification thereby retaining 41.7 % activity. Since lipases are hydrophobic in nature and display strong interaction with hydrophobic supports, hydrophobic interaction column has been used in the purification of many lipases that were reviewed in (Sharma et al. 2001). Likewise, in the current work, hydrophobic column chromatography using Sepharose CL-4B was used as the final step of purification. Finally, 4.17-fold purification, thereby retaining 17.4% activity, was achieved with specific activity of 712.6 U/mg. Ji et al. reported 4.3 fold purification of an organic solvent tolerant lipase from *Pseudomonas aeruginosa* LX1 with specific activity of 156 U/mg (Ji et al. 2010), which is significantly lower than that of LS133. Likewise, the specific activity of LS133 is higher than that of lipases from *Streptomyces rimosus* (659.2 U/mg) (Abrami et al.

1999) and *Pseudomonas fluorescens* MTCC 2421 (424.04 U/mg) (Chakraborty and Paulraj et al. 2009). The molecular mass of LS133 was determined by SDS-PAGE.

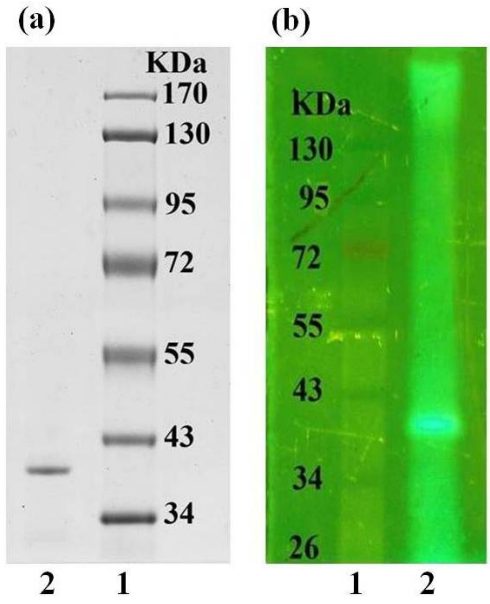

Fig. 3.1: SDS PAGE and zymagrophy of LS133. (a) SDS-PAGE of LS133 lipase. Lane 1, Sigma protein molecular mass marker; lane 2, Phenyl Sepharose CL-4B purified lipase. (b) Zymography using MUF- heptanoate as a substrate. Lane 1, molecular mass marker; lane 2, activity of the purified lipase.

The protein migrated as a single band (Fig. 3.1a). Its molecular mass was estimated to be 39.8 kDa which is higher than that of lipase (27.5 kDa) from *Streptomyces rimosus* (Abrami et al. 1999) and lower than that of lipase (50 kDa) from *Streptomyces cinnamomeus* (Sommer et al. 1997). Zymography with MUF-

heptanoate as the substrate yielded only one band (a signing fluorescence band in Fig. 3.1b), with the same molecular mass.

3.2.2 Analysis of N terminal amino acid sequence

The first fifteen residues of N-terminal amino acid sequence of LS133 were determined to be AIPLRQTLNFQAXYQ. When compared with the sequences of other *Streptomyces* lipases (*Streptomyces coelicolor* A3(2), *Streptomyces pristinaespiralis* ATCC 25486, *Streptomyces albus* J1074, *Streptomyces* sp. AA4, *Streptomyces avermitilias*, and *Streptomyces clavuligerus*) deposited in the National Centre for Biotechnology Information (NCBI) database, no perfect homology was found. As can be noted in Table 3.2, the N-terminal amino acid sequence of lipase LS133 showed highest identity (40%) to that of lipase from *Streptomyces* sp. AA4 and *Streptomyces clavuligerus.* LS133 had some closely comparable enzymatic features as that of SCO7513 lipase (~33kDa) from *Streptomyces coelicolor* A3(2) (Cote and Shareck 2007), with which N-terminal sequence homology was 26.7%. Temperature optimality (45-55 ^{0}C) and pH optimality (8.5) of SCO7513 were nearly close to that of LS133. In regard to substrate specificity, both LS133 and SCO7513 lipases were selective for substrate with longer acyl chains (C_{16}).

Table 3.2: Alignment of N-terminal sequences of LS133 with other lipases from *Streptomyces*[a]

Lipase source	N-terminal amino acid sequence															Identity (%)
S. sp. CS133	**A**	**I**	**P**	**L**	**R**	**Q**	**T**	**L**	**N**	**F**	**Q**	**A**	**X**	**Y**	**Q**	100
S. coelicolor A3(2)	**A**	**I**	**P**	**L**	T	P	W	N	L	D	K	S	W	P	E	26.7
S. pristinaespiralis 25486	**A**	**I**	**P**	**L**	T	G	W	N	L	D	K	T	W	P	E	26.7
S. albus J1074	**A**	**I**	**P**	**L**	T	P	W	N	L	D	K	T	W	P	E	26.7
S. sp. AA4	**A**	S	**P**	**L**	**R**	A	**T**	**L**	E	D	L	A	G	L	P	40.0
S. avermitilias	G	N	V	**L**	**R**	**Q**	**T**	M	I	D	S	L	Y	A	P	26.7
S. clavuligerus	**A**	F	M	S	N	**Q**	S	F	**N**	**F**	**Q**	**A**	G	A	P	40.0

[a]The identical amino acid residues of LS133 and the other compared enzymes are marked with bold letters.

3.2.3 Effect of pH and temperature on lipase activity and stability

Fig. 3.2a depicts the effect of pH on activity of LS133 at various pH values. LS133 was found to exhibit maximum activity at pH 7.5. It was stable in the adequate range of pH values (5.0-8.5), retaining more than 95 % of its original activity (Fig. 3.2b). On the other hand, significant reduction in the lipase activity was observed in the low acidic range (< pH 4.0) and at high alkaline range (> pH 10.0). LS133 is stable in mild acidic to mild alkali range, unlike the acidic-only stability (pH 3.0-6.0) observed in lipase A from *Geotrichum* sp. SYBC (Cai et al. 2009), which might be a desirable property for various industrial processes. pH stability of LS133 was comparable to that of other *Streptomyces* lipases including *Streptomyces coelicolor* A3 (pH 6.0-9.0) (Cote and Shareck 2007), *Streptomyces rimosus* R6-554W (pH 4.0-10.0) (Abrami et al. 1999), and *Streptomyces fradiae*

var. k11 (pH 4.0-10.0) (Zhang et al. 2008). Fig. 3.2c depicts the effect of temperature on activity of LS133. It showed maximum activity at 40 °C as lipase from *Pseudomonas aeruginosa* LX1 (Ji et al. 2010). Abrami et al. and Cote et al. previously reported an extracellular lipase from *Streptomyces rimosus* (Abrami et al. 1999) and SCO7513 lipase from *Streptomyces coelicolor* A3(2) (Cote and Shareck 2007) with optimal temperatures at 45°C and 45-55°C, respectively. Thermostability of LS133 was analyzed for various temperatures by measuring the residual activities at interval of 30 min for 2 h (Fig. 3.2d). The enzyme was highly stable at 50 °C, as lipase from *Pseudomonas aeruginosa* LX1 while the thermal stability profile at the other higher investigated temperatures (60, 70, and 80 °C) showed that the enzyme retained 65 %, 45 % and 21 % of the activity, respectively, for only 30 min implying LS133 is not stable above 50 °C.

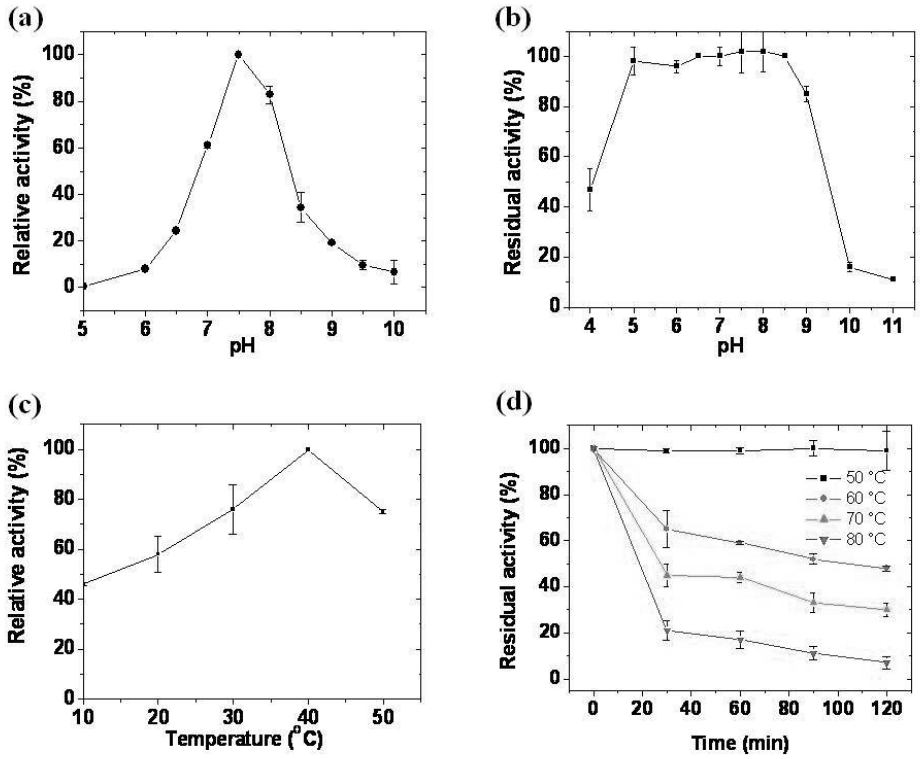

Fig. 3.2: Effect of pH and temperature on activity and stability of LS133: (a) pH optimality. Optimal pH was determined by assessing the enzyme activity at pH 5.0-10.0; (b) pH stability. Stable pH range was determined by measuring the residual enzyme activities after incubating the enzyme at various pH buffers (4.0-11.0) for 1 h at 40 °C.; (c) Temperature optimality. Optimal temperature was determined by performing the enzyme assay in different temperature ranging from 10-50 °C; and (d) Temperature stability. Stable temperature range was determined by measuring the residual enzyme activities after incubating the lipase in various temperatures at (50 – 70 °C) at pH 8.0.

3.2.4 Effect of various chemicals on lipase activity

Effects of different metal ions, reducing agents, and oxidizing agents on the activity of LS133 are shown in Table 3.3.

Table 3.3: Effect of various chemicals on the stability of LS133[a]

Metal ions	**Residual activity (%)**		
	(1 mM)	**(5mM)**	**(10 mM)**
None	100 ± 0	100± 0	100 ± 0
NaCl	103 ± 2.1	104± 2.2	106 ± 2.4
$MnSO_4$	102 ± 5.2	102± 3.1	103 ± 2.9
$CoSO_2$	102 ± 3.8	91± 2.6	80 ± 1.8
$CaCl_2$	100 ± 5.8	97± 4.1	96 ± 3.7
$FeSO_4$	85 ± 5.0	78± 3.9	70 ± 4.9
$CuSO_4$	85 ± 2.3	67± 4.8	56 ± 6.1
$ZnSO_4$	62 ± 7.5	39± 3.9	23 ± 3.7
Modulators	**(1 mM)**	**(5mM)**	**(10 mM)**
EDTA	110 ± 1.3	107± 2.2	99 ± 7.2
Sodium perborate	99 ± 4.8	85± 3.4	88 ± 5.7
H_2O_2	94 ± 7.9	76± 4.9	56 ± 3.9
Mercaptoethanol	103 ± 4.8	102± 3.2	97 ± 4.5
PMSF	98 ± 3.9	65± 3.6	40 ± 2.7

[a] The lipase was pre-incubated with the metal ions and modulators for 1 h at 40 °C before measuring the residual activity. The presented results are the average of three repeated experiments with ± standard deviation from the mean.

Among the considered metal ions, Zn^{2+}, Cu^{2+} and Fe^{2+} (in the descending order of inhibitory effect) showed noticeable effects at the considered concentrations of 1 mM, 5mM, and 10 mM while Co^{2+} managed to minimally inhibit (around 10 %

and 20 %) at the higher concentrations of 5 mM and 10 mM. The inhibitory activity of transition metal cations (Fe, Cu, and Zn and Co) at higher concentration may be due to the direct inhibition of the catalytic site and/or formation of complexes between metal ions and ionized fatty acids (Chakraborty and Paulraj 2009). On the other hand Mn^{2+} and Na^{+} marginally enhanced the activity at all of the considered concentrations. In general, lipases were understood to be stimulated in the presence of Ca^{2+}. This effect has been suggested to be due to the formation of long-chain fatty acid calcium salts (Kim et al. 2005). However, unlike such effect, the activity of LS133 was slightly reduced in the presence of $Ca^{2+.}$ It is interesting to note that EDTA, a metal ion chelator, stimulated the lipase activity at the lower concentrations of 1 mM and 5mM while the activity at the higher concentration (10 mM) was unaltered. Similar behavior of EDTA was observed in a lipase from *Amycolatopsis mediterranei* DSM 43304 (Dheenan et al. 2011). Since the presence of EDTA did not abolish the activity it can be said that LS133 is not a metalloprotein. Oxidizing agents such as sodium perborate and H_2O_2 had less inhibitory effects when they were applied in the lower concentration. However, when the concentration was raised to 10 mM, sodium perborate and H_2O_2 inhibited around 12% and 44% of the activity, respectively. Reducing agents are often cited to be lipase inhibitors. Mercaptoethanol, however, stimulated LS133 activity (at lower concentration of 1mM), though marginally. Dheeman et al. and Soliman et al. also observed the similar effect of Mercaptoethanol on lipase from *amycolatopis*

mediterranei (Dheeman et al. 2011) and *geobacillus thermoleovorans* (Soliman et al. 2007), respectively. PMSF significantly inhibited the activity of LS133, especially when used in the higher concentration of 10mM, indicating the involvement of serine in its catalytic activity.

3.2.5 Effect of detergents on lipase activity

Effect of different nonionic, ionic, and zwitterionic detergents on the lipase activity was investigated (Table 3. 4).

Table 3.4: Effect of detergents on the activity of LS133

Detergents	Concentration (%)	Relative activity (%)
None	–	100 ± 0
Chaps	0.5	75 ± 5.3
N lauryl sarcosine	0.5	75 ± 0.3
Deoxy cholic acid	0.5	67 ± 4.0
Tween 80	0.5	147 ± 6.5
Tween 20	0.5	194 ± 5.6
SDS	0.5	89 ± 1.9
Triton X-100	0.0625	394 ± 1.39
Triton X-100	0.125	469 ± 1.74
Triton X-100	0.25	459 ± 8.36
Triton X-100	0.5	376 ± 8.01
Triton X-100	1	269 ± 7.33

Among the tested nonionic detergents, the highest enhancement in the enzyme activity was observed in presence of Triton-X 100, followed by Tween-20 and Tween-80. The non-ionic detergents seem to weaken the hydrophobic interaction

within the protein causing disaggregation and thus stimulating its activity. Even the lowest considered concentration (0.0625%) of Triton X-100 enhanced the activity by more than 294%. Nawani et al. also observed the similar effect of Triton X-100, Tween-20 and tween-80 on activity of a lipase purified from *Bacillus* sp. (Nawani et al. 2007). On the other hand, the lipase activity was slightly decreased in the presence of three anionic detergents (SDS, N-lauryl sarcosine, and deoxy cholic acid) and zwitterionic CHAPS. This can be attributed to the fact that anionic detergents act upon the disulphide linkages and cause inactivation of protein (Borkar et al. 2009). Since triton X-100 had the best effect on the lipase activation, lipase activity was further examined by varying its concentrations and it was noted that the optimum stimulation can be achieved by lowering its concentration to around 0.125 % (v/v).

3.2.6 Substrate specificity and determination of kinetic parameters

The activity of LS133 towards various substrates was investigated with *p*-NP alkanoate esters for varying alkyl chain lengths. Following were the observation: (C_2; 11 ± 4.10), (C_4; 12 ± 1.80), (C_{10}; 56 ± 4.20), (C_{14}; 76 ± 1.81), (C_{16}; 100 ± 0.0) and (C_{18}; 81±2.70). Note that the results are presented in the format (C_x;y) where y is the relative activity (in %) for the varying alkyl chain length x. The highest hydrolysis rate was obtained with *p*-NP palmitate (C_{16}) as in the case of a lipase from *Streptomyces coelicolor* (Cote and Shareck 2007). A lipase from

Streptomyces rimosus (Abrami et al. 1999), however, showed its preference for the mid acyl chain lengh (C_8-C_{12}). Ji et al., Peng et al., and Gaur et al. reported three lipases from *Pseudomonas aeruginosa* which also showed their preference for the longer acyl chain length (C_{16}) (Ji et al. 2010; Peng et al. 2010; Gaur et al. 2008).

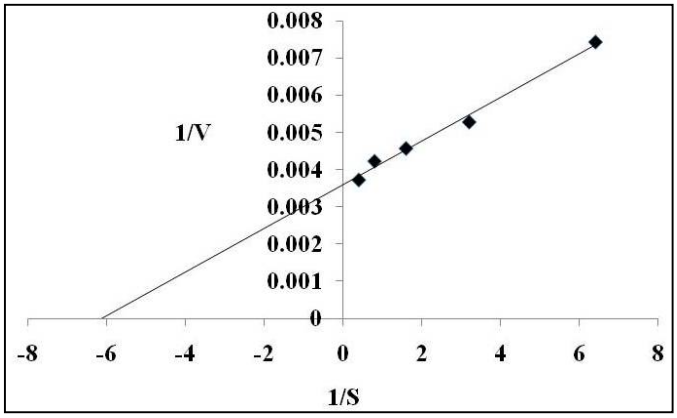

Fig. 3.3: Lineweaver-Burk plot of purified LS133 lipase using *p*-NP palmitate as a substrate.

The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) of LS133 for the substrate *p*-NP palmitate were determined to be 0.152 mM and 270.2 mmol min^{-1} mg^{-1}, respectively, based on the Lineweaver-Burk plot in Fig. 3.3. The ratio V_{max}/K_m defines a measure of the catalytic efficiency of an enzyme-substrate pair. The catalytic efficiency of the lipase LS133 was found to be approximately 70-fold and 49-fold efficient than that of lipases from *Amycolatopis mediterranei* DSM 43304 (Dheeman et al. 2011) and *Ralstonia* (Yoo et al. 2011), respectively.

3.2.7 Positional specificity

Lipases have selectivity to the position of the ester bond in triglyceride. Based on such selectivity, lipases can be categorized into either position specific or non-specific. Positional specificity of LS133 was investigated using triolein as a substrate. It hydrolyzed triolein and released not only 1,3 diolein but also 1,2-diolein as the end product (Fig. 3.4).

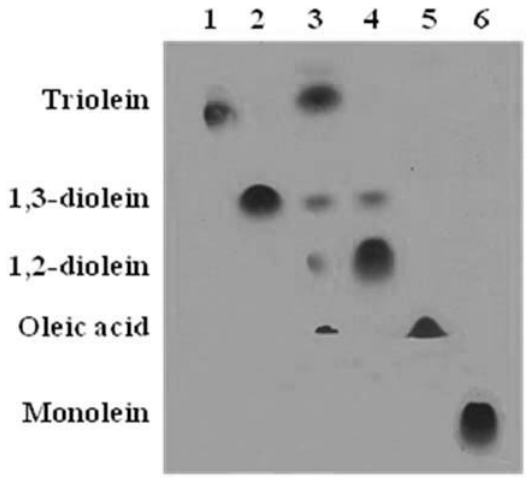

Fig. 3.4: Positional specificity of LS133. Marked lanes have the following meaning: Lane 1, triolein; Lane 2, 1,3 diolein; Lane 3, hydrolyzed product of triolein; Lane 4, 1,2 diolein; Lane 5, oleic acid; and Lane 6, monolein.

This indicated that LS133 is non position specific. Lipases from *Streptomyces rimosus* (Lescic et al. 2001), *Bacillus thermoleovarans* ID-1 (Lee et al. 2001), and *Pseudomonas aeruginosa* (Ogino et al. 2000) were also reported to be non-positional specific.

3.2.8 Effect of organic solvents on lipase stability

Organic solvents with various *log P* values, ranging from -1.37 to 5.6, were selected to examine organic solvent tolerance of LS133, where *log P* value for a solvent is a logarithm of the partition coefficient that provides a measure of differential solubility of the solvent in water and n-octanol. Table 3.5a presents the effect of those organic solvents on lipase activity in term of residual activity.

Table 3.5a: Effects of various organic solvents on the stability of LS133[a]

Solvents (25%, v/v)	Log *P*	Residual activity (%)
None		100 ± 0
Decane	5.6	94 ± 5.9
Octane	4.9	173 ± 6.7
Heptane	4.0	155 ± 4.1
Hexane	3.5	179 ± 6.2
Xylene	3.1	130 ± 5.2
Toulene	2.5	154 ± 4.4
Benzene	2.0	161 ± 7.2
Dichloromethane	1.25	129 ± 5.1
Diethylether	0.87	123 ± 3.8
Ethyl acetate	0.71	49 ± 2.0
Isopropanol	0.074	24 ± 5.8
Acetone	-0.23	41 ± 0.8
Ethanol	-0.24	48 ± 4.6
Acetonitrile	-0.34	29 ± 0.1
Methanol	-0.76	80 ± 3.0
DMSO	-1.37	159 ± 0.7

[a]25 % (v/v) of organic solvents were added to the enzyme solution and incubated for 48 h in a rotary shaker (180 rpm) at 30 °C

Activity of LS133 was noted to be significantly enhanced in the presence of organic solvents having *log P* greater than or equal to 0.87 (except for the case of decane in which the activity was minimally inhibited). For example, in the particular cases of hexane (*log P* 3.5) and octane (*log P* 4.9), the residual activities were 179 % and 173 %, respectively.

Lipase from *Ralstonia* (Yoo et al. 2011) is noted to be unstable in all of the aforesaid organic solvents. If the lipase stability in presence of various organic solvents is considered, polar (water miscible) solvents, in general, are more destabilizing than their non-polar counterparts. The results, enhancement in the lipase activity in presence of non-polar solvents benzene (log P 2), toluene (log P 2.5), Xylene (log 3.1), hexane (log 3.5), hectane (log 4.0) and octane (log P 4.9) are in good agreement with those general understandings. The increase in activity of LS133 in the aforesaid non-polar solvents could be due to the hydrophobicity of these solvents. In particular, those hydrophobic solvents could not easily strip the essential water molecule associated with the lipase, which is necessary for the active conformation. On the other hand, in the presence of solvents having *log* p within the range 0.71 to -0.34, LS133 significantly lost its activity. For, example in the case of acetonitrile, the activity fell to as low as 29%. Similar trend of the activity loss was previously noticed for the lipase from *Pseudomonas aeruginosa* LX1 (Ji et al. 2010). Most importantly, LS133 was adequately stable in methanol

retaining more than two-third of its activity for 48 h. Since methanol is one of the popularly used organic media for biodiesel production, such stability is highly desirable. Motivated with such desirable methanol tolerance property of LS133 at a typical methanol concentration of 25 % (v/v), the level of tolerance at various methanol concentrations was examined (Table 3.5b). It is quite interesting to note that the activity of LS133 was not only perfectly stable for the duration of 24 h but also was enhanced for the broad range of methanol concentrations, 5 % to 30 % (v/v). In a recent work (Yang et al. 2009), it has been shown that different microbial lipases like *Candida antarctica* lipase B (Cal B), *Candida rugosa* lipase, and *Pseudomonas cepacia* loose their activity (as quickly as 30 min) even in lower concentration of methanol (as low as 10 %). In comparison to such results the methanol tolerance offered by LS133 is very promising.

Table 3.5b: Effect of methanol on the stability of LS133[b]

MeOH % (v/v)	**Residual activity %**
None	100 ± 0
5	115 ± 5.3
10	116 ± 2.5
20	117 ± 1.0
30	107 ± 4.2
40	53 ± 1.0
50	23 ± 0.2

[b]Different concentration of methanol were added to enzyme solution and incubated for 24 h in a rotary shaker (180 rpm) at 30 °C

3.2.9 Application of the purified lipase in biodiesel production

LS133 catalyzed transesterification reaction of both soybean oil and olive oil in methanol (Fig. 3.5), though it preferred soybean oil over olive oil.

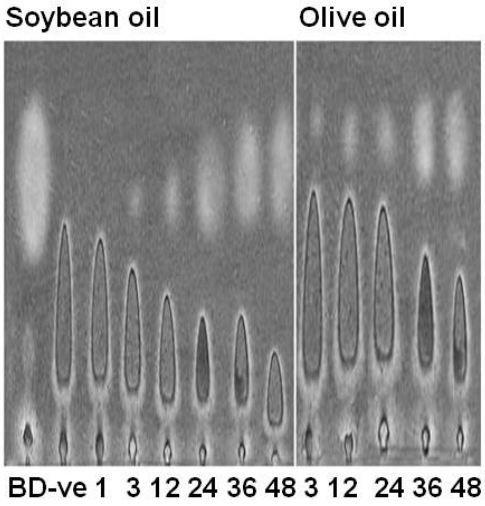

Fig. 3.5: TLC analysis of LS133 catalyzed biodiesel (BD) production using methanol and soybean/olive oil. The gray spots in the figure are the BD spots. Negative control corresponds to the absence of LS133 in the reaction medium. Numeric values at the bottom of the figures are time in unit of h.

The production nearly reached its maximum at around 24 h in the case of soybean oil while it took additional 12 h in case of olive oil. Upon comparing the required reaction time for various microbial lipases, which is reported to be in the range 5-72 h (Bajaj et al. 2010), one can say that even though the reported time for LS133 is not the fastest among others but it is comparable in some cases and significantly

faster in many cases. In particular it is significantly faster than the time reported in two recent works (Kumari et al. 2009; Yang et al. 2009), 60 h and 72 h respectively, for transesterification of Jatropha and soybean oil in methanol using a lipase extracted from *Enterobacter aerogenes* and *Pseudomonas fluorescens,* respectively.

It is noteworthy to mention that both reaction rate and conversion ratio of any enzymatic transesterification reactions depend primarily on various factors, such as methanol concentration, amount of catalyst, temperature, pH, agitation speed and so on**.** The values selected in this current work (those reported in the method section) were based on the preliminary optimization procedure carried according to one variable at a time (OVAT) approach. However, such optimization may not always be able to infer the exact non-linear correlation involved among the multiple variables and may depict false optimality (huge deviation from the actual optimality). Hence, as a future work we are looking forward to optimize those parameters and extend the current subjective analysis (TLC plate based analysis) of the biodiesel production with quantitative analysis incorporating gas chromatography.

3.3 Conclusion

A lipase with molecular weight of about 39.8 kDa was purified from *Streptomyces* sp. CS133 and its biochemical characteristics were investigated which revealed numerous industrially important characteristics including stability in both acidic and alkaline pH, resistance to various detergents, oxidizing agents, reducing agents, and organic solvents. Its organic solvent tolerance capability was exploited to seek application in enzymatic biodiesel production. The lipase was found to catalyze biodiesel synthesis from various vegetable oils, such as soybean and olive oil, in methanol.

4. An Acidic Xylanolytic Enzyme

With the aim of producing an enzyme suitable for the enzymatic hydrolysis of cellulosic agricultural waste, we have produced and purified, an acidic xylanase, referred to as EX624 from the *Streptomyces* sp. CS624. In what follows, we report the methods that we followed for the purification and biochemical characterization of EX624 and discuss the results highlighting its peculiar characteristics.

4.1 Materials and methods

4.1.1 Materials

All the reagents and chemicals used for the study were obtained from Sigma (St. Louis, USA). Standard xylooligosaccharides (xylose, xylobiose, xylotriose, and xylotetraose) were purchased from Megazyme (Ireland). TLC silica gel 60 plates were purchased from E-Merck (Darmstadt, Germany). EDTA was obtained from Roche Applied Science (Mannheim, Germany). Agro-residues, such as corncobs and wheat bran, were purchased from the local market. Sephadex G75 and Tris acryl gel were obtained from Pharmacia (Uppasala, Sweden) and IBF (Villeneuve-la-Garenne, France), respectively.

4.1.2 Microbial strain and cultivation

A bacterial strain CS624 was isolated from the soil samples collected from Cheonnam province, Korea. It was identified as *Streptomyces* based on

morpholigical and 16S rDNA sequences as described in our previous paper (Mander et al. 2011). The strain was examined for its ability to produce xylanase enzyme by growing it at 37°C for 48 h on xylan agar plate (containing (in g/l) : xylan, 5; yeast extract, 0.5; tryptone, 10; KH_2PO_4, 7.5; K_2HPO_4, 1.5; $MgSO_4$, 0.5; and agar, 10). The plate was then stained with congo red solution (0.5% w/v) prepared in distilled water for 15 minutes and then destained with 1M NaCl as described in (Ninawe et al. 2006).

4.1.3 Culture medium for xylanase production

For the production of xylanase, strain CS624 was streaked on OSYM agar plate and incubated for a week at 28 °C and transferred to seed culture medium as previously described in our recent paper (Mander et al. 2011). Effects of various agro wastes and residues as a carbon source for the xylanase production was assessed by transferring the preculture (4% v/v) in the xylanase producing medium which consisted of the following (in g/l): any of the agro-waste substrates among wheat bran, corn cob, banana peel, citrus peel, and orange peel, 10; yeast extract, 0.5, tryptone, 10, KH_2PO_4, 7.5; K_2HPO_4, 1.5 and MgSO4, 0.5. The culture medium was further cultivated in a shaking incubator (180 rpm) at 28 °C for 90 h.

4.1.4 Purification of xylanase

The culture broth was harvested and centrifuged at 6000 × g for 1 h at 4°C to obtain the supernatant. Solid $(NH_4)_2SO_4$ was added with stirring to the supernatant

to give 30-80% saturation to precipitate the proteins. Precipitated proteins were recovered by centrifugation at 6000 × *g* for 1 h at 4 °C. The precipitate, suspended in 10 mM citrate buffer (pH 6.0), was dialyzed against the same buffer and then concentrated by ultra-filtration using YM 30 membrane (Milipore Corp., USA). The resulting concentrated sample was loaded on CM Tris acryl column (10 × 1cm) equilibrated with 10mM citrate buffer (pH 6.0). The bound proteins were eluted by gradient of same buffer containing 0 to 1M KCl at flow rate of 30 ml/h. The active xylanase fractions with high specific activities were pooled, concentrated, and then purified using gel filtration with Sephadex G-75 column (25 x 1 cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.0). Proteins were eluted in the same buffer at elution speed of 30 ml/h. After monitoring the enzyme purity by gel electrophoresis and zymogram analysis, various biochemical characteristics of the enzyme were investigated.

4.1.5 Protein determination and xylanase activity assay

Protein concentration was determined as described in previous chapter. The xylanase activity was determined by measuring the release of reducing sugar from the soluble xylan using 3,5-dinitrosalicyclic (DNS) method described by Miller (Miller 1959). The standard assay mixture contained 100 µl of appropriately diluted enzyme and 100 µl of 1% (w/v) beechwood xylan prepared in 10mM citrate buffer (pH 6.0). The enzyme and substrate mixture was then incubated at 50°C for

10 min. After the incubation, the reaction mixture was taken out from the water bath and 100 µl of DNS reagent was added and boiled for 10 min, and cooled by immersing in ice water bath. The amount of reduced sugar released from xylan was measured by absorbance at 540 nm. One unit of xylanase activity is defined as the amount of enzyme that releases 1 µmol of xylose equivalent reducing sugar per minute.

4.1.6 Determination of molecular mass

The molecular mass of the purified enzyme was determined by SDS-PAGE as described in Chapter 3. Zymography was carried as described in (Hung et al. 2011), but with some minor modifications as briefly discussed below. Samples without heat treatment were applied to SDS-PAGE with 12% acrylamide gel containing 0.5% (w/v) beechwood xylan. Upon completing electrophoresis, the gel was washed twice with 10 mM citric buffer (pH 6.0) containing 25% isopropanol for 30 min and then soaked in the citrate buffer for 15 min. To carry out the enzymatic reaction, the gel was incubated in the citrate buffer for 10 min at 50 °C. The gel was then stained with 0.2% (w/v) Congo red solution for 10 min at room temperature and destained with 1.0 M NaCl. Then the gel was fixed by dipping it into 0.5% acetic acid solution.

4.1.7 Effect of pH on xylanase activity and stability

Various pH buffer systems (10 mM), citric acid-sodium citrate buffer (pH 4.0-6.0), Tris-HCl buffer (pH7.0-9.0), sodium bicarbonate-sodium hydroxide buffer (pH 10.0-11.0) and potassium chloride-sodium hydroxide (pH 12-13), were used to evaluate the effect of pH on the xylanase activity and stability. Xylanase activity against those aforesaid pH buffers was measured following the standard assay protocol and optimum pH (at which the highest activity was measured) was determined. Similarly, for analyzing the stability of xylanase, the enzyme solution was preincubated in the aforesaid pH buffers (20 mM) for 1 h at 40 °C. The residual enzyme activity was determined by measuring the activity at the previously determined optimum pH following the standard assay protocol. The xylanase activity (prior to the incubation in the buffer) was considered to be 100 %.

4.1.8 Effect of buffer concentration on xylanase activity

The effect of buffer concentrations on xylanase activity was detected with citric acid and sodium citrate pH buffers (4.0 to 7.5) at different concentrations of buffer ranging from 5 to 60 mM following the standard assay protocol.

4.1.9 Effect of temperature on xylanase activity and stability

Effect of temperature on xylanase activity was determined by incubating the enzyme along with substrate at different temperature (40-70 °C) in citrate buffer

(10 mM) for the duration of 10 min. Thermal stability of the xylanase was evaluated by measuring the residual activities after incubating the enzyme solution at various temperatures (10~80°C) for 1h. The residual activity was measured according to the standard assay method as described earlier.

4.1.10 Effect of metal ions and reagents on xylanase activity

The effect of various metal ions on xylanase activity was determined by incubating the enzyme at 60 °C following the standard assay method along with 1mM or 10 mM of the following in the reaction mixture: NaCl, KCl, $FeSO_4$, $CoSO_4$, $CuSO_4$, $MnSO_4$, $ZnSO_4$, and $MgCl_2$. Likewise, effect of the following other chemicals on the activity was determined in the same way: enzyme inhibitor EDTA, detergent SDS, and reducing agent β-mercaptoethanol (β-Met). Enzyme activity measured in the absence of the metal ions or chemicals was considered to be 100%.

4.1.11 Substrate specificity and determination of kinetic parameters

The substrate specificity of the xylanase for several substrates was determined after incubating the 100 µl of suitably diluted enzyme at 60°C for 10 min in citrate buffer (10 mM, pH 6.0) containing one of the following substrates (1% w/v): beechwood xylan, birchwood xylan, carboxymethyl cellulose (CMC) and Avicel. Reactions were terminated by adding 100 µl DNS. For a given amount of the purified xylanase, the amount of released reducing sugar was estimated as described above.

The kinetic constants, Michaelis constant (K_m) and the maximum reaction rate (V_{max}), of the purified xylanase were determined using the Lineweaver Burk equation after incubating it in citrate buffer (10 mM, pH 6.0), containing 1-10 mg ml^{-1} of beechwood xylan or birchwood xylan, at 60 °C for 10 min.

4.1.12 Analysis of hydrolysis product

Hydrolytic product analysis was carried out using TLC as describe by Ninawe (Ninawe et al. 2008). For the enzymatic hydrolysis of birchwood xylan and beechwood xylan, the reaction mixture consisting of 20 mg of each substrate in 2.0 ml of 10 mM citrate buffer (pH 6.0) was incubated with 200 µl of suitably diluted enzyme at 40°C for 48h. At regular time intervals, aliquots were withdrawn, boiled for 5 min and centrifuged at 10,000 rpm. The supernatant obtained were analyzed qualitatively by spotting them on the silica gel plates. A solvent system containing chloroform:acetic acid:water (6:7:1 v/v) was used to separate the end products. The chromatogram was developed by spraying the solution of concentrated H_2SO_4:C_2H_5OH (5:95 v/v) and heating the plate at 100°C for few minutes. A mixture of xylooligosaccharides consisting xylose (X1), xylobiose (X2), xylotriose (X3) and xylotetraose (X4) was used as the standard as in (Yan et al. 2009).

4.1.13 Biodegradation of agricultural lingocellulosic waste

To determine the xylanase capacity to degrade agricultural lignocellulosic wastes, agro wastes (wheat bran and corn cob) were first washed with distilled water and

then dried completely. For enzymatic biodegradation, corn cob (20 mg) or wheat bran (20 mg) was treated with enzyme solution (700 U/ml) at 40°C for 2.5 h. Agro wastes treated with buffer only (without enzyme) were considered as control. After reaction, the samples were centrifuged at 3000 rpm for 5 min, the supernatant was discarded and the precipitates were collected and dried in hot air oven at 55°C until completely dried. Structures of lignocellulosic materials treated after enzymatic hydrolysis was analyzed by scanning electron microscope (SEM) analysis (Wang et al. 2012).

4.2 Results and Discussion

4.2.1 Strain identification

The 16S rDNA gene sequence analysis of CS624 was performed and it showed 100 % sequence homology to *Streptomyces griseofuscus* (Accession no. AB184206), *Streptomyces murinus* (Accession no. AB184155) and *Streptomyces costaricanus* (Accession no. AY999910). A phylogenetic tree constructed via the neighbor-joining method for homologus family of *Streptomyces* sp. CS624 is presented in Fig. 4.1.

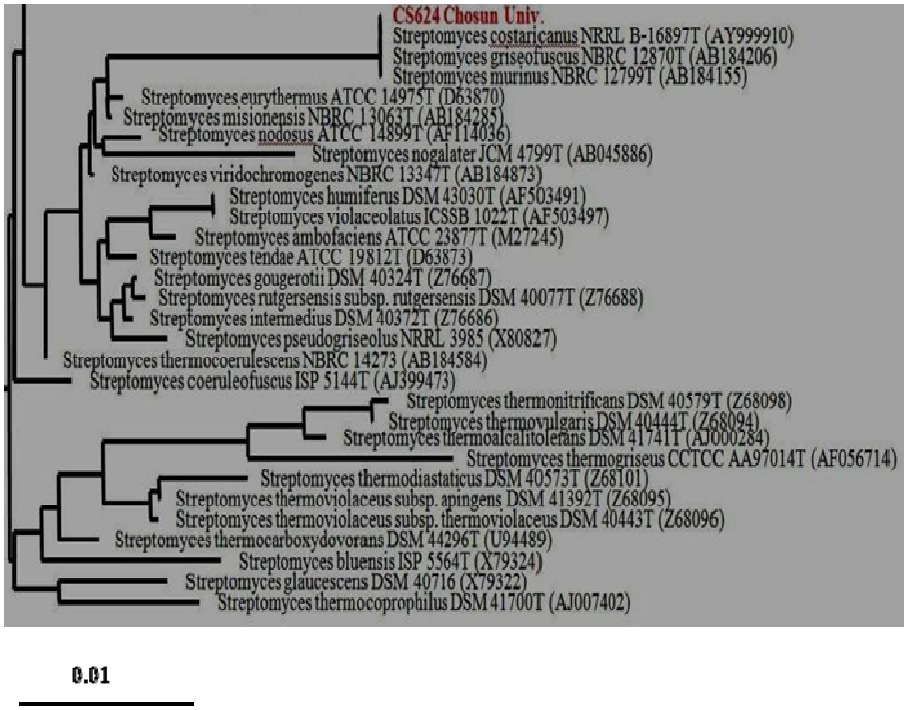

Fig. 4.1: Phylogenetic tree of *Streptomyces* sp CS624 and related *Streptomyces* strain based on 16 S rDNA sequence analysis. The bar represents 0.01nucleotide substitution per site.

The strain is identified as a xylanase producer since it showed clear lytic zones surrounding the colonies against red back ground, as shown in Fig. 4.2, when it was grown on the xylan agar plate at 37°C for 48 h and stained with congo red solution. Strain CS624 was further tested for xylanase production in liquid culture medium containing various economic agro residues and wastes including wheat bran, corn cob, and banana peels.

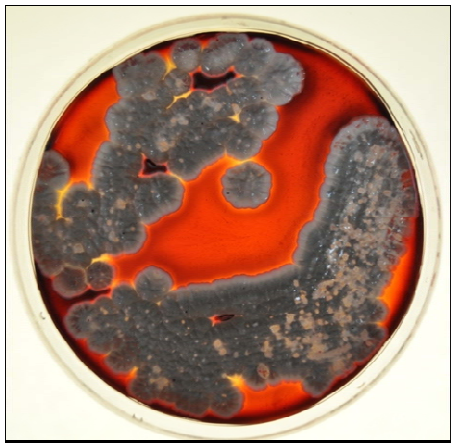

Fig 4.2: Clearing haloes around colonies of CS624 by congo-red plate assay. CS624 strain was grown on xylan agar plate containing 1% (w/v) beechwood xylan. After 48h incubation at 37°C, plate was flooded with congo red solution and destained with 1% NaCl.

4.2.2 Xylanase production

To reduce the cost of xylanase production, it is desirable to use inexpensive agro wastes and residues as the carbon source in the xylanase production medium rather than the commercially available xylan which are generally expensive. Various agro wastes such as corn cob, wheat bran, orange peel, banana peel, and citrus peel are accessed for such purpose and their effect in xylanase yield is compared to that of the commercial beechwood xylan. As can be noted in the Table 4.1, among the considered agro wastes as the carbon source, wheat bran yielded the highest xylanase production (3276 U/ml) while orange peel yielded the lowest xylanase production (1003 U/ml).

Table 4.1: Effect of agro waste as a carbon source on xylanase production from *Streptomyces* sp. CS624

Substrates (1% w/v)	**Xylanase yield (U/ml)**
Beechwood xylan	3752
Corn cob	2153
Wheat bran	3276
Orange peel	1003
Banana peel	1100
Citrus peel	1160

It is interesting to note that the effect of wheat bran as a carbon source is nearly comparable to that of the commercial beechwood xylan (3752 U/ml). Similar to our case, wheat bran has been reported as an ideally suitable substrate for other microorganisms including some *Streptomyces* strains: *Streptomyces* sp. QG-11-3 *and Streptomyces* sp. RCK -2010 (Beg et al. 2000 ; Kumar et al. 2012). Such a suitability of the wheat bran as the substrate in the xylanase producing medium is due to the complete nutritious feed (containing various soluble sugars) that it contains which are helpful for the initiation of growth and replication of microorganisms and remain loose even under moist conditions providing a large surface area. In addition, higher xylanase production on wheat bran may be due to low lignin and silica content (Kumar et al. 2012).

4.2.3 Purification of xylanase

A summary for the purification of EX624 xylanase from *Streptomyces* sp. CS624 strain is presented in Table 4.2.

Table 4.2: Summary of purification of xylanase from Streptomyces CS624

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Fold
A. sulfate fraction	6.3	104665	16613	100	1
CM Tris acryl	2.48	78184	31526	74.7	1.9
Sephadex G75	0.41	25180	61415	24	3.7

EX624 was purified to 3.7 fold with a recovery of 24 % after two step of purification using column chromatography. The molecular mass of EX624 was determined to be 40 kDa by SDS-PAGE as shown in Fig. 4.3a.

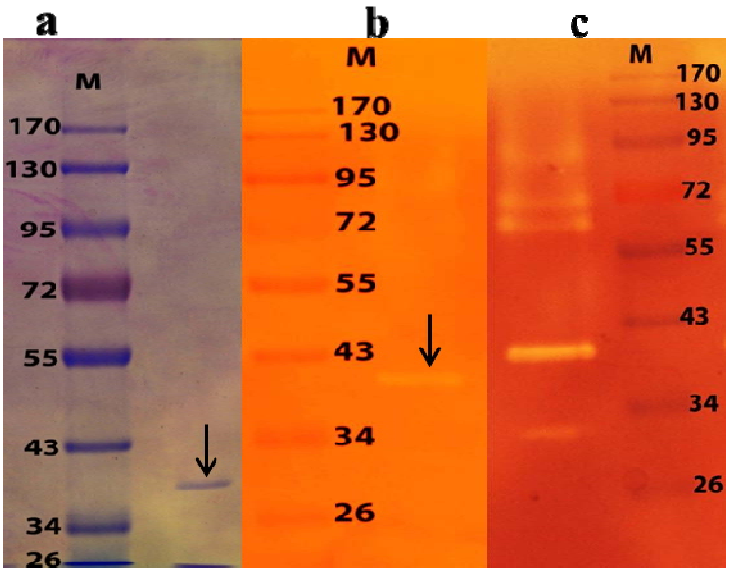

Fig 4.3: SDS-PAGE and Zymograph of EX624. (a) SDS-PAGE analysis of the purified EX624, (b) Zymograph analysis of the purified enzyme, (c) Zymograph showing multiple form of xylanases in the crude enzyme. Lane M represents standard protein molecular weight markers.

Microbial xylanases have been reported to have their molecular mass within the average range of 11-85kDa (Ninawe et al. 2008). Zymogram analysis revealed the presence of a zone of hydrolysis which corresponds to the SDS-PAGE band of the purified xylanase Fig. 4.3b. On the other hand, zymogram analysis of the partially purified xylanase showed multiple bands, among which the 40kDa band was prominent as can be noted in Fig. 4.3c. The other bands were minor and comprised of both high and low molecular mass xylanases within the range 30 to 90 kDa. This indicated that *Streptomyces* sp. CS624 was producer of multiple forms of

xylanases. Multiple xylanases could be due to several factors such as different mRNA processing, partial proteolysis or differences in the degree of amidation and glycosylation (Lenardoviz et al. 2002). Different microorganisms have been reported to produce multiple xylanases, as in our case, for example *Bacillus* sp. strain 41M-1 (Bataillon et al. 2000) and *Gracilibacillus* sp. TSCPVG (Giridhar and Chandra 2010).

4.2.4 Effect of pH and temperature on xylanase activity and stability

Purified EX624 exhibited optimal activity at 60°C and pH 6.0 (Fig. 4.4a and Fig. 4.4c). Several other xylanases from *Streptomyces,* for example *Streptomyces cyaneus* SN32 (Ninawe et al. 2008), *Streptomyces olivaceoviridis* E-86 (Kaneko et al. 2000) and *Streptomyces* sp. K37 (Mansour et al. 2003), have been reported to have the same optimal pH and temperature. The thermal stability of the enzyme was maintained above 80% for the duration of 1h at temperatures ≤ 60°C (Fig. 4.4d). EX624 retained over 70% of its activity in the pH range 5.0-10.0, but its activity decreased sharply outside this range (Fig. 4.4b). Buffer concentration was found to have completely opposite effects in the lower and higher value of considered pH range (4.0-7.5). Increase in the buffer concentration was found to have negative effect in the lower pH, while it had positive effect in the higher pH (Fig. 4.5).

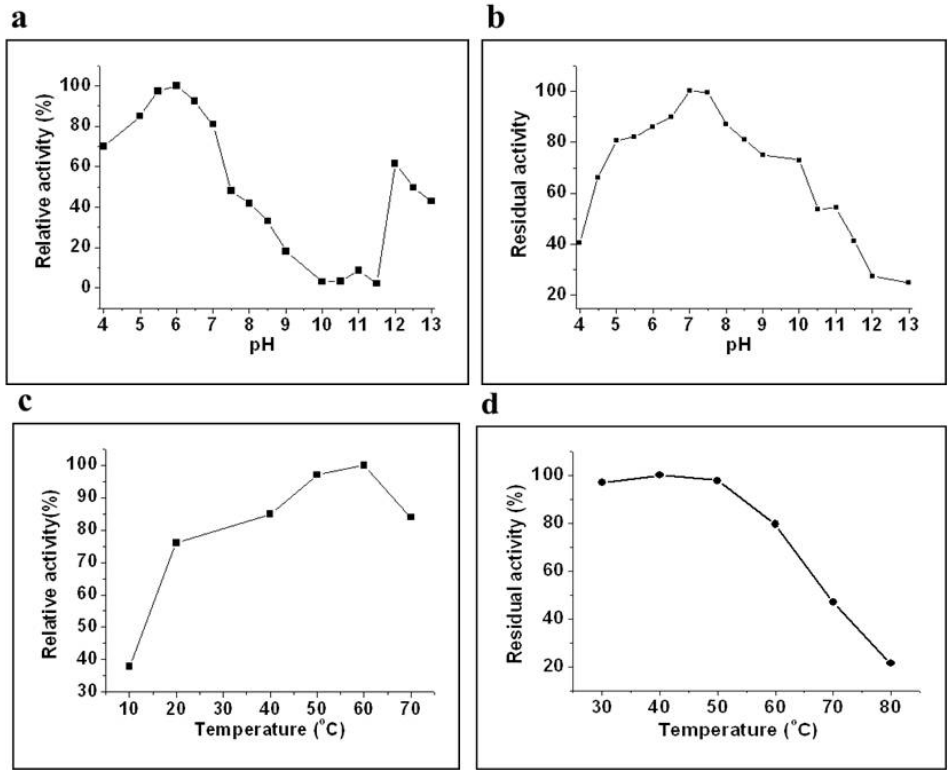

Fig 4.4: Effect of pH and temperature on EX624 xylanase activity: (a) Optimal pH, (b) pH stability, (c) Optimal temperature, and (d) Thermal stability.

Fig 4.5: Effect of buffer concentration on EX624 xylanase activity.

4.2.5 Effect of metal ions

Effect of metal ions and modulators on activity of EX624 is shown in Table 4.3. The xylanase activity of EX624 was significantly enhanced by the metal ions Fe^{2+}, Co^{2+} and Ca^{2+} at both the higher and lower concentration of 1mM and 10 mM, while it was significantly inhibited by Zn^{2+} and Mg^{2+}. Xylanase from *Streptomyces matensis* DW67 has also been reported to enhance the xylolytic activity (Yan et al. 2009) in the presence of Fe^{2+} and Co^{2+} ions in the reaction medium. On the other hand, $Cu^{2+,}$ Mn^{2+} and reducing agent β-Met enhanced the activity of EX624 at their lower concentration of 1mM. However, they inhibited the activity at their higher concentration of 10 mM.

Protein disulfide reducing agent β-Met has been understood to be the enhancer of the xylanase activity, as previously reported in the case of *Streptomyces megaporus* DSM 41476 (Qiu et al. 2010), and *Streptomyces matensis* (Yan et al. 2009). SDS and EDTA significantly inhibited the activity of EX624. Inhibitory effect of EDTA has been reported earlier in the case of the xylanase produced from several *Streptomyces* strains including *Streptomyces matensis.*

Table 4.3: Effect of metal ions and modulators on EX624 activity

Chemicals	Relative activity	
	1 mM	10 mM
NaCl	87.0	94.6
$FeSO_4$	108.4	383.9
$CoSO_4$	245.5	107.0
$CaCl_2$	127.3	161.7
$CuSO_4$	138.3	89.6
$MnSO_4$	121.1	68.5
$ZnSO_4$	54.9	63.1
$MgCl_2$	39.6	48.7
KCl	96.4	107.7
EDTA	35.4	28.9
B-Me	103.9	74.2
SDS	88.6	58.4
None	100	100

4.2.6 Substrate specificity and determination of kinetic parameters

The hydrolytic activity of EX624 xylanase on various substrates was examined. EX624 xylanase exhibited high activities of 399996 U/mg and 37831 U/mg on the beechwood xylan and birchwood xylan, respectively. Activities towards the Avicel substrate was negligible (~3 %) in comparison to the beechwood xylan and birchwood xylan. EX624 did not show any activity towards CMC. This suggests that EX624 xylanase is cellulase-free. Thus, it would be useful in pulp and paper industry as it avoids cellulase degradation. Cellulase-free xylanase from different microorganisms have been reported earlier (Yan et al. 2009; Qiu et al. 2010; Hung et al. 2011). The k_m and V_{max} values of EX624 xylanase for beechwood xylan were 5.61 mM and 74.626 mmol min^{-1} mg^{-1}, while for the case of birchwood xylan they

were 9.79 mM and 57.471 mmol min^{-1} mg^{-1}, respectively. Lower K_m and higher V_{max} value for the beechwood xylan indicates the preference of EX624 xylanase towards beechwood xylan over birchwood xylan.

4.2.7 Analysis of hydrolysis product

The hydrolysis products of beechwood xylan and birchwood xylan formed by the action of EX624 were analyzed using TLC. The time course analysis of the degradation products of beechwood and birchwood xylan are shown in Fig 4.6a and Fig. 4.6b, respectively.

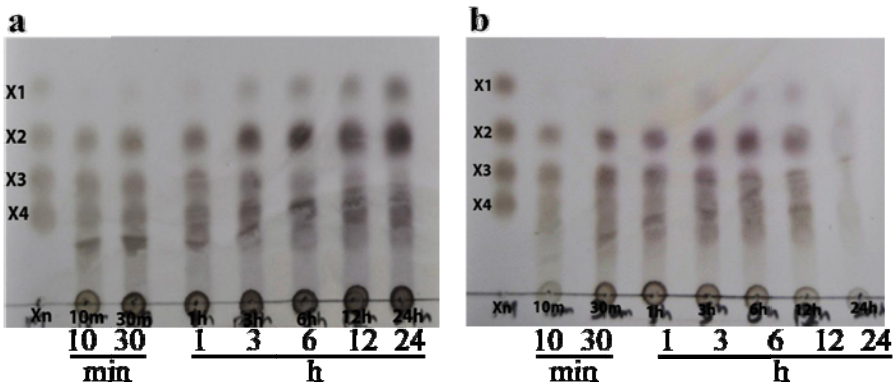

Fig 4.6: Time course of hydrolysis of two different xylans: (a) Beechwood xylan, and (b) Birchwood xylan. The reaction mixture consisted of 20 mg of each substrate in 2.0 ml of 10 mM citrate buffer (pH 6.0) and 200 µl of suitably diluted enzyme was incubated at 40°C for 24h. Time of incubation (h or min) and xylooligosaccharides are indicated in the figure.

After 10 min of incubation, the EX624 xylanases liberated mainly X2, followed by X3 and X4 in both the cases of hydrolysis of beechwood xylan and birchwood xylan. After 30 min of reaction time, traces of X1 were visible, and became more prominent as the reaction time increased. In addition, as the reaction time increased to 12 h, X3 and X4 were slightly degraded and converted into X1 and X2. Production of X2 was greater than that of X1. However, the rate of X1 and X2 production in case of birchwood xylan was declined after 24 h incubation which may be due to decrease level of accessible hydrolytic sites in the xylan chain (Chapla et al. 2011). The hydrolysis of birchwood xylan by a xylanase from *Gracibalillus* sp. TSCPVG with X2 and X1 as the major hydrolytic products, as in our case, has been reported earlier (Giridhar and Chandra 2010). The reported hydrolysis pattern suggested that EX624 is an endoxylanase which can be potentially applied to produce xylooligosaccharides with various xylose units. Further detailed studies on the hydrolysis of agricultural residues by EX624 are underway.

In order to understand the potential of the EX624 xylanase to degrade agriculture waste, the structure changes in the corn cob and wheat bran after the enzymatic hydrolysis were observed by using SEM (Fig. 4.7). Enzymatic hydrolysis disrupts the lignocellulosic structure by dissolving the hemicelluloses; morphological changes such as holes, cracks and peelings were observed.

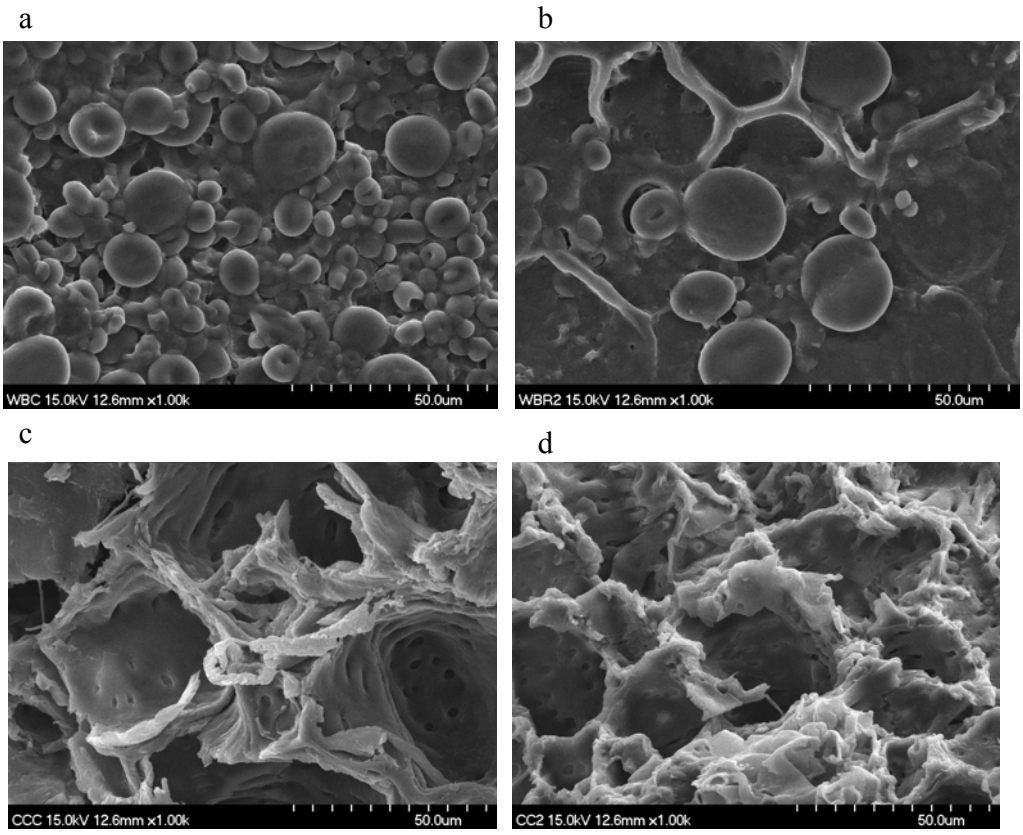

Fig 4.7: SEM microgram (1000 magnification) of hydrolyzed wheat bran and corn cob. (a) Wheat brean treated with buffer. (b) Wheat bran after enzymatic hydrolysis (treated with enzyme 1000 U/ml). (c) Corn cob treated with buffer. (d) Corn cob after enzymatic hydrolysis (treated with enzyme 1000 U/ml)

In case of corn cob treated with enzyme there were many ruptures found as well as flakes and filaments of material detached from the surface (Fig. 4.7 a). In contrast, untreated corn cob showed smoother surface (Fig. 4.7 b). Untreated wheat bran exhibited rigid and ordered fibrils with smoother surfaces (Fig. 4.7 a). While in

enzymatically hydrolyzed wheat bran, the hemicellulose was removed and the material structure was changed with unwrapped fibers on the surface (Fig 4.7 b). This qualitative study attributed to the involvement of xylanase not only in xylan hydrolysis but also helps in fiber modification.

4.3 Conclusion

Agricultural wastes and residues are mostly organic biomass, which through clean microbial technology, can be utilized in various value added applications. In this current work, EX624 xylanase was economically produced by growing *Streptomyces* sp. CS624 in a culture media supplemented with a readily available and inexpensive agricultural residue, wheat bran. Interestingly, the yielded xylanase for the case of using wheat bran was found to be comparable, though slightly low, to that of the case of using commercially available expensive beechwood xylan. Biochemical characterization revealed that EX624 xylanase was cellulase-free, stable in broad range of acidic and alkaline pH, and adequately theromstable. In addition, enzymatic hydrolysis of beechwood and birchwood xylan using the purified EX624 xylanase verified that it can be potentially used for xylooligosachharide production.

5. Conclusion

In this dissertation, three different EC3 enzymes, viz. a fibrinolytic metalloprotease, an organic-solvent tolerant lipase, and an acidic xylanase, have been produced from two strains of Korean soil bacteria (selected from several hundreds of strains) which were identified to be belonging to *Streptomyces* genus based on morphological and 16 rDNA sequence analysis.

The produced enzymes were purified to homogeneity using several chromatography techniques. In particular, the metalloprotease (18 kDa) and lipase (39.8 kDa) were purified using gel-permeation and hydrophobic interaction column chromatography with the final specific activity of 1060 U/mg and 712.6 U/mg, respectively, while the xylanase (40 kDa) was purified employing ion exchange and gel filtration column chromatography with the final specific activity of 61,415 U/mg. The purified metalloprotease and lipase were noted to have a low N-terminal amino acid sequence similarity to that of other proteases and lipases from *Streptomyces.*

The purified protease, lipase and xylanase were noted to have similar thermostability characteristics, while their pH stability ranges were different, as can be noted in Table 5.1. Among the various tested substrates, the protease, lipase and xylanase were found to show the highest catalytic efficiency towards the *N*-succinyl-Ala-Ala-Pro-Phe-pNA, p-nPP, and beechwood xylan, respectively (Table 5.2).

Table 5.1: pH and temperature profile of the purified enzymes

	Optimum pH	pH stability	Optimum temperature	Temperature stability
Protease FES624	7.0	6.0-8.0	60°C	≤ 50°C
Lipase LS133	7.5	5.0-9.0	40°C	≤ 50°C
Xylanase EX624	6.0	4.5-10.0	60°C	≤ 50°C

Table 5.2: Kinetic parameters of the purified enzymes

	K_m (mM)	V_{max} (mmol min-1 mg-1)	Substrate
Protease FES624	0.218	84.03	*N*-succinyl-Ala-Ala-Pro-Phe-pNA
Lipase LS133	0.152	270.2	*p*-nPP
Xylanase EX624	5.61	74.626	beechwood xylan

Effects of various metal ions, enzyme inhibitors, modulators, oxidizing agents, and reducing agents were also examined and presented.

In addition, the suitability of those three enzymes in enzymatic fibrinolysis, enzymatic transesterification, and enzymatic hydrolysis of cellulosic agricultural waste (rich in xylan) is demonstrated via lab scale experiments.

References

Abrami M, Lescis I, Korica T, Vitale J, Saenger W, Pigac J (1999) Purification and properties of extracellular lipase from *Streptomyces rimosus*. Enzyme Microb Technol 25:522–529.

Agrebi R, Haddar A, Hmidet N, Jellouli K, Manni L, Nasri M (2009) BSF1 fibrinolytic enzyme from a marine bacterium *Bacillus subtilis* A26: Purification, biochemical and molecular characterization. Process Biochem 44:1252-1259.

Arpigny JL, Jaeger KE (1999) Bacterial lipolytic enzymes: classification and properties. Biochem J 343:177-183.

Astrup T, Mullertz S (1952) The fibrin plate method for estimating fibrinolytic activity. Arch Biochem Biophys 40:346-351.

Bae HJ, Kim HJ, Kim YS (2007) Production of recombinant xylanase in plants and its potential for pulp biobleaching applications. Bioresour Technol 99: 3513-3519.

Bajaj A, Lohan P, Jha PN, Mehrotra R (2010) Biodiesel production through lipase catalyzed transesterification: an overview. J Mol Catal B Enzym 62:9–14.

Bastawde KB, Puntambekar US, Gokhale DV (1994) Optimization of cellulose-free xylanase production by novel yeast strain. J Ind Microbiol 13: 220-224.

Bataillon M, cardinali APN, Castillon N, Duchiron F (2000) Purification and characterization of a moderately thermostable xylanase from *Bacillus* sp. strain SPS-0. Enzyme Microb Technol 26:187-92

Beg QK, Bhushan B, Kapoor M, Hoodal GS (2000) Effect of amino acids on production of xylanase and pectinase from *Streptomyces sp. QG-11-3*. World J Microbiol Biotechnol 16: 211-213

Beg QK, Kapoor M, L. Mahajan, Hoondal GS (2001) Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56: 326-338.

Bono F, Savi P, Tuing A, Maftouch M, Pereillo JM, Capdevielle J, et al. (1996) Purification and characterization of a novel protease from culture filtrated of *Streptomyces* sp. FEMS. Microbiol Lett 141:213-220.

Bradford MM (1996) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254.

Borkar PS, Bodade RG, Rao SR, Khobragade CN (2009) Purification and characterization of extracellular lipase from a new strain – *Pseudomonas aeruninosa* SRT 9. Braz J Microbiol 40:358–366.

Bugg TDH (2004) Intdoduciton to enzyme and coenzyme chemistry. Second edition. Blackwell Publishing ltd, Oxford, UK.

Cai YJ, Wang L, Liao XR, Ding YR, Sun J (2009) Purification and partial characterization of two new cold–adapted lipases from mesophilic *Geotrichum* sp. SYBC WU-3. Process Biochem 44:786–790.

Canakci M (2007) The potential of restaurant waste lipids as biodiesel feedstocks. Bioresour Technol 98:183–190.

Cha WS, Park SS, Kim SJ, Choi D (2010) Biochemical and enzymatic properties of a fibrinolytic enzyme from *Pleorotus eryngii* cultivated under solid-state conditions using corn cob. Bioresour Technol 101:6475-6481.

Chakraborty K, Paulraj L (2009) Purification and characterization of an extracellular lipase from *Pseudomonas fluorescens* MTCC 2421. J.Agric. Food Chem 57-3859-3866.

Chapla D, Pandit P, Shah A (2011) Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. Bioresour Technol 115:215-212

Chapla D, Divecha J, Madamwar D, Shah A (2010) Utilization of agro-industrial waste for xylanase production by Aspergillus foetidus MTCC 4898 under solid state fermentation and its application in saccharification. Biochem Eng J 49: 361-369.

Chitte RR, Dey S (2000) Potent fibrinolytic enzyme from a thermophilic *Streptomyces megasoprous* strain SD 5. Lett Appl Microbiol 31:405-410.

Cho SS, Park DJ, Simkhada JR, Hong JH, Sohng JK, Lee OH, Yoo JC (2011) A neutral lipase applicable in biodiesel production from a newly isolated *Streptomyces* sp. CS326. Bioprocess Biosyst Eng 35:227-234.

Choi DB, Cha WS, Park N, Kim HW, Lee JH, Park JS, et al. (2011) Purification and characterization of a novel fibrinolytic enzyme from fruiting bodies of Korean *Cordyceps militaris*. Bioresour Technol 102:3279-3285.

Choi NS, Song JJ, Chung DM, Kim YJ, Maeng PJ, Kim SH (2009) Purification and characterization of a novel thermoacid-stable fibrinolytic enzyme from *Staphylococcus* sp. strain AJ isolated from Korean salt-fermented anchovy-*joet*. J Ind Microbiol Biot 36:417-426.

Chung DM, Choi NS, Maeng PJ, Chun HK, Kim SH (2010) Purification and characterization of a novel fibrinolytic enzyme from Chive (*Allium tuberosum*). Food Sci Biotechnol 19:697-702.

Cote A, Shareck F (2007) Cloning, purification and characterization of two lipases from *Sreptomyces coelicolor* A3(2). Enzym and Microbiol Technol 2007;42:381–388.

De Sousa JS, Cavalcanti-Oliveira ED, Aranda DA, Freire DM. Application of lipase from the physic nut (*Jatropha curcas* L.) to a new hybrid (enzyme/chemical) hydroesterification process for biodiesel production. J Mol Catal B Enzym 2010;65:133–137.

Demir BS, Tükel SS (2010) Purification and characterization of lipase from *Spirulina platensis*. J Mol Catal B: Enzym 64:123–128.

Demirbas A. Progress and recent trends in biodiesel fuels. Energ Convers Manage 50:14–34.

Deng Z, Wang S, Li Q, Ji X, Zhang L (2010) Hong M. Purification and characterization of a novel fibrinolytic enzyme from the polychaete, *Neanthes japonica* (Iznka). Bioresour Technol 101:1954-1960.

Dheeman DS, Henehan GT, Frias JM (2011) Purification and properties of *Amycolatopis mediterranei* DSM 43304 lipase and its potential in flavor ester synthesis. Bioresour Technol 102:3373–3379.

Doukyu N, Ogino H (2010) Organic solvent-tolerant enzymes. Biochem Eng J 48:270-282.

Gaur R, Gupta A, Khare SK (2008) Purification and characterization of lipase from solvent tolerant *Pseudomonas aeruginosa* PseA. Process Biochem: 43:1040–1046.

Georgogianni KG, Katsoulidis AP, Pomonis PJ, Kontominas MG (2009). Transesterification of soybean frying oil to biodiesel using heterogeneous catalysts. Fuel Process Technol 90:671–676.

Giridhar PV, Chandra TS (2010) Production of novel halo-alkali-thermo-stable xylanase by a newly isolated moderately halophilic and alkali-tolerant *Gracibacillus* sp. SCPVG. Process Bichem 45:1730-1737.

Giron ME, Salazar AM, Aguilar I, Perez JC, Sanchez EE, Aricha-Pinango CL, et al (2008) Hemmorrhagic, coagulant and fibrin(ogeno)lytic activities of crude venom and fractions from mapanare (*Bothrops colombiensis*) snakes. Comp Biochem Physiol C Toxicol Pharmacol 147:113-121.

Hahn BS, Cho SY, Ahn MY, Kim YS (2001) Purification and characterization of a plasmin-like protease from *Tenodera sinensis* (Chinese mantis). Insect Biochem Mol Biol 31:573-581.

Huber GW, Iborra S, Corma A (2006) Synthesis of transportation fuels from biomass: chemistry, catalysts, and engineering. Chem Rev 2006;106:4044–4098.

Hung KS, Liu SM, Tzoua WS, Lin FP, Chorng-Liang Pan CL, Fang TY, Kuang-Hui Sun KH, Tang SJ (2011) Characterization of a novel GH10 thermostable, halophilic xylanase from the marine bacterium *Thermoanaerobacterium saccharolyticum* NTOU1. Process Biochem 46:1257-1263.

Ji Q, Xiao S, He B, Liu X (2010) Purification and characterization of an organic solvent–tolerant lipase from *Pseudomonas aeruginosa* LX1 and its application for biodiesel production. J Mol Catal B: Enzym 66:264–269.

Joshi R, Jan S, Wu Y, MacMahon S (2008) Global inequalities in access to cardiovascular health care. J Am Coll of Cardiol 2008; 52:1817-1825.

Kaneko S, Kuno A, Muramatsu M, Iwamatsu S, Kusakabe I, Hayashi K (2000) Purification and characterization of a family G/11 beta-xylanase from *Streptomyces olivaceoviridis* E-86. Biosci Biotechnol Biochem 64:447-451.

Kim JS, Kim JE, Choi BS, Park SE, Sapkota K, Kim S, et al. (2008) Purification and characterization of fibrinolytic metalloprotease from *Perenniporia Fraxinea* mycelia. Mycol Res 112:990-998.

Kim KR, Kwon DY, Yoon Sh, Kim WY, Kim KH (2005) Purification, refolding, and characterization of recombinant *Pseudomonas Xuorescens* lipase. Protein Expr Purif 39:124-129.

Kim SB, Lee DW, Cheigh CI, Choe EA, Lee SJ, Hong YH, et al. (2006) Purification and characterization of a fibrinolytic subtilisin-like protease of *Bacillus subtilis* TP-6 from an Indonesian fermented soybean, Tempeh. J Ind Microbiol Biotechnol 33:436-444.

Kim YJ, Park JU, Seo MJ, Kim MJ, Lee HH, Jin SH, et al. (2010) Purification and biochemical characteristics of fibrinolytic enzyme from *Streptomyces corcohrussi* JK-20. Journal of Life Science 20:838-844.

Kumar A, Gupta R, Sheivastava B, Khasa YP, Kuhad RC (2012) Xylanase production from an alkalophilic actinomycete isolate Streptomyces sp. RCK-201, its characterization and application in saccharification of second generation biomass. J Mol Catal B: Enzym 74:170-177.

Kumari A, Mahapatra P, Garlapati VK, Banerjee R (2009) Enzymatic transesterification of Jatropha oil. Biotechnol Biofuels 2:1.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:280-285.

Lee DW , Kim HW, Lee KW, Kim BC, Choe EA, Lee HS, et al. (2001) Purification and characterization of two distinct thermostable lipases from the gram-positive thermophilic bacterium *Bacillus thermoleovorans* ID-1 W. Enzyme Microb Technol 9:363–371.

Lee JS, Bai HS, Park SS (2006) Purification and characterization of two novel fibrinolytic proteases from mushroom, *Fomitella fraxinea*. Microbio Biotechnol 16:264-271.

Lee SY, Kim JS, Kim JE, Sapkota K, Shen MH, Kim S, et al. (2005) Purification and characterization of fibrinolytic enzyme from cultured mycelia of *Armillaria mellea*. Protein Expr Purif 43:10-17.

Lenartovicz V, Marques De Souza CG, Moreira FG, Peralta RM (2002) Temperature effect in the production of multiple xylanases by *Aspergillus fumigatus*. J Basic Microbiol 42:388-95.

Lescic I, Vukelic B, Elenkov MM, Saenger W, Abramic M. (2001) Substrate specificity and effects of water-miscible solvents on the activity and stability of extracellular lipase from *Streptomyces rimosus.* Enzyme Microb Technol 29: 548–553.

Lineweaver M, Burk D (1934) The determination of enzyme dissociation constants. J Am Chem Soc 56:658-666.

Liu XL, Du XL, Lu FP, Zheng XQ, Xiao J (2005) Purification and characterization of a novel fibrinolytic enzyme from *Rhizopus chinensis* 12. Appl Microbiol Biotechnol 67:209-214.

Lu F, Lu Z, Bie X, Yao Z, Wang Y, Lu Y, et al. (2010) Purification and characterization of a novel anticoagulant and fibrinolytic enzyme produced by endophytic bacterium *Paenibacillus polymyxa* EJS-3. Thrombosis Research 126:349-355.

Mander P, Cho SS, Simkhada JR, Choi YH, Yoo JC (2011) A low molecular weight chymotrypsin-like novel fibrinolytic enzyme from *Streptomyces* sp. CS624. Process Biochem 46: 1449-1455.

Mansour FA, Shereif AA, Nour el-Dein MM, Abou-Dobara MI, Ball AS (2003) Purification and characterization of xylanase from a thermophilic *Streptomyces* sp. K37. Acta Microbiol Pol 52 :159-172.

Mayfield CI, Williams ST, Ruddick SM, Hatfield HL (1972). Studies on the ecology of actinomycetes in soil. IV: observations on the form and growth of streptomycetes in soil. Soil Biol. Biochem., 4: 79-91.

Meher LC, Sagar DV, Naik SN (2006) Technical aspects of biodiesel production by transesterification-a review. Renew Sust Energ Rev 10:248–268.

Miller GH (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Biochem 31:426-428.

Nakajima N, Mihara H, Sumi H (1993) Characterization of potent fibrinolytic enzymes in earthworm, *Lumbricus rubellus*. Biosci Biotech Biochem 57:1726-1730.

Nascimento RP, Coelho RRR, Marques S, Alves L, Girio FM, Boc EPS, Amaral-Collaco MT (2002) Production and partial characterization of xylanase from

Streptomyces sp. strain AMT-3 isolated from Brazilian cerrado soil. Enzym Microbial Technol 31: 549-555.

Nawani N, Kaur J (2007) Studies on lipolytic isoenzymes from a thermophilic *Bacillus sp*.: production, purification and biochemical characterization. Enzym Microbiol Technol 40:881–887.

Nielsen PM, Brask J, Fjerbeak (2008) Enzymatic biodiesel production: Technical and economical considerations. Eur J Lipid Sci Technol 110:692–700.

Ninawe S, Kapoor M, Kuhad RC (2008) Purification and characterization of extracellular xylanase from *Streptomyces cyaneus* SN 32. Bioresour Technol 99:1252-1258.

Ninawe S, La l R, Kuhad RC (2006) Isolation of three xylanase-producing strains of actinomycetes and their identification using molecular methods. Curr Microbiol 53: 178–182.

Oda K, Murao S (1974) Purification and some properties of acid proteinase A and B of *Scytalidium lignicolum* ATCC 24568. Agric Biol Chem 1974; 38:2435-2444.

Ogino H, Nakagawa S, Shinya K, Muto T, Fujimura N, Yasuda M, Ishikawa H (2000) Purification and characterization of organic solvent-stable lipase from organic solvent-tolerant *Pseudomonas aeruginosa* LST-03. J Biosci Bioeng 89:451–457.

Parawira W (2009) Biotechnological production of biodiesel fuel using biocatalysed transesterification: a review. Crit Rev Biotechnol 29: 82–93

Peng R, Lin J, Wei D (2010) Purification and characterization of an organic solvent–tolerant lipase from *Pseudomonas aeruginosa* CS–2. Appl Biochem Biotechnol 162:733–743.

Peng Y, Yang X, Zhang Y (2005) Microbial fibrinolytic enzymes: an overview of source, production, properties and thrombolytic activity in *vivo*. Appl Microbiol Biotechnol 69:126-132.

Qiu Z, Shi P, Luo H, Bai Y, Yuan T, Yang P, Liu S, and Yao B (2010) A xylanase with broad pH and temperature adaptability from *Streptomyces megasporus* DSM 41476, and its potential application in brewing industry. Enzym Microbial Technol 46: 506-512.

Rao MB, Tanksale AM, Ghathe MS, Deshpande VV (1998) Molecular and biotechnology aspects of Microbial Proteases. Microbiol Mol Biol Rev 62:597-635.

Robles-Medina A, Gonzelez-Moreno PA, Esteban-Cerdan L, Molina-Grima E (2009) Biocatalysis: Towards ever greener biodiesel production. Biotech Adv 27:398–408.

Sharma R, Chisti Y, Banerjee UC (2001) Production, purification, characterization, and applications of lipase. Biotechnol Adv 19:627–662.

Simkhada JR, Mander P, Cho SS, Yoo JC (2010) A novel fibrinolytic protease from *Streptomyces* sp. CS684. Process Biochem 2010; 45:88-93.

Soliman NA, Knoll M, Abdel-Fattah YR, Schmid RD, Lange S (2007) Molecular cloning and characterization of thermostable esterase and lipase from *Geobacillus thermoleovorans* YN isolated from desert soil in Egypt. Process Biochem 42:1090–1100.

Sommer P, Bormann C, Gotz F (1997) Genetic and biochemical characterization of a new extracellular lipase from *Streptomyces cinnamomeus.* Appl Environ Microb 63:3553–3560.

Subramaniyan S, Prema P (2002) Biotechnology of microbial xylanases: enzymology, molecular biology and applications. Critical Rev Biotechnol 22:33-46.

Sumi H, Nakajima N, Yatagai C (1995) A unique strong fibrinolytic enzyme (Katsuwokinase) in skipjack 'shiokara', a Japanese traditional fermented food. Comp Biochem Phys B 112:543-547.

Taneja K, Gupta S, Kuhad RC (2002) Properties and application of a partially purified alkaline xylanase from an alkalophilic fungus *Aspregillus nidulans* KK-99. Bioresour Technol 85: 39-42.

Uesugi Y, Usuki H, Iwabuchi M, Hatanaka T (2011) Highly potent fibrinolytic serine protease from *Streptomyece*. Enzyme Microb Technol 48:7-12.

Uusitalo JM, Nevalainen KMH, Harkki AM, Knowles JKC, Penttila ME (1991) Enzyme production by recombinant *Trichoderma reesei* strains. J Bioechnol 17:35-50.

Vazquez MJ, Alonso JL, Dominguez H, Parajo JC (2000) Xylooligosaccharides: manufacture and applications. Trends Food Sci Technol 11: 387-393.

Wang CT, Ji BP, Li B, Nout R, Li PL, Ji H, et al. (2006) Purification and characterization of a fibrinolytic enzyme of *Bacillus subtilis* DC33, isolated from Chinese traditional douch. J Ind Microbiol Biotechnol 33:750-758.

Wang H, Peng G, Wang X, Wang X, Cui Z (2012). Degrading capability and activity of extracellular xylanase secreted by a composite microbial system XDC-2. African J Biotechnol 34:8476-8483

Wang J, Wang M, Wang Y (1999) Purification and characterization of a novel fibrinolytic enzyme from *Streptomyces* sp. Chin J Biotechnol 1999;15:83-89.

Wejse PL, Ingvorsen Kjeld, Mortensen KK (2003) Xylanase production by a novel halophilic bacterium increased 20-fold by response surface methodology. Enzym Microbial Technol 32: 721-727.

Winkler UK, Stuckman M (1979) Glycogen, hyaluronate and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens.* J Bacteriol 138:663–670.

Wu B, Wu L, Chen D, Yang Z, Luo M (2009) Purification and characterization of a novel fibrinolytic protease from *Fusarium* sp. CPCC 480097. J Ind Microbiol Biotechnol 36:451-459.

Yan Q, Hao S, Jiang Z, Zhai Q, Chen W (2009) Properties of a xylanase from *Streptomyces matensis* being suitable for xyloologosaccharides production. J Mol Catal B: Enzym 58:72-77.

Yang J, Zhang B, Yan Y (2009) Cloning and expression of *Pseudomonas fluorescens* 26-2 lipase gene in *Pichia pastoris* and characterizing for transesterification. Appl Biochem Biotechnol 159:355–365.

Yang KS, Sohn JH, Kim HK (2009) Catalytic properties of lipase from *Photobacterium Lipolyticum* for biodiesel production containing a high methanol concentration. J Biosci Bioeng 107:599–604.

Yoo HA, Simkhada JR, Cho SS, Park DH, Kim SW, Seong CN, et al (2011) A novel alkaline lipase from *Ralstonia* with potential application in biodiesel production. Bioresour Technol 102:6104–6111.

Yoo JC, Kim JH, Ha JW, Park NS, Sohng JK, Lee JW, et al. (2007) Production and biological activity of laidlomycin, anti-MRSA/VRE antibiotic from *Streptomyces* sp. CS684. J Microbiol 45:6–10.

Zhang Y, Wisner A, Xiong Y, Bon C (1995) A novel plasminogen activator from snake venom. J Biol Chem 270:10246-10255.

Zhang Y, Meng K, Wang Y, Luo H, Yang p, Shi P, et al. (2008) A novel proteolysis–resistant lipase from keratinolytic *Streptomyces fradiae* var K11. Enzym and Microbiol Technol 42:246–352.

Acknowledgement

First and foremost I would like to thank my supervisor Prof. Dr. Jin Cheol Yoo for his excellent guidance, understanding, and help throughout this study. This thesis would not have been possible without his patience and encouragement. I sincerely appreciate invaluable academic support I received from him throughout this thesis.

I would like to express my grateful thanks to my thesis committee members: Prof. Dr. Hong Joon Hee, Prof. Dr. Woo Eun Rhan, and Prof. Dr. Choi Hong Seok for their valuable comments and critical suggestion during my thesis study. My deepest gratitude towards Prof. Kim Sung Jun, chairperson of my thesis committee for giving me his valuable time and comments and helped me to improve my thesis in many ways.

Special thanks to my seniors Dr. Jay Ram Simkhada and Dr, Cho Seun Sik for teaching me and helping me throughout my study. I would also like to thank rest of my lab members Choi Yun Hee, Kim Okh, Park Da jung, Park Jin Hee, Pradeep, Arifur for their support and friendly environment to work and learn.

Finally my gratitude goes to my family: my mom and dad, sister and extended family. Thanks for all their motivation, encouragement and their support throughout not only this study and also all through my life. Lastly I express my special thanks to my dear husband Subodh Pudasaini, for his constant support, love

care and guidance. Without him I would have never been able to complete my thesis.

저작물 이용 허락서

학 과	약학과	학 번	20097723	과 정	박사
성 명	한글: 푸남 맨더 영문 : Poonam Mander				
주 소	광주광역시 동구 서석동 375번지 조선대학교 약품미생물학실				
연락처	E-MAIL : Poonam_mander@hotmail.com				
논문제목	한글 : 한국 토양세균이 분비하는 단백분해효소, 지질분해효소, 자일란분해효소의 정제, 생화학적 특성분석 및 산업적 응용 연구 영어 : Purification Biochemical Characterization and Corroborated Applications of Protease, Lipase and Xylanase from Korean Soil Bacteria				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(o) 반대( )

2012년 07월 10일

저작자: 맨더 푸남 (서명 또는 인)

조선대학교 총장 귀하