ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MICROBIAL, CELLULAR AND MOLECULAR BIOLOGY

Characterization of Aspartic Protease Enzyme from Fungi and Bacteria and its Potential Application for Cheese Production

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This is to certify that the thesis prepared by Jermen Mamo, entitled: **"Characterization of Aspartic Protease Enzyme from Fungi and Bacteria and its Potential Application for Cheese Production"** and submitted in fulfillment of the requirements for the Degree of Doctor of Philosophy (Applied Microbiology) complies with the regulations of the University and meets the accepted standards with respect to originality and quality.

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Declaration

I declare that this dissertation submitted for the PhD Degree in Applied Microbiology at Microbial, Cellular and Molecular Biology, Addis Ababa University, entitled as "**Characterization of Aspartic Protease Enzyme from Fungi and Bacteria and its Potential Application for Cheese Production**" is my original work and has not previously been submitted in any form for another degree, diploma or an award at this or any other University, and that all resources of materials used in this dissertation have been duly acknowledged.

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Characterization of Aspartic Protease Enzyme from Fungi and Bacteria and its Potential Application for Cheese Production

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Abstract

Cheese is a dairy product processed through milk clotting using rennet enzyme (chymosin) (EC 3.4.23.4). Chymosin is a complex enzyme produced by animals, plants or microorganisms. Traditionally, it is extracted from the fourth stomach (abomasum) of young ruminants. However, only 20-30% of the world demand for milk-clotting enzymes is covered by calf rennet, indicating that the milk-clotting enzymes derived from animals are not sufficient to cover worldwide cheese production. This necessitates the search for alternative sources for calf rennet substitutes such as microbial aspartic proteases. In this study, a total of 237 (188 fungal and 49 bacterial) isolates were tested for milk-clotting enzyme production in primary and secondary screening techniques. After the secondary screening, 17 potential fungal and 14 bacterial isolates were successfully identified using, a combination of phenotypic and molecular techniques. The physicochemical parameter and media composition for potential fungus (*Aspergillus oryzae* DRDFS13) under SSF were optimized by one-factor-at-a-time and Response Surface Methodology (RSM) whereas the culture profile of the potential bacterium (*Bacillus subtilis* SMDFS 2B) was studied under partially optimized conditions. The enzyme from *A. oryzae* DRDFS13 was characterized molecularly and biochemically after purification by size-exclusion (SEC) and ion-exchange (IEC) chromatography. The enzyme from *B. subtilis* SMDFS 2B was characterized biochemically after partial purification by

dialysis. Furthermore, the aspartic protease gene from *A. oryzae* DRDFS13 was characterized by cloning into *Pichia pastoris* using pGAPZαA as a vector and *E. coli* K12 for gene amplification. Finally a partially purified enzyme from *A. oryzae* DRDFS13 and *B. subtilis* SMDFS 2B used for Danbo cheese production using commercial rennet as a control. The Danbo cheeses produced using fungal enzyme (E1), bacterial enzyme (E2) and commercial rennet (C) were analyzed for body property, organoleptic characteristics, proximate and mineral composition when fresh and after 2 months of ripening. Seventeen fungi isolates were identified into different strains under the genera *Aspergillus, Fusarium,* and *Pleurotus*, whereas all the 14 bacterial isolates were identified to different strains under genus *Bacillus.* Moreover, *A. oryzae* DRDFS13 and *B. subtilis* SMDFS2B that showed enhanced MCA were selected for further study. The result from optimization and culture profile determination for *A. oryzae* DRDFS13 and *B. subtilis* SMDFS2B increased the MCA by 2.7 and 5.3 fold, respectively. The purified enzyme from *A. oryzae* DRDFS 13, IEC fraction A_8 was exhibited a purification fold, specific activity, and yield of 6.20, 183.50 U/mg and 9.2%, respectively. The molecular weight of IEC A_8 was 40 kDa, however, its MW was decreased to 30 KDa upon deglycosylation assay which infers that the protein is glycosylated. Inhibition study of IEC A_8 with pepstatin A caused a 94 % inhibition on MCA. The dialyzed enzyme from *A.oryzae* DRDFS13 was shown maximum MCA at 60 ^{o}C and pH 5.0 with stability at pH 4.5-6.5 and temperature 35-45 ^{0}C. The partial purification of the crude enzyme from *B. subtilis* SMDFS 2B was increased its MCA by 2.0 fold. The dialyzed enzyme showed the highest MCA at 55 ^{o}C and pH 5.5 with stability at pH 4-6 and temperature 35 ^{o}C- 40 ^{o}C. The enzyme also showed the lowest residual MCA in the presence of EDTA (7.94%) and pepstatin-A (26.71%). The aspartic protease gene cloned into pGAPZαA (later pMKAP) was

successfully expressed in *P. pastoris* and showed the highest MCA (190.47 U/mL) at pH 5 on the 6th day of incubation time. The recombinant protein has a MW between 32-46 kDa. Furthermore, the overall organoleptic characteristics, proximate composition, and mineral composition obtained from Danbo cheese made of *A. oryzae* DRDFS13 were closer to the control cheese produced using commercial rennet as compared to the bacterial enzyme. Therefore, the results from the present study confirmed that the enzyme from *A. oryzae* DRDFS 13 purified by an ion-exchange chromatography is an aspartic protease and could be used as a substitute for rennet enzyme in cheese production. This may open the way for applications of the enzymes in the food and dairy industries. Finally, the results from cheese production revealed that the fungal enzyme from *A. oryzae* DRDFS 13 is more appropriate for Danbo cheese production than the bacterial enzyme from *Bacillus subtilis* SMDFS 2B.

Keywords: *Aspergillus*, *Bacillus*, Danbo cheese, Milk-clotting activity, Milk-clotting protease, Minerals, Solid-state fermentation, Submerged fermentation

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List of Abbreviations

A·	Angstrum
AFEA	Austrian Federal Environment Agency
AGP	Aspergillopepsin
ANN	Artificial neutral network
ANOVA	Analysis of Variance
AOAC	Association of Official Agricultural Chemists
Aps	Aspartic proteases
Asp	Aspartate
ATPS	Aqueous two-phase systems
BCA	Bicinchoninic acid
BLAST	Basic local alignment search tool
BSA	bovine serum albumin
CA	Clotting Activity
CAR	Calf rennet
CAGR	Compound annual growth rate
CD	Colony diameter
cDNA	Complementary Dioxyribnuicleic Acid
CR	Commercial rennet
Cys	Cysteine
CSA	Central Statistics Agency
CTAB	Cetyl trimethylammonium bromide
CYA	CzapeK Dox Agar
D	Dilution factor
DBE	Dialized Bacterial Enzyme
DDA	Dairy development Agency
DDE	Dairy Development Enterprise
DEAE	Diethylaminoethyl
DFE	Dialyzed Fungal Enzyme
DIFP	Diisopropyl fluorophosphate

DM	Dry matter
dNTPs	Deoxyribose nucleotide triphosphates
DNS	Dinitro salicyclic calorimetric
EC	Enzyme Comission
EDTA	Ethylene-diamine-tetra-acetic acid
EGTA	Ethyleneglycol-bis(β-aminoethyl ether)-N-N-N-N-tetraacetic acid
Endo-H	endo-β-N-acetylglucosamine-H
FAA	Free amino acids
FDA	Food and Drug Administration
g	Relative centrifugal force/G-Force
gdfs	Gram of dried fermented substrates
gDNA	Genomic Deoxyribonucleic acid
GlcN	Glucosamine
GRAS	Generally Regarded as Safe
GMP	Good manufacturing practice
HCl	Hydrochloric acid
HCN	Hydrogen cyanide
HIV	Human-Immuno Virus
His	Histidine
HPLC-FLD	High-performance liquid chromatography- fluorescence detector
HUT	Hemoglobin units tyrosine
IEC	Ion-exchange chromatography
IA	Iodoacetamide
ILAP	Irpex Lacteus Aspartic Protease
IMCU	International Milk Clotting Units
ITS	Internal Transcribed Spacer Region
IZD	Inhibition Zone Diameter
KDa	Kilo Daltons
k-casein	Kappa casein
Km	Concentration of substrate

LAB	Lactic acid bacteria
MAP	*Mucor miehei* Aspartic protease
MCA	Milk Clotting Activity
MCE	Milk-Clotting Enzyme
MCP	Milk-Clotting Protease
MEA	Malt Extract Agar
MEGA7	Molecular evolutionary genetics analysis version 7
Met	Methionine
ms	milliseconds
MW	Molecular weight
NCIUBM	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
NCBI	National Centre for Biotechnology Information
N-glycosylation	an oligosaccharide (glycan) attached to a nitrogen atom
OD	Optical density
O-glycosilation	The attachment of a sugar molecule to the oxygen atom
OTA	Ochratoxin A
PA	Protease Activity/Proteolytic Activity
pCMB	p-Chloromercuribenzoic acid
PCR	Polymerase chain reaction
PDA	Potato-Dextrose Agar
PDB	Potato Dextrose Broth
Phe	phenyl
pI	Isoelectric point
PMSF	Phenyl-methane Sulphonyl Fluoride
PYCG	Peptone Yeast extract Casein Glucose
REA	Relative enzyme activity
RBF	Radial basis function
Rf	Relative migration distance
RNA	Ribonucleic acid

rpm	Revolution per minute
rRNA	Ribosomal Ribonucleic acid
RSM	Response surface methodology
RU	Rennin units
SAS	Statistical analysis software
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SMA	Skim-milk Agar
SmF	Submerged-fermentation
SN	soluble nitrogen
SSF	Solid-State Fermentation
SU	Soxhlet Unit
TTA	Titrable acidity
TAE	Tris-acetate EDTA
TCA	Trichloroacetic acid
TP	Total protein
TPE	Tris-phosphate EDTA
TVFAs	Total volatile fatty acids
UF	Ultra filtrated
Vmax	Maximum rate of reaction
UV	Ultraviolet
WB	Wheat bran
WHO	World Health Organization
W/V	Weight by volume
W/W	Weight by weight

Chapter 1

1. General Introduction

Enzymes are proteins produced by living organisms that effectively catalyze biochemical reactions. They have substantial advantages over chemical catalysts in their specificity, high catalytic activity, and ability to work at moderate temperatures (Chandran *et al*., 2005).

Proteases are enzymes that catalyze proteolysis in cells and extracted for the large scale industrial application in food, laundry detergents, leather treatment, bioremediation processes and the pharmaceutical industry (Chandran *et al*., 2005). They are classified into acid, alkaline and neutral proteases depending on the optimal pH at which they are active (Chandran *et al*., 2005).

They are one of the three largest groups of industrial enzymes and account for about 60% of the sales worldwide (Mandujano-gonzález *et al*., 2016). They are used to improve the taste, texture, and appearance of the food products (Mandujano-gonzález *et al*., 2016). Several proteases are used to produce biological detergents, meat tenderization agents and milk clotting agents in dairy industries (chymosin and pepsin)(Benlounissi *et al*., 2014). Acidic proteases with high activity and stability at acidic pH have important industrial applications, specifically as milk-coagulating agents for cheese-processing and as flavor enhancers in other food industries (Mandujano-gonzález *et al*., 2016).

Rennet is a complex enzyme produced by animals, plants or microorganisms used to clot milk in the process of cheese making (Baskar *et al.*, 2014; Talib *et al.*, 2011). In the early time, rennets are obtained from the fourth stomach (abomasum) of young

ruminants. Chymosin is the active component of calf rennet that helps the casein micelles to consequently aggregate to form cheese curd (Hang *et al*., 2017).

The rennet from the young calf is high in rennin (chymosin) (EC 3.4.23.4) composition, whereas rennet from the older animals is rich in pepsin. Rennet coagulated cheese represents the major type (~75%) of cheese and calf rennet has been the most widely used milk-clotting enzyme preparations for the production of rennet coagulated cheese (Amer *et al*., 2015; Ayana *et al*., 2015; Nasr *et al*., 2016).

The lack of animal-derived milk-clotting enzyme encouraged a search for calf rennet substitute. Only 20-30% of the world demand for milk-clotting enzymes is covered by calf rennet (Ayana *et al*., 2015). Therefore, the increased demand has prompted research in the production of milk-clotting enzyme from microorganisms (Ayana *et al*., 2015). Microorganisms are excellent sources of rennin and other protease enzymes. Many studies showed that suitable coagulants, including proteinases from microorganisms such as *Mucor miehei*, *Rhizomucor miehei*, *Mucor pucillus* and *Cryphonectria parasitica* (formerly *Endothia parasitica*), have become more popular in the production of cheeses (Ozcan and Vapur, 2013).

Microbial milk-clotting enzymes are used as a substitute for calf rennet in cheese making industry (Baskar *et al*., 2014). Most of the microbial extracellular enzymes perform a similar activity like animal chymosin (rennet) and appropriate for cheese production (Jacob *et al*., 2011). In cheese making, chymosin (E.C.3.4.23.4) is frequently substituted by aspartic protease produced from *Mucor miehei* for a high ratio of milk clotting activity/proteolytic activity (MCA/PA). Therefore aspartic protease is essential to substitute calf rennet (Garcia, *et al.*, 2005). The rapid growth, the limited space required for cultivation and the comfort of genetical manipulation to

generate new enzymes with altered properties makes microbes desirable for protease enzyme production (Rao *et al*., 1998).

Although various groups of microorganisms are capable of producing aspartic protease (Baskar *et al*., 2014; Jacob *et al*., 2011), most of the cheese-making enzymes are produced from fungal sources. Milk-clotting enzymes of fungal origins have gained major importance as they are used in more than one-third of the dairy industry worldwide (Khademi *et al*., 2013).

At present more than 100 fungal sources are implicated with coagulant enzyme production. The three most dominant fungal species are *Rhizomucor miehei, Rhizomucor pusillus,* and *Cryphonectria parasitica,* which have been used for large scale enzyme production (Jacob *et al*., 2011). Apart from the fungi, bacteria have the potential for large scale production of milk-clotting enzymes (Amer *et al.*, 2015).

Most plant rennets have proved unsuitable because they impart a bitter taste to the cheese. Microbial rennet appears to be more promising because its production is cheaper, biochemical diversity is greater, and genetic modification is easier (El-Tanboly *et al*., 2013). Microbial rennet is used for one-third of all the cheese consumed worldwide at present (Baskar *et al*., 2014).

Milk-clotting is the basic step in the production of all types of cheeses and based on this, all cheese varieties (>2000 types) are classified into three superfamilies. These include rennet coagulated, acid coagulated, and a combination of heat and acid coagulated cheeses (Nasr *et al*., 2016). Milk-clotting enzymes are one of the most important raw materials in the cheese industry for impacting and regulating milk coagulation. Even though all milk-clotting enzymes used in cheese production belong

to aspartic proteinases they have significant differences that greatly modify their value for cheese making (Tabayehnejad *et al*., 2012).

The price of industrial enzymes is increasing from time to time. The food and beverage enzymes represented 29% of enzyme business and bio-business sales by the industry (Kumar *et al*., 2014). The world enzyme market is expected to grow at 6.8% per year. Bakery enzymes represent a relevant segment of the food and beverage industry. According to a research report by the Freedonia Group, the enzymes market for baked goods in 2020 is expected to be around $ 9.0 billion of which about 35% represents food enzymes (Kumar *et al*., 2014).

1.1. Statement of the problem

Ethiopia is one of the largest cattle producing countries in Africa. The total milk production in Ethiopia is 3.1 billion liters per annum; with average per capita milk consumption is estimated to be about 19 kg/year (Central Statistical Agency, 2017; Central Statisticsal Agency, 2017; Tadesse, Mihret *et al*., 2017). However, it is lower than the 200 liters per capita per annum recommended by WHO. Although it is lower than the average per capita consumption of milk in other African countries which is 27 kg/year and 100 kg/year to the world per capita consumption (Tadesse Mihret *et al*., 2017).

Demand for milk and milk products especially in urban areas of Ethiopia are mostly met with imported products worth about 3.1 million USD in the year 2001 to USD 9.3 million in the year 2008, which is about 300% growth as a function of time. In terms of quantity, it has increased from 1,716 tons (2001) to 2,087 tons (2007) with steady increases every year (Zelalem Yilma *et al*., 2011).

All these shortcomings notwithstanding, there is a great potential for the development of the dairy industry in the country. This includes the development of modern cheese manufacturing industries with modern cheese production technology using rennet enzymes. The newly expanding dairy industry in Ethiopia is barely using commercial rennet enzymes imported from abroad with a high price for ripening and speedy processing of the product.

This necessitates the search for cheap enzymes from microbes. Therefore, the present work was initiated with the isolation of the microbial strains potentially endowed with aspartic protease enzymes that can be process optimized and purified and applied for commercial Danbo cheese production.

1.2. Objectives of the study

The general objective of the study is to produce, optimize, purify and characterize aspartic protease enzyme from fungi and bacteria applicable for commercial cheese production.

Therefore, the specific objectives of the present project are:

- To isolate, screen and identify effective aspartic protease enzyme-producing fungal and bacterial isolates from soil and cow dung.
- To produce microbial aspartic protease enzyme from fungi and bacteria using solid-state and submerged fermentations
- To optimize substrate and another physical parameter for maximum production of microbial aspartic protease.
- To purify and characterize aspartic protease in order to know its optimum activity parameters.
- To study the expression and catalytic activity of an aspartic protease gene from the *A. oryzae* DRDFS13 strain into *P. pastoris*.
- To analyze the aflatoxin content of crude enzyme from the potential fungal strain.
- To apply partially purified microbial aspartic protease enzyme for Danbo cheese production and analyze the nutritional value, sensorial quality, and mineral analysis.

Chapter 2

2. Literature review

2.1. Background

Proteases are enzymes that catalyze proteolysis in cells and extracted for large scale in the food industry, laundry detergents, leather treatment, bioremediation processes and the pharmaceutical industry (Chandran *et al*., 2005). They are one of the three largest groups of industrial enzymes and account for about 60% of the worldwide enzyme sale (Chandran *et al*., 2005). They are used to improve the taste, texture, and appearance of the food products. According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NCIUBM), they are classified into class 3 of the hydrolases and the subclass 3.4 of the peptide hydrolases or peptidases. The term "peptidase" (exopeptidase) is as synonymous with "peptide hydrolase" for any of the enzyme which hydrolyzes peptide bonds according to the NCIUBM (Yegin and Dekker, 2013). At present, the term "peptidase" is used as synonymous with "protease" and "proteinase" (endopeptidases), the latter two still preferred by many scientists (Chandran *et al*., 2005; Yegin and Dekker, 2013).

However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Chandran *et al*., 2005).

Plants, animals, and microorganisms are endowed with the ability to produce proteases. However, the ever-increasing demand for the enzyme in the world increased the interest of microbial proteases. Microbial protease is chosen for other

sources because they possess almost all the characteristics desired for their biotechnological applications (Chandran *et al.*, 2005).

The Novo industry of Denmark is among the major protease producers of the world, which accounts for 40% of the market share of these enzymes. It manufactures three brands such as Aquaderm, NUE, and pyrase which are used for soaking, dehairing, and bating industries, respectively (Chandran *et al.*, 2005).

2.2. Classification of proteases

Proteases are generally categorized into two major groups depending on their site of action, i.e., exopeptidases and endopeptidases. Exopeptidases are that protease which cleaves the peptide bond proximal to the amino or carboxy termini of the substrate (cleave N- or C-terminal peptide bonds of a polypeptide chain), whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (cleave internal peptide bonds) (Yegin and Dekker 2013). Proteases are also classified into acid, alkaline and neutral proteases depending on the optimal pH at which they are active (Chandran *et al*., 2005).

Proteases are divided into four groups based on their catalytic action: serine, aspartic, cysteine, and metalloproteases as showen in Table 2.1 (Sumantha *et al*., 2006). However, recently, three new systems have been defined: the threonine-based proteasome system, the glutamate-glutamine system of eqolisin, and the serine–glutamate aspartate system of sedolisin (Dunn, 2010).

2.2.1. Serine proteases

Serine proteases (EC 3.4.21), contain a serine group in their active site. They are abundant and common among viruses, bacteria, and eukaryotes. They are found in the exopeptidase, endopeptidase, oligopeptidase, and omega-peptidase groups (Theron

and Divol, 2014). *Bacillus* produces most of the commercial neutral and alkaline proteases. Other bacteria such as *Thermus caldophilus* and *Desulfurococcus mucosus, Streptomyces, Aeromonas,* and *Escherichia,* and the fungus *Aspergillus oryzae* similarly produce several serine proteases (Chandran *et al*., 2005).

2.2.2. Cysteine/thiol proteases

Cysteine proteases (EC 3.4.22), occur in both prokaryotes and eukaryotes. There are about 20 families of cysteine proteases. Cysteine protease activity can be determined on dyad consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues differ among the families. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine (Rao *et al*. 1998; Theron and Divol, 2014) (Table 2.1).

Cysteine proteases are broadly divided into four groups based on their side chain specificity: (i) papain-like, (ii) trypsin-like with a preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Papain cysteine proteases have neutral pH optima, except a few lysosomal proteases, are maximally active at acidic pH (Rao *et al*. 1998). They are not so widely distributed unlike serine and aspartic proteinases (Chandran *et al.*, 2005).

2.2.3. Aspartic proteases

Aspartic proteinases (EC 3.4.23) or aspartic proteinases are endopeptidases having two aspartic acid residues (Asp32 and Asp215, pepsin numbering) within their active site (Yegin *et al*., 2011). It is commonly known as acidic proteases (Rao *et al*., 1998). They are grouped into three families, i.e., pepsin (A1), retropepsin (A2), and enzymes from para-retroviruses (A3), and are placed in clan AA. Most of the aspartic proteases

show the best activity at low pH (pH 3 to 4) and have isoelectric points in the range of pH 3 to 4.5 (Rao *et al*., 1998) (Table 2.1).

Microbial aspartic proteases can be broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor spp* such as *Mucor miehei*, *M. pusillus* and *Endothia parasitica* (Rao *et al*., 1998; Sumantha., 2006). They are specific to aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin (Rao *et al*., 1998; Yegin *et al*., 2011).

Acid proteases represent an important group of enzymes, widely used in food, beverage and pharmaceutical industries. For most of these applications, the enzymatic preparation must be at least partially purified and free of substances that could change the characteristics of the product or the process (Silva *et al*., 2011).

2.2.4. Metalloproteases

Metalloproteases (EC 3.4.24) are the most diverse types of proteases. They contain enzymes from different origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria. They require divalent metal ion for their activity. About 30 families of metalloproteases have been recognized, of which 17 are endopeptidases, 12 are exopeptidases, and 1 (M3) contains both endo- and exo-peptidases (Rao *et al*., 1998; Theron and Divol, 2014) (Table 2.1).

Table 2.1: The characteristics of four types of protease (Sumantha *et al*., 2006)

Properties	EC NO	Molar mass range/KDa	pH optimum	Temperature optimum (^{0}C)	Metal requirement(s)	Active Amino acid(s)	Major inhibitor(s)	Major sources
Aspartyl or carboxyl protease	3.4.23	30-45	3-5	40-55	Ca^{2+}	Aspartate or Cysteine	Pepstatine	*Aspergillus,Mucor, Endothia,Rhizopus, Pencillium, Neurospora*, Animal tissue (stomach)
Cysteine or thiol protease	3.4.22	34-35	2-3	40-55	-	Aspartate or Cysteine	Iodoacetamide, pCMB	*Asapergillus*, Stem of pine apple (*Ananas comorus*), latex of fig tree (*Ficus sp),* Papaya (*Carica papaya*), *Streptococcus*, *Clostrdium*
Metallo-protease	3.4.24	19-37	5-7	65-85	Zn^{2+}, Ca^{2+}	Phenylalanine or leucine	Chelating agent such as EDTA, EGTA	*Bacillus, Aspergillus, Pencillium,Pseudomonas, Streptomyces*
Serine protease	3.4.21	18-35	6-11	50-70	Ca^{2+}	Serine, histidine and aspartate	PMSF, DIFP, EDTA, Soybean trypsin inhibitor, Phosphate buffers, Indole, Phenol, tri-amino acetic acid	*Bacillus, Aspergillus*, Animal tissue (gut), *Tritirachium album* (thermostable)

2.3. Mechanism of action of Aspartic protease

Aspartic proteases display a wide range of activities and linked to a variety of physiological functions including mammalian digestion of nutrients (e.g. chymosin, pepsin A), defense against pathogens, virulent yeasts (e.g. candidapepsins), metastasis of breast cancer (e.g. cathepsin D), pollen-pistil interactions (e.g. cardosin A), control of blood pressure (e.g. renin), haemoglobin degradation by parasites (e.g. plasmepsins) and maturation of HIV proteins (retropepsin)(Vega-Herná, 2007).

Structurally, they belong to the A1 pepsin family and are synthesized as preproenzymes similar to other pepsin enzymes. In general, the active enzymes consist of a single peptide chain of about 320-360. They depend on their aspartic acid residues for their catalytic activity (Vega-Herná, 2007). It has a molecular masses between 30–45 kDa (Vishwanatha *et al*., 2009).

APs are active at acidic pH, and the optimum pH of each aspartic protease is determined by the electrostatic potential at the active site, which in turn is determined by the position and orientation of all residues near the active site (Vega-Herná,2007).

2.4. Microbial source of aspartic protease

Microorganisms are excellent source of enzymes due to their wide biochemical diversity and susceptibility to genetic manipulation. About 40% of the total global enzyme sales are from microbial sources (Rao *et al*., 1998; Sumantha *et al*., 2006). Aspartic protease has been found in molds and yeasts, but rarely in bacteria (Sumantha *et al*., 2006).

2.4.1. Fungal aspartic proteases

It has been shown that fungi produce a wider range of enzymes than do bacteria(Rao *et al*., 1998). For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases that are active in a wide range of pH (pH 4 to 11) with broad substrate specificity (Rao *et al*., 1998). Fungal acid protease have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheese-making industry due to their narrow pH and temperature specificities (Rao *et al*., 1998).

Fungal aspartic proteases (Aps) have been used as milk-clotting enzymes in the dairy industry for about 30 years due to the worldwide shortage of calf chymosin. Most of

these extracellular fungal aspartic proteases are produced from *Aspergillus* species. These include *A. oryzae, A. fumigatus, A. saitoi, A. awamori* and *A. niger* (Theron and Divol, 2014). The enzymes, marketed under the trade names Rennilase®, Fromase®, Novoren®, Marzyme®, Hannilase®, Marzyme® and Suparen® produced by *M. miehei*, *M. pusillus* and *Cryphonectria* (*Endothia*) *parasitica*, are widely used for the production of different kinds of cheese (Vega-Herná, 2007). The aspartic acid extracted from *Botrytis cinerea* has other industrial applications (Sluyter *et al*., 2013). Jacob *et al*., (2011) reported that milk-clotting enzymes from more than 100 fungal sources are used for cheese production worldwide.

2.4.1.1. Mucorpepsin

Mucorpepsin is the aspartic protease (Aps) extracellularly produced by the two closely related species of zygomycetes, *Mucor pusillus* and *Mucor miehei* but now reclassified in the genus *Rhizomucor*. It possesses comparatively high milk-clotting activities (Vega-Herná, 2007). It is one of the microbial aspartic proteinases that were discovered in the 1960s during the shortage of calf chymosin (Teruhiko and Nishiyama, 2013). This enzyme was termed with different names such as *Mucor pusillus* pepsin (rennin), *Mucor miehei* pepsin (rennin), and aspartic proteinases of *M. pusillus* and *M. miehei* (Teruhiko and Nishiyama, 2013). The aspartic protease from *M. miehei* (MAP, EC 3.4.23.23) is the most glycosylated of the APs enzymes. This enzyme preferentially cleaves the Phe105-Met106 bond of κ-casein; this destabilizes casein micelles to cause coagulation of milk. The milk-clotting activity can be determined based on the time required for clotting a 10% solution of skim-milk powder (Teruhiko and Nishiyama, 2013).

The optimum pH for mucorpepsin is at about pH 4, even though the pH varies with the substrate, and shows the highest levels of thermal stability among the Aps (Teruhiko and Nishiyama, 2013). It is now the major microbial milk-clotting enzyme used in the cheese industry. The high thermal stability leads the activity of the enzyme to persist the cooking temperature of the curd and may cause off-flavor in cheese during long maturation periods (Teruhiko and Nishiyama, 2013; Vega-Herná, 2007).

2.4.1.2. Aspergillopepsin I and II

Aspergillopepsins are extracellular proteinases secreted by fungal mycelia. Aspergillopepsin I was isolated from *Aspergillus saitoi*, a microorganism used in the fermentation of the traditional Japanese liquors awamori and shochu (Ichishima, 2013). The enzyme was formerly known as aspergillopeptidase A. Aspergillopepsin I is assigned to the aspartic proteinase family (I) in the pepsin superfamily. It is found in a variety of species within the genus of imperfect fungi *Aspergillus* and has been known by a variety of names (Ichishima, 2013).

Aspergillopepsin I from *A. saitoi* consists of 325 amino acid residues. It shows two forms of activity at acidic pH, pepsin-like catalytic function and trypsinogen activating activity like enteropeptidase. Aspergillopepsin A from *A. awamori* is 52% identical to endothiapepsin. The nucleotide sequence of the coding region of the proctase B cDNA from *A. niger* var. *macrosporus* also shares a high identity with that of the genes for aspergillopepsins I from *A. awamori* (98%) and *A. saitoi* (95%) (Ichishima, 2013). The molecular mass of Aspergillopepsin A from *A. niger* is 43 kDa and inhibited by pepstatin. The optimum pH of Aspergillopepsin I for milk casein digestion is the pH range of 2.5-3.0 and stable over the pH range of 2.5-6.0 (Ichishima, 2013).

Aspergillopepsin II is a non-pepsin-type acid proteinase, resistant to the inhibitors of ordinary pepsin-type aspartic proteinases such as pepstatin, with optimum pH between 1.8-2.6 and optimum temperature of about 70 ^{o}C for casein degradation. Aspergillopepsin I and II are usually present as the major and minor components of the commercial crude enzyme powder named Proctase (AGP). AGP is a food grade and inexpensive enzymatic preparation that has been found to be very active at wine pH and at temperatures at which grape proteins are in an unfolded state (Marangon *et al*., 2012).

2.4.1.3. Endothiapepsin

The chestnut blight fungus *Endothia parasitica* produces an aspartic protease, endothiapepsin, with milk-clotting properties similar to calf rennet (Vega-Herná, 2007). Like most aspartic proteinases, endothiapepsin has an acid pH optimum and a low pI of around 5.5. It is strongly inhibited by pepstatin A. Endothiapepsin is a single-chain proteinase with 330 amino acids and has a molecular mass of 33.8 kDa (Cooper, 2013). It can be used as fungal rennet in cheese production and has been marketed by Pfizer under the brand name Sure-Curds and more recently Suparens (Cooper, 2013).

This enzyme is predominantly suitable for the production of Emmental and Italian style cheeses due to its very high thermolability. Different varieties of cheeses including Cheddar, Swiss, Colby and Italian varieties produced with partially purified enzymes from *Endothia parasitica* are considered to be equal or superior to control cheeses made with animal rennet (Vega-Herná, 2007).

2.4.1.4. Pencillopepsin

It is a proteolytic enzyme obtained from an unidentified species of a filamentous imperfect fungus of the genus *Penicillium* (Hofmann, 2013). It was first named as mold kinase, which activates trypsinogen at low pH (pH optimum 3.4). A similar enzyme from *Aspergillus oryzae* was named as trypsinogen kinase. The purification and characterization of the enzyme from *Penicillium janthinellum* were later renamed as peptidase A. Finally the name was changed to penicillopepsin. Penicillopepsin-JT1, a single-chain enzyme with 323 amino acyl residues.

2.4.1.5. Rhizopuspepsin

It is an acid protease extracted from *Rhizopus chinensis* that has provided a very useful model system for the application of a wide variety of experimental and theoretical studies (Dunn, 2013). It has optimal activity at pH 2.9 to 4.5, depending on the different types of substrates. Its cleavages occurred most readily between aromatic or bulky hydrophobic residues. The sequence analysis of a crystalline preparation containing both the pI 5.1 and 5.8 isozymes, shows the presence of two divergent amino acid residues at eight positions.

2.4.1.6. Irpex lacteus protease (ILAP)

An aspartic protease produced from the wood-decaying basidiomycete *Irpex lacteus* (ILAP) has high milk-clotting activity and considered as a noble rennet substitute. ILAP contains 340 amino acid residues with a molecular mass of 35 kDa. It has a high content of serine and threonine residues (48 and 54, respectively) accounting for 30% of the protein residues. It lacks the three-disulfide bridges that are generally present in most pepsin-type Aps. ILAP is active at pH 3.0 and is inhibited by pepstatin (Vega-Herná, 2007).

ILAP is a pepsin-like enzyme extensively distributed in nature. The overall structure is similar to pepsin and other aspartic proteases. Unlike the other aspartic proteinases, ILAP needs hydrophobic residues both in the P1 or P10 site and also in the P4 and/or P3 site(s) for secondary interactions (Fujimoto *et al*., 2004).

2.4.1.7. Saccharopepsin

The activity of saccharopepsin in yeast was discovered as early as 1917 under the name 'Hefepepsin'. It is termed as proteinase A or proteinase yscA (Winther *et al*., 2013). The name saccharopepsin is desirable because it distinctively identifies this protease by its origin (Saccharomyces) and type (pepsin like). The specificity of Saccharopepsin resembles that of pepsin and cathepsin D. It is by far the most abundant aspartic protease in yeast (Winther *et al*., 2013).

The activity of saccharopepsin can be measured using acid-denatured hemoglobin. It's *in vivo* activity can be detected indirectly on the agar plate using an overlay assay for carboxypeptidase Y activity, with N-acetyl-DL-phenylalanyl-β-naphthyl ester as substrate. Therefore, the activity of saccharopepsin secreted from yeast can be monitored as cleared zones on agar plates containing 1% non-fat dry milk. The mature active saccharopepsin has a molecular mass of 42 kDa.

2.4.1.8. Production of milk-clotting enzymes from *Aspergillus*

Different fungi and bacteria have been used for the industrial level of milk-clotting proteases production. Table 2.2 shows some of the well-known microbes as reported by Jacob *et al*., (2011).

Fungi (*Aspergillus spp*)

The genus *Aspergillus* is found worldwide and consists of more than 180 officially recognized species, and comprises a particularly important group of filamentous

ascomycete species. Most of the members are useful microorganisms in nature for the degradation of plant polysaccharides and are important industrial microorganisms for the large scale production of both homologous and heterologous enzymes (Vishwanatha, 2009). According to the List of the Food and Drug Administration (FDA) in the United States *Aspergillus oryzae* and *Aspergillus niger* are Generally Recognized as Safe organism (GRAS) (Kumar *et al*., 2014). *A. oryzae* is generally recognized as a non-pathogenic fungus and non-productivity of aflatoxin has been well established in the industrial strains (Ichishima, 2018). The safety of *A. oryzae* is also supported by the World Health Organization (Ichishima, 2018; Kumura *et al*., 2017).

A. oryzae has been used for centuries in the food fermentation industry and no strains of *A. oryzae* are known to produce aflatoxin. The genes encoding the pathway enzymes for aflatoxin biosynthesis are clustered within a 75 Kb region of DNA in *A. flavus*. Strains of *A. oryzae* also have the aflatoxin biosynthetic cluster but it does not appear to be functional. Compared to the *A. flavus* sequence, the *A. oryzae* genes contained deletions, frameshift mutations, and base-pair substitutions (Payne *et al*., 2006).

Aspergilli play an important role in the production of industrial enzymes due to their high capacity for producing and secreting extracellular enzymes. *Aspergillus* species are also important microorganisms in the fermented food industry and produce a variety of amylases and proteases. *Aspergillus* species specially GRAS-designated strains, produce and secrete a variety of enzymes including α-amylases, proteases, glucoamylases, cellulases, pectinases, xylanases and other hemicellulases (Vishwanatha, 2009).

Aspergillus oryzae is the major producers of aspartic protease for they possess several encoding genes such as pepA (Gomi *et al*., 1993). Aspartic protease genes from fungi may be expressed in yeast for large scale fermentation as it is reported that yeast is good expression hosts for genes of fungal origin (Sun *et al*., 2018; Yegin and Fernandez-Lahore, 2013). The authors reported that the methylotrophic yeasts such as *Pichia pastoris* are widely used as expression platforms for recombinant proteins for basic research and industrial applications.

The methylotrophic yeast *Pichia pastoris*, currently reclassified as *Komagataella pastoris*, has become a substantial workhorse for biotechnology, especially for heterologous protein production (Ahmad *et al*., 2014). *P. pastoris* have many advantages in yielding a high-level expression of recombinant proteins, protein processing and are characterized by post-translational modifications (Kangwa *et al*., 2018; Luo *et al*., 2016). The post-translation modification associated with higher eukaryotes such as processing of signal sequence, folding, disulfide bridge formation, certain types of lipid addition and O- and N-linked glycosylation (Cereghino and Cregg, 2000). It can also be cultivated on cheap media with low-level proteins and has been accepted as a safe and effective expression system by the U.S. Food and Drug Administration (FDA) (Luo *et al*., 2016). *P. pastoris* has been shown great achievement in the large-scale production of recombinant protein.

The expression of the aspartic protease in *P. pastoris* is achieved by cloning the protease gene into the expression vector pGAPZα-A. pGAPZαA is chosen as an expression vector since it is designated for high-level constitutive expression in *P. pastoris* (Cereghino and Cregg, 2000). The pGAPZαA was created when the methanol-regulated AOX1 promoter was replaced with a constitutive, glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter in the vector. The

advantage of using the GAP promoter is that there is no need to shift cultures from one carbon source to another as methanol is not required for induction of the enzyme. It is established that genetic manipulation in filamentous fungi is more complex than in yeast and bacteria and so necessitates expression of the desired gene from filamentous fungi into a suitable host(Cereghino and Cregg, 2000).

Table 2.2: Microbial sources of milk-clotting aspartic proteases (Jacob *et al*., 2011).

Microorganisms	Properties
Pleurotus sojur-caju (white-rot fungi)	Clotting activity under cheese-making conditions
Mucor bacilliformis	High structural similarity to bovine chymosin, Lower thermostability than *Rhizoucor meihei* protease
Thermoascus aurantiacus	Enzymatic hydrolysis of bovine casein differed largely from Proteolysis patterns generated by bovine chymosin
Thermomucor indicae-seudaticae N31	The crude enzymatic extract showed high milk clotting and low proteolytic activity and low thermostability
Metschnikowia reukaufii	Milk-clotting activity, successfully cloned into *Escherichia coli*
Myxococcus xanthus	Molecular mass:40 kDa, Highest clotting activity at pH 6 and 37^0C, acceptable yield and properties of the curd in cheesemaking experiments, successfully cloned into *Escherichia coli*
Enterococcus faecalis	Similar electrophoretic patterns of hydrolyzed k-casein as *Rhizomucor miehie* effectively applied for camembert cheese manufacture
Nocardiopsis sp.	Milk-clotting ability of extracellular extracts, optimization of enzyme yield by fermentation conditions
Bacillus subtilis	The ratio of milk clotting to proteolytic activity is comparable with commercial fungal protease but high thermostability
Bacillus liceniformis	Shows typical milk-clotting kinetics

2.4.2. Bacteria

It has been widely assumed that bacteria do not produce milk-clotting enzymes due to a few types of research conducted on them compared to the fungi (Ding *et al*., 2012). However, *Bacillus* is known for the production of numerous proteases during the

fermentation process (Guleria *et al*., 2016). The capacity of some *Bacillus* strains to produce and secrete large quantities of extracellular enzymes has made them among the most important industrial enzyme producers (Guleria *et al*., 2016).

Several *Bacillus* strains like *Bacillus licheniformis* (Akcan, 2012), *Bacillus subtilis* (Dutt *et al*., 2009), *Bacillus amyloliquefaciens* (Guleria *et al*., 2016), *Bacillus sphaericus* (El-bendary *et al*., 2007), and *Bacillus subtilis* natto (Shieh *et al*., 2009) have been reported for milk-clotting enzyme production.

The milk-clotting enzyme produced from *B. subtilis* KU710517 isolated from marine sponge showed the highest (581.8 U/ml) milk-clotting activity. The partially purified milk-clotting enzymes from these bacteria showed the optimal temperature and pH at 85 ^{o}C and 5.0 respectively. This enzyme is thermally stable at 45 ^{o}C and retained 100% activity after 90 min. However, at 60 ^{o}C, the enzyme loses about 70% of its original activity after 30 min (Hala *et al*., 2016).

B. subtilis, B. mesentricus and *B. cereus* are potential sources of milk clotting enzymes among several bacterial species (Dutt *et al*., 2008). The milk-clotting activity (MCA) and milk-clotting activity/proteolytic activity (MCA/PA) ratio of the crude enzyme was comparable with those of Pfizer microbial rennin and *Mucor* rennin (Shieh *et al*., 2009).

B. subtilis (natto) Takahashi, a commercial natto starter, is commonly used to prepare fermented soybean product-natto, which is a traditional Japanese food for more than 1,000 years. *B. subtilis* is one of the most investigated microbial groups because they can produce varieties of biotechnological interesting substances. It is known to secrete several proteases during the fermentation process (Wu *et al.*, 2013).

Some *Bacillus* species such as *B. subtilis* and *B. licheniformis* are Generally Regarded as Safe (GRAS) based on FDA (Kumar *et al*., 2014; Lim, 2019; Nair and Jayachandran, 2019). *B. subtilis* CU1 was non-toxic, safe and well-tolerated in the clinical subjects without undesirable physiological effects on markers of liver and kidney function, complete blood counts, hemodynamic parameters, and vital signs (Lefevre *et al*., 2017). The genus *Bacillus* especially *B. subtilis* are being used as probiotics (Hong *et al*., 2008). The use of *B. subtilis* is also approved for use as a food supplement in at least one European country (Italy)(Hong *et al*., 2008).

2.5. Production of microbial aspartic protease

Different investigators reported that the fermentation parameters and the medium components for microbial aspartic protease production are widely varied (Foda *et al*, 2012). The production of extracellular protease by microorganisms is significantly influenced by media components, especially carbon and nitrogen sources, and physical parameters such as temperature, pH, incubation time, agitation, and inoculum density. The composition of media is one of the most important factors for the industrial production of enzymes to minimize the cost of production. Therefore, the use of local substrates in growth media composition can significantly decrease the cost of enzyme production (Siala *et al*., 2012).

2.5.1. Isolation and primary screening of proteolytic microbes

Isolation of potential microbial strain is the first step in the production of an industrial enzyme (Sumantha *et al*., 2006). An easy, efficient and rapid method of screening protease producing microorganisms is vital to save time and to reduce the cost of enzyme production (Saran *et al*., 2007). The organisms living in protein-rich soil have the potential of producing higher amounts of proteolytic enzymes (Sandhya *et al*.,

2005). Screening of proteolytic microorganisms by plate assay methods using different agar media containing gelatin, casein, bovine serum albumin, and hemoglobin is very common (Sumantha *et al*., 2006).

Several agar plate assays has been used to detect milk-clotting protease producing microbes (Sumantha *et al*., 2006). Agar Reese's medium containing (0.5% casein as protein substrate) is used for screening of microbes capable of producing milk-clotting enzymes (Khademi *et al*., 2013a). The plates are then incubated at 35 ^{o}C for 2 days to allow the growth of the microbes. The clear zone that showed enzyme activity was examined by flooding with 5% Trichloroacetic acid (TCA) (Khademi *et al*., 2013a).

Acid protease producing microbes are also screened using a qualitative plate medium (Fazilat, 2016), skim-milk agar media (Saran *et al*., 2007). *B. amyloliquefaciens* D4 was screened from the soil of Tibetan Plateau in China by using casein/agar plates containing (g/L); casein 10, skim-milk powder 6.25, peptone 2.5, glucose 10, yeast extract 1, Agar 20, pH=7) (He *et al*., 2012). The zone of hydrolysis was observed by flooding 10% tannic acid into the milk agar plate (Saran *et al*., 2007a). The hydrolysis zone was also observed by staining the plates with Coomassie Brilliant Blue R-250 for 2 h followed by destaining overnight (Fazilat, 2016).

Proteolytic activity of milk-clotting enzymes is also determined by flooding bromocresol green reagent on casein/skimmed milk agar plates. A few amounts of bromocresol green dye (0.0015%) are incorporated with the substrate agar plates before autoclaving to detect the proteolytic activity of bacteria. The activity appeared as a colorless zone, while the rest of the plates is greenish-blue in color which is pH-dependent (Vijayaraghavan *et al*., 2013). Similarly, milk clotting bacteria have also

been detected by supplementing 1% (w/v) bovine serum albumin (BSA), 1% hemoglobin and/ or 1% gelatin into the solid media (Vermelho *et al.*, 1996).

Microbial protease production is improved by optimizing process parameters, such as production media composition, pH, volume (in case of SmF), moisture content (in case of SSF), concentration of mineral salts, age and size of the inoculum, fermentation time and temperature, carbon, organic and inorganic nitrogen supplement (Sumantha *et al*., 2006). Nitrogen sources like casein, soybean meal, gelatin, corn steep liquor, brewer's yeast and carbohydrate sources like starch, ground barley or lactose-containing media are commonly used for protease production. During media preparation, care must be taken with a substrate that induces or repress protease production. The production of protease in general and aspartic protease, in particular, are repressed by high carbohydrate concentrations, free amino acids, isoleucine, and proline while proteins and peptides induce protease production in several groups of microorganisms like *Mucor miehei* (Sumantha *et al*., 2006).

Cheap nitrogen sources such as corn steep liquor are used for the production of a thermostable acid protease by a strain of *A. niger* F2078 (Sumantha *et al.*, 2006). Acid protease is also produced using sweet potato residue. Sunflower meal is another low-cost substrate which is successfully used for protease production. Additives such as vitamins (like biotin), growth promoters (1-naphthyl acetic acid), etc. enhance protease production (Sumantha *et al.*, 2006).

2.5.2. Fermentation

The types of fermentation either solid or submerged significantly affect the growth of microorganisms as well as their enzyme production. Molds grown under solid-state

fermentation produce more fruiting bodies which has a positive influence on enzyme production (Sumantha *et al*., 2006).

2.5.2.1. Solid-state fermentation

The term solid-state fermentation (SSF) is applied for the processes in which insoluble materials in water are used for microbial growth. Under the circumstances, the quantity of water should not exceed the capacity of saturation of the solid bed in which the microorganisms grow (Aguilar *et al*., 2008).

It involves microbial modification of a solid, un-dissolved substrate into a moist solid with little or no free water. The concentration of solids in SSF is usually high, whereas the content of water is frequently low. SSF is less expensive than SmF in terms of cost (Sumantha *et al*., 2006). In solid-state fermentation, the solid substrate provides essential nutrients and support for the growth of microbes. Many factors such as the type of substrate, particle size, water level/humidity, and temperature can influence the growth and microbial activity during SSF (Araújo *et al*., 2015).

SSF is more efficient than submerged fermentation with regard to production cost. The product can be recovered in a highly concentrated form as compared to those obtained from submerged fermentation (Sumantha *et al*., 2006). SSF has several advantages over submerged fermentation. These include high volumetric productivity, relatively high concentration of the product, less effluent generated and less expensive technology and skill (Renge *et al*., 2012). However, the scale-up and optimization of SSF processes are complex and demand intensive research for commercial production (Foda *et al*., 2012; Gais *et al*., 2009).

SSF technique is particularly suitable for the production of fungal enzymes. Industrial enzyme production by molds is usually carried out by SSF because of high yields and

low energy consumption (Foda *et al*., 2012; Gais *et al*., 2009). SSF is a time-tested, cost-effective method for the production of enzymes from fungi (Soares *et al*., 2016). The fungus possesses more secretory proteinase genes that function in acidic pH and grows on cheap agricultural by-products such as wheat bran, rice bran, bagasse, etc. where amino acids and sugars are initially deficient that drive the organism to have a number of hydrolytic enzymes and transport-related gene families to utilize external nutrient resources for its growth (Ferea *et al*., 1999; Vishwanatha *et al*., 2009).

The cultivation parameters and the medium constituents in SSF greatly affect the ratio of milk-clotting activity to the proteolytic activity of the enzyme production (Jacob *et al*., 2011). It is suitable for fungi growth using agricultural by-products as substrates and requirement of the less sterile environment and skill and recovery of concentrated and more stable enzymes than those obtained in submerged fermentation (Soares *et al*., 2016).

The choice of substrate depends on many factors, which is primarily associated with the cost and availability of the substrate. Other factors such as particle size and the level of moisture are also vital for selecting substrates in SSF. Substrates with smaller particle sizes have a larger surface area for the proliferation of the microbes, but fine substrates adversely affect the growth and efficiency of the respiration and poor enzyme production. Larger particles provide more efficient aeration and respiration but they reduce the surface area (Renge *et al*., 2012).

Agro-industrial by-products like sunflower meal, coffee hulls, soybean meal, rice bran and husks, corn bran, yam residue, and wheat bran have been considered to be suitable substrates for the production of milk-clotting proteases (Araújo *et al*., 2015). However, wheat bran is a cheap source of energy for the production of the enzyme in

SSF (Zhang *et al*., 2013). *Aspergillus sp.* and *M. pusillus* produce higher protease yields in SSF using wheat bran as the solid media with 50% moisture content than in SmF (Sumantha *et al*., 2006).

The production of extracellular proteases is also affected by the physical factors; such as temperature, pH, incubation time and inoculum density. Pallavi *et al*., (2012) showed that crude enzymatic extract produced by *A. oryzae* NCIM 1032 exhibited high milk- clotting activity (MCA) and low proteolytic activity (PA) after 120 h of fermentation using a mixture of wheat bran and rice bran (7:3) on SSF. The extracellular acid protease produced from *A. niger* on wheat bran showed optimal milk-clotting activity at pH 5.5 and 45 ^{0}C in the presence of 0.01 M $CaCl_2$ (Fazouane-Naimi *et al*., 2010).

Another study showed that *R. miehei* NRRL 2034 showed the highest MCA on wheat bran compared to twelve industrial by-products under SSF (Foda *et al*., 2012). Sathya *et al*., (2009) also showed high milk-clotting activity and enzyme yield of a local isolate of *M. circinelloides* on dhal husk with the supplementation of sucrose and yeast extract as carbon and nitrogen source, respectively.

Generally, the search for local and low-cost substrates, together with optimization of cultivation conditions, is necessary to significantly minimize the cost of enzyme production in the context of traditional "one-variable-at-a-time" strategic operation in biotechnology (Siala *et al*., 2012).

2.5.2.2. Submerged fermentation (SmF)

SmF is the cultivation of microbes on a substrate, which is either dissolved or remains suspended in an aqueous medium. Industrial enzymes can be produced using

submerged fermentations. It involves the growth of carefully screened microorganisms (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen (Renge *et al*., 2012). The addition of a nitrogen-rich medium with glucose enhances protease production in submerged fermentation (Sumantha *et al*., 2006).

There are different types of SmFs, such as batch, fed-batch, and continuous; but continuous cultures produce higher enzyme units than batch fermentations. The relatively new experimental designs such as radial basis function (RBF), artificial neural network (ANN), and response surface methodology (RSM) have been used to study the relationship between various interacting parameters for enzyme production (Sumantha *et al*., 2006).

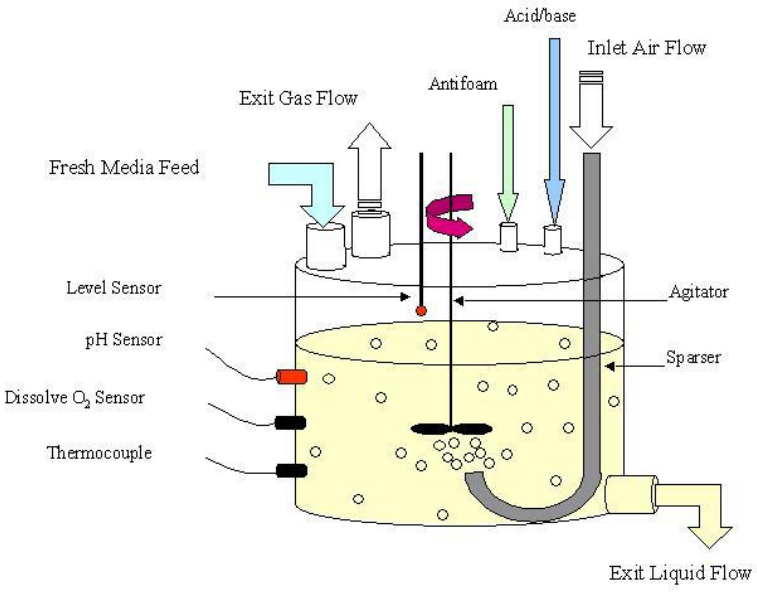


Figure 2.1: Typical fermenter (Renge *et al*., 2012)

All *Bacillus sp.* best produce proteases in submerged fermentation (Sandhya *et al*., 2005). *B. amyloliquefaciens* JNU002 showed high milk-clotting activity (4969 SU/mL) and low proteolytic activity (4.02 U/mL) in submerged fermentation containing glucose, 16.2 g/L and wheat bran 30 g/L (Ding *et al*., 2012). Hang *et al*.,

(2016), reported that 3%–6% (w/v) wheat bran in broth media raised the milk-clotting activity (MCA) of the enzyme extracted from *Paenibacillus sp.* Strain BD3526.

Thermomucor indicae-seudaticae N31 showed milk-clotting activity (MCA) of 60.5 U/mL using a 4% wheat bran and 0.3% saline solution under submerged fermentation (Silva *et al*., 2014). Yegin *et al*. (2013) also showed the production of extracellular milk-clotting enzymes from *Mucor mucedo* DSM 809 in submerged fermentation.

2.5.3. Extraction and purification of microbial aspartic protease

There is a wide range of techniques available for the recovery of enzymes from the fermented substrate. The method of choice for enzyme recovery depends on the source, i.e. intracellular or extracellular, the scale of operation and enzyme stability (Sumantha *et al*., 2006).

2.5.3.1. Crude enzyme leaching (extraction)

I. Intracellular enzymes

A small percentage of enzymes that are firmly bound with the membrane (integral proteins) are called intracellular enzymes. Intracellular enzymes can be liberated from the cell by cellular disintegration techniques, such as pressure homogenization, grinding in the presence of alumina in a mortar and pestle (Sumantha *et al*., 2006).

II. Extracellular enzymes

Most of the secreted enzymes are extracellular for they are bound on peripheral membrane proteins and can be released into the culture filtrate by certain extraction techniques. Extracellular enzymes are undertaken in either a batch or continuous process using different solvents. Some of the solvents are water, aqueous buffers, diluted solutions of salts (e.g. 0.9% sodium chloride), 1% glycerol, or diluted (0.1%)

solutions of non-ionic detergents such as Triton X-100, Tween 20, Tween 40, Tween 60, and Tween 80 (Sumantha *et al*., 2006).

The duration of the extraction is about 50–60 min. The solvent-to-solid ratio ranges from 1 to 8 and the extraction temperature is around 30 ^{o}C. Thereafter, the extracts are adjusted to temperatures of 4–10 ^{o}C to avoid denaturation, proteolysis and microbial growth. The extraction of the enzyme can be carried out at pH close to 7, but pH far from the pI (isoelectric pH/point) of the enzyme is recommended to avoid precipitation. Acid protease from *Rhizopus oligosporus* is best extracted from fermented rice bran at pH 7, though it is most stable at pH 4 (Sumantha *et al*., 2006).

The enzyme extraction from solid-state fermentation is undertaken by taking a known quantity of solid medium in 100 mL (1:10 ratio of Bran-solvent w/v) of distilled water or distilled water containing 0.1M NaCl and allowed to stay for 60 min at 4 ^{o}C to minimize autolysis (Fernandez-lahore *et al*., 1998). Then the crude enzyme is extracted by shaking the flask on a rotary shaker at 220 rpm for 1 h at 30 ^{o}C and filtered using filter paper.

The filtrate is centrifuged at 10,000 g for 10 min at 4 ^{0}C to recover the supernatant as a crude enzyme. In submerged fermentation, the crude enzyme is extracted by directly filtering the media through Whatman No 1 filter paper (90 mm) and centrifuging at 10,000 g for 10 min at 4 ^{o}C. Then the resultant filtrate is directly used for enzyme assay (Garcia *et al*., 2005).

2.5.3.2. Enzyme purification

Enzyme production by SSF or SmF consists of two major stages: (a) upstream operations and the cultivation process which leads to enzyme biosynthesis and (b) downstream processing which aids the efficient recovery and purification of the

enzyme product (Yegin *et al*., 2011). Enzyme purification is a complex process that uses several methods sequentially to get a final pure enzyme (Sumantha *et al*., 2006). It is to recover the enzyme in it's active, clarified and concentrated form which includes a high degree of purity, high protease activity and high reproducibility (Sumantha *et al*., 2006; Yegin *et al*., 2011).

When proteases are produced in SmF, separation of the biomass is performed either by centrifugation or filtration, and the subsequently clarified broth is concentrated by ultrafiltration (Yegin *et al*., 2011). However, in SSF the enzyme has to be extracted out of the fermented solid mass by "leaching" via the addition of a known volume of an appropriate solvent. The use of sodium chloride solution for leaching operations is more efficient than a non-ionic detergent solution (Triton X-100; Tween 80) or distilled water (Fernandez-Lahore *et al*., 1998). Then the protease enzyme is further purified by various chromatographic and adsorption techniques for adequate separation between the product and other contaminants. The combination of various methods such as salt and acid precipitation, ion-exchange chromatography and size-exclusion chromatography are used as a common strategy to purify protease enzyme (Yegin *et al*., 2011).

The first step in protease purification is a separation of the cell debris from the crude enzyme by either differential sedimentation or precipitation. After the removal of nucleic acid and debris from the cell extract, then the supernatant containing the enzyme can be further exposed to the process of removal of unwanted contaminants such as small organic and inorganic molecules, other proteins and water by salt (ammonium sulfate) or solvent (acetone/ethanol) precipitation. Then salts are removed from the enzyme extract by dialysis against its corresponding buffer

(Sumantha *et al*., 2006). Ultrafiltration is a technique used to separate proteins by passing water and other small molecules through a semi-permeable membrane, thereby concentrating the protein molecules in the solution. This technique is faster and easier to handle than the two-step process of precipitation and dialysis (Sumantha *et al*., 2006).

Chromatographic techniques such as ion exchange and gel filtration significantly increase the specific activity of the enzyme. Such purification procedures are also efficient methods to determine the molecular mass of the enzyme protein using a protein with a known molecular mass as a reference standard (Sumantha *et al*., 2006). Dye-affinity membrane chromatography is another technique that is used for the purification of neutral protease. Another type of affinity chromatography is purifying the enzyme by using its inhibitor as a ligand. proteases can be purified using activated charcoal and hydrogen peroxide, followed by acetone precipitation (Sumantha *et al*., 2006).

Protease purification by partitioning in aqueous two-phase systems (ATPS) is also possible for small scale enzyme production. In this method, polyethylene glycol/salt systems are used to partition and purify proteases. The partitioning of protease by ATPS depends on the surface properties (such as the size, charge, and hydrophobicity) of the enzyme and the physicochemical traits of the two phases. This system is influenced by factors such as type of polymers, polymer molecular mass and concentration, the type of phase forming salt and its concentration, pH, buffer, ion strength and temperature (Peričin *et al*., 2009).

Purification of protease enzyme increases its activity on protein substrates. The purification of the milk-clotting enzyme from *R. miehei* by ultrafiltration, ammonium

sulfate fractionation and Sephacryl S-300 chromatography improved its activity by 220.29-fold (14444.2 U/mg protein) (El-Bendary *et al*., 2007). The two-step purification of the extracellular milk-clotting enzyme from *R. miehie* by ion exchange and affinity chromatography also showed a 2 fold increase in milk-clotting activity/ protease activity (MCA/PA) ratio (Preetha and Boopathy, 1997). In another study, the purification of aspartyl proteinase from *M. mucedo* DSM 809 by ion-exchange chromatography (IEC) and size exclusion chromatography (SEC) revealed that the active fraction eluted at 43% of the gradient development with 36% activity yield and a concentration factor of 4 (Yegin *et al*., 2012).

2.5.3.3. Molecular weight determination

Although protein analysis technologies are developing fast, the current standard method for protein sizing is still the denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Goetz *et al*., 2004). SDS is an anionic detergent with a net negative charge. It binds to most soluble protein molecules in aqueous solutions over a wide pH range. The amount of bound SDS is proportional to the size of the molecules. The SDS eliminates most of the complex secondary, tertiary or quaternary structure of proteins, which is one requirement for protein sizing by SDS-PAGE (Goetz *et al*., 2004).

SDS is also essential to reduce protein disulfide bridges before the proteins adopt the random-coil configuration necessary for separation by size. This is achieved with reducing agents such as 2-mercaptoethanol or dithiothreitol. In addition, SDS also confers a negative charge to the polypeptide, which is proportional to its length and utilized to separate the protein in an electrical field within polyacrylamide gels. The

polyacrylamide forms porous gels allowing separating molecules by size (Goetz *et al*., 2004).

A polyacrylamide gel with a certain acrylamide concentration restrains larger molecules from migrating as fast as smaller molecules. Because the charge-to-mass ratio is nearly the same among SDS-denatured polypeptides, the final separation of proteins is dependent almost entirely on the differences in molecular weight (MW) of polypeptides. In a gel of uniform pore size, the relative migration distance of a protein (Rf) is negatively proportional to the logarithm of its MW. If proteins of known MW are run simultaneously with the unknowns, the relationship between Rf and MW can be plotted to determine the MWs of the unknown proteins (Goetz *et al*., 2004).

Glycosylation, including N- and O-glycosylation is a post-translational modification that is found on almost 50% of secreted and transmembrane proteins of eukaryotes and to a lesser extent in prokaryotes (Goettig, 2016). It can potentially affect biochemical properties of proteins including, stability, solubility, intracellular trafficking, activity, pharmacokinetics, and antigenicity (Wang *et al*., 2008). It is important for proper folding; oligomerization and solubility of eukaryotic proteins, as it significantly prolongs the stability and half-life time by inhibiting proteolysis (Goettig, 2016), and protecting molecular damage from free radicals, and maintaining thermal stability of proteins (Wang *et al*., 2008).

2.5.3.4. Milk-clotting activity (MCA) test

MCA is the most essential characteristics of enzymes used for cheese production. It is the measure of the capability of the milk-clotting enzyme for specific K-casein hydrolysis. The Soxhlet method, which is still used in traditional cheese-making, represents a conventional method to determine the total MCA of a rennet enzyme.

The clotting strength in Soxhlet units refers to the volume of raw milk which can be clotted by one volume unit of the enzyme in 40 min at 35 ^{o}C (Jacob *et al*., 2011). The MCA of microbial rennet enzyme is determined by the addition of 0.5 ml of the crude enzyme into 5 mL of reconstituted skim milk (10% skim milk in 10 mM $CaCl_2$, pH= 6.5) pre-incubated at 35 ^{o}C for 10 min. Then, the clotting time is recorded starting from the addition of the enzyme until the formation of the first particles.

The MCA is calculated as SU= (2400×5×D)/(T×0.5)

- Where T is milk-clotting time (s), and
- D is the dilution factor of the enzyme extract.

One Soxhlet unit (SU) of milk-clotting activity was defined as the amount of enzyme required to clot 1mL of the substrate within 40 min at 35 ^{o}C (Arima *et al*., 1970).

MCA is also determined with a little modification according to the method of Yegin *et al*., (2012) and expressed in terms of Soxhlet units (SU). In order to perform the assay, 0.1 ml of the sample enzyme is added to a glass test tube containing 1 mL of reconstituted skim milk solution (10 g skim-milk powder dissolved in 100 mL of 0.01 M $CaCl_2$ solution) pre-incubated at 35 ^{o}C for 10 min. The mixture is then mixed well and the clotting time t (s) is measured with a chronometer. The clotting activity is calculated using the following formula:

SU= (2400 x1xD)/(0.1x T)

- Where D is the dilution factor and
- T is the clotting time in seconds. One Soxhlet unit is defined as the amount of enzyme that clots 1 ml of the substrate in 40 min at 35 ^{o}C (Yegin *et al*., 2012).

The MCA assay is also carried out with slight differences from the previous methods (Daudi and Mukhtar, 2015). In this method, the enzyme is added to the milk substrate pre-incubated at 35 ^{0}C and mixed by inversion. The mixture is kept at 35 ^{o}C and the endpoint has been marked by the appearance of milk clots. The amount of enzyme-producing clotting in one minute is equal to 200 RU (Rennin units). Finally, the clotting time of a particular milk-clotting enzyme is compared with that of an international reference enzyme and standardized reconstituted skim milk (32 ^{o}C, pH 6.5) used as a substrate. The capacity of the milk-clotting enzymes is expressed in International Milk Clotting Units (IMCU).

2.5.3.5. Protease assay

Protease assay is most frequently undertaken using hemoglobin or casein that is completely soluble in the buffer. Casein precipitates below pH 6, so it is used at neutral to alkaline pHs. Hemoglobin must be denatured either by treatment with acid (if the assay is at acidic pH) or urea (neutral to alkaline pH assay) before using for protease assay (Sumantha *et al*., 2006).

Casein is the recommended substrate for protease assay, as it represents the most standard casein preparation available. Assay for proteases generally involves incubating the enzyme with its substrate for a specific time period, stopping the reaction with TCA and measuring the absorbance of the solubilized peptide. A tyrosine standard curve can be used for this purpose (Sumantha *et al*., 2006).

During protease assay, protease digests casein substrate leads to the liberation of tyrosine along with other amino acids and peptide fragments. The free tyrosine then reacts with Folin and Ciocalteus Phenol reagents and produces a blue-colored chromophore, which is quantified as an absorbance value on the spectrophotometer

(Cupp-Enyard, 2008). An enzyme with the highest protease activity is implicated with the release of more tyrosine from casein substrates that develop high chromophores. The absorbance value is recorded and compared with the tyrosine standard curve to correlate the changes in absorbance with the amount of tyrosine in micromoles. Finally, the activity of the protease enzyme is determined in terms of units from the standard curve, which is the amount in µmol of tyrosine equivalent released from casein per minute (Cupp-Enyard, 2008).

The proteolytic activity of the sample enzyme is assayed by adding 0.5 mL of enzyme extract into 2.5 mL of 1% (w/v) alkali-soluble casein in 0.02 M potassium phosphate buffer (pH 6.5). The reaction mixture is incubated in a water bath at 35 ^{o}C for 10 min. Then, the reaction is terminated by adding 2.5 mL of 0.44 M trichloroacetic acid (TCA) to the mixture. The precipitate formed is removed by filtration or centrifugation at 10,000 *g* for 10 min. A 1-ml volume of three times diluted 2N Folin/phenol reagent and 2.5 mL of 0.55 M sodium carbonate solutions are added to 1 ml of the filtrate.

This is further incubated at 35 ^{o}C for 20 min for color development and the optical density (OD) is measured at 660. One unit (1 U) of enzyme activity is defined as the amount of enzyme that liberated 1 µg of tyrosine per 1 mL in 1 min (Arima *et al*., 1970). Sodium carbonate is added to regulate any pH drop created by the addition of the Folin's reagent.

Protease activity is also determined by the azocasein digestion method (Yegin *et al*., 2013). In this method, 20 µl of azocasein substrate (5% w/v azocasein solution in 200 mM Tris-HCl buffer, pH 7.5), 20 µl enzyme sample and 460 µl of 50 mM Tris HCl buffer, pH 7.5, are mixed to make a final volume of 500 µl. Then 500 µl of TCA

(10% w/v) is added after the mixture is incubated at 37 ^{o}C for 30 min, and the samples are kept on cold ice for 15 min. Then, 800 µl of supernatant is added to a tube containing 200 µl of NaOH (1.8 N) after centrifugation at 7000 rpm for 10 min. The absorbance is then measured at 420 nm (Yegin *et al*., 2013).

Protease activity is also measured by hemoglobin units' tyrosine (HUT) assay using denatured and completely soluble hemoglobin as a substrate. In this method, at the end of incubation of the enzyme with hemoglobin, the undigested protein is precipitated with an equal volume of 10 % mass per volume ratio of TCA, and filtered through Whatman # 42 filter paper. The absorbance of the filtrate is determined at 280 nm and compared with 0 to 75 µg/mL of the tyrosine standard curve. One HUT unit is defined as the amount of enzyme-producing an absorbance at 280 nm, equivalent to 1.1 µg/mL of tyrosine per minute (1.10 µg/mL of tyrosine = 0.0084 A units) (Sumantha *et al*., 2006).

2.6. Application of microbial aspartic protease

Several proteases of microbial origin are used to produce biological detergents, meat tenderization agents and milk clotting agents in dairy industries (Chymosin and pepsin) (Benlounissi *et al*., 2014). Acidic proteases with high activity and stability at acidic pH have important industrial applications, specifically as milk-coagulating agents for cheese-processing and as flavor enhancers in other food industries (Mandujano-gonzález *et al*., 2016).

2.6.1. Cheese production

The major application of acid proteases in the dairy industry is to manufacture cheese. Traditionally different varieties of cheese are industrially produced by using calf rennet (Amer *et al*., 2015). Among which, Danbo cheese is semi-hard cheese

characterized by surface ripening with few round pea-sized holes, distinctive flavor and is consumed after ripening for 2–3 months but a few variants are allowed to mature for up to 12 months (Sorensen and Benfeldt, 2001). The flavour of Danbo cheese originates from smear bacteria that develop on the surface during the first weeks of maturation (Madsen, 2001). It is originated and most commonly consumed cheese in Denmark, and comprises about 13.2% of total Danish cheese production (Ryssel *et al*., 2001).

In cheese making, the primary function of calf rennet or aspartic protease is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) in a two-phase process to generate para-K-casein and macro peptides (Crabbe, 2004; Rao *et al*., 1998; Yegin *et al*., 2011). Proteinase from *Cryphonectria parasitica* cleaves the S_{104}-F_{105} bond. Rennin can also hydrolyze other milk proteins (αs1-, αs2-, β-caseins, and α-lactalbumin) at a lower rate (Crabbe, 2004).

Chymosin is preferred due to its high specificity for casein which is very crucial for cheese making. The aspartic proteases produced by microbes such as *Mucor miehie, Bacillus subtilis,* and *Endothia parasitica* which is GRAS (Generally regarded as safe) are gradually replacing chymosin in cheese making (Rao *et al*., 1998).

The second phase is non-enzymatic in which para-K-casein and other casein aggregates which eventually lead to gel formation under the influence of Ca^{2+}. These two steps of milk-clotting activity overlap when the aggregation of micelles starts before the end of the enzymatic process (Crabbe, 2004). Aspartic protease enzymes (APs) (EC3.4.23.X) are more active at acidic pH (Benlounissi *et al*., 2014), and the ones extracted from fungi are accepted as an alternative for chymosin in the dairy

industry (Yegin and Dekker, 2013). For instance, about 60% of cheese in USA is manufactured using fungal enzyme sources (Hala *et al*., 2016).

The production of UF (ultra-filtrated) white soft cheese with fungal rennin (1mL fungal rennin/100 mL milk) from *Rhizomucor miehei* NRRL 2034 perform as good as, and in some parameters better than calf rennet cheese (Abbas *et al*., 2015). The fungal rennin- treated cheese showed higher soluble nitrogen (SN), total volatile fatty acids (TVFAs), tyrosine and tryptophan than the calf rennet cheese. Furthermore, the fungal rennet cheese imparts soft body, smooth texture, and desirable taste. The production of miniature cheddar-type cheeses using microbial rennet from *B. amyloliquefaciens* (MCE) and calf rennet (CAR) did not show a significant difference in gross composition with the exception of pH (An, He, Gao, Zhao, & Zhang, 2014).

Similarly, ochratoxin free extracellular acid protease from *A. niger* FFB1 showed similar basic characteristics (pH 4.5, acid taste, white color) as marketed cheeses to the ones produced using calf rennet (Fazouane-Naimi *et al*., 2010). According to Lee and Nikraz, (2015), Turkish white brined cheese produced from microbial rennet of *Rhizomucor miehei*, showed similar physicochemical characteristics and concentration of free amino acids (FAA) to that produced using calf rennet after 90 days of ripening period. The yield, chemical and organoleptic properties of domiati cheese produced by *M. mucedo* KP736529 enzyme were better than the cheese produced by commercial calf rennet (Ayana *et al*., 2015).

Milk-clotting acid protease enzymes produced by GRAS *B. subtilis* are gradually replacing chymosin in cheese making industry (Chen *et al*., 2010; Rao *et al*., 1998). Microbial aspartic protease produced from *B. amyloliquefaciens* is also used for the production of miniature cheddar-type cheeses (An *et al*., 2014).

The purified protease obtained from *Bacillus sp.* P45 was used to produce cream cheese enriched with chia and quinoa flour. The result showed the cheese product displayed better quality with high water retention (99.0%), low syneresis process, adequate sanitary conditions, and highly stable and viable technological characteristics. Moreover, the results implied that the purified enzyme can be an alternative coagulant for the development of innovative biotechnological processes, such as the development of new dairy products with functional ingredients (Lazzaroni *et al*., 2016).

A comparative study on the milk-clotting activity of proteinase produced by *B. subtilis* var, natto, *Rhizopus oligosporus*, and commercial rennet, showed the commercial rennet had the higher viscosity and curd tension with the shortest clotting time than the curd produced using proteinase from *Rhizopus*. The enzyme produced by *B. subtilis* had the highest proteolytic activity, while the commercial rennet had the highest milk-clotting activity (Chen *et al*., 2010).

2.7. The global market for enzymes

Currently, about 4000 enzymes are known, of which nearly 200 microbial enzymes are used for commercial purposes. However, only about 20 enzymes are produced on a truly industrial scale. The world enzyme supply is monopolized by about 12 major producers and 400 minor suppliers (Kumar *et al*., 2014). According to a report from Austrian Federal Environment Agency (AFEA. 2002), about 158 enzymes are used in the food industry, 64 enzymes in various technical application and 57 enzymes in the animal feed industry, of which 24 enzymes are applicable across all these three sectors (Kumar *et al*., 2014).

Proteases constitute the largest selling segment with about 60% market share while carbohydrases, phytases, and lipases are other fastest-growing segments covering about 70% market share with proteases. The world market for industrial enzymes is projected to record a compound annual growth rate (CAGR) of approximately 4.7% for the period spanning from 2012-2014 (Kumar *et al*., 2014). A survey on world enzyme sales assigns 31% for food enzymes, 6% for feed enzymes and the remaining for technical enzymes. The sales for the food and beverage enzymes segment have been estimated at about $ 1 billion in 2010. In particular, milk and dairy had the highest sales within this segment.

Enzymes used in food processing are typically sold as enzyme preparations that contain enzyme-catalyzed metabolites and added substances such as stabilizers. All these materials are expected to be safe under the guidance of good manufacturing practice (GMP) (Kumar *et al*., 2014). Microbes are one of the largest and useful sources of many enzymes. About 150 industrial processes use enzymes or whole microbial cell catalysts. Only about nine recombinant microorganisms are issued as Generally Recognized as Safe (GRAS) based on FDA regulations from a relatively small number of bacterial and fungal species primarily *A. oryzae, A. niger*, *B. subtilis* and *B. licheniformis* (Kumar *et al*., 2014).

2.7.1. The market value of food enzymes

According to the market report published by Transparency Market Research (2013), the food additives market revenue was $28.2 billion in 2011 and is expected to reach $36.1 billion in 2018, growing at a CAGR of 3.6% from 2012 to 2018. Among the main industrial enzyme producers, Novozymes S/A occupies 47% of the market, DuPont 21%, and DSM 6% (Kumar *et al*., 2014). Furthermore, the food and beverage

enzymes represented 29% of enzyme business and bio-business sales by the industry. The world enzyme market is expected to grow at 6.8% per year. Bakery enzymes represent a relevant segment of the food and beverage industry. According to a research report by The Freedonia Group, the enzymes market for baked goods in 2020 is expected to be around $ 9.0 billion of which about 35% represents food enzymes (Kumar *et al*., 2014).

2.8. Dairy production in Ethiopia

Ethiopia possesses the largest cattle population in Africa with an estimation of 59.5 million (Central Statistics Agency, 2017). The milk production systems in Ethiopia are classified into urban, peri-urban and rural. The urban system producing about 35 million liters of milk annually of which only 7.6 % is processed into butter and Ayib (Ethiopian cottage cheese).The peri-urban milk production system includes smallholder and commercial dairy farms are located in the proximity of Addis Ababa and other regional towns (Zelalem Yilma, *et al*., 2011). The National average per-capita milk consumption is 19 kg/year as compared to 27 kg for other African countries and 100 kg for the world per capita consumption (Tadesse Mihret *et al*., 2017).

Organized milk collection and processing were introduced in the country mainly in Addis Ababa in the 1960s. Only one milk processing plant was functional in 1960, while processing and distribution in Addis Ababa were run by a government agency - Sholla Dairy. The agency was renamed DDA (Dairy development Agency) and later, DDE (Dairy Development Enterprise), but currently, it has been privatized and named Sholla or '*Lame*' (Amharic for 'my cow') (Zelalem Yilma, *et al*., 2011). With the political change, the numerous private dairy farms were merged and nationalized and

DDE became the only government processing plant that collected all the milk from these farms and its capacity grew to 60,000 liters per day. DDE under the new name of Lame (Sholla), is now a private company, operating with 25 collection centers located around Addis Ababa, 13 of the centers are near Selale, 5 close to Holetta, and 7 around Debre Birhan (Zelalem Yilma, *et al*., 2011). About 88% of milk and dairy marketing systems practiced through the informal sector. Only about 2% of milk sold in the formal market in Ethiopia which is much less than that sold in neighboring countries: 15 % in Kenya and 5 % in Uganda. The formal system was dominated by DDE, which functions as a milk producer, collector, and processor (Tadesse Mihret, *et al*., 2017).

Ethiopia is not known to export dairy products. However, some insignificant quantities of milk and butter are exported to a few countries. Butter is mainly exported to Djibouti and South Africa (targeting the Ethiopians in Diaspora), while milk is solely exported to Somalia from the South Eastern Region of the country. However, the country spent more money on importing milk and milk products from different countries. The import value, which was more than $5.6 million in 2005, also increased to $10.3 million in 2009 indicating that Ethiopia is a net importer with a net traded value worth negative 29, 034 000 USD (Zelalem Yilma, *et al*., 2011).

Therefore any effort applied to the production of pure aspartic protease enzyme from local microbes which is a candidate for substituting rennet enzyme is valuable to reduce the foreign currency spent on importing dairy products.

CHAPTER-3

3. Materials and Methods

3.1. Description of the Study Area

A total of 67 soil and dung samples were collected from Hawassa University (7°03'27.3"N 38°29'56.3"E), Lake Hawassa (7°03'14.0"N 38°26'11.4"E), Wondogenet College of Forestry and Natural Resource (7°06’03.3” N 38°37’36.2” E), Lake Ziway (8°01'42.6"N 38°50'28.5"E), Dire dawa: (9°37'14.9"N 41°50'28.9"E) and Samara (11°48'09.3"N 40°59'26.8"E) as shown in Figure 3.1 and Table 3.1.

3.2. Sample collection

Five hundred grams of soil (5–10 cm below the surface) and cow dung samples were collected aseptically from the dairy farm area located at Wondogenet, Hawassa, Ziway, Samera and Dire Dawa, Ethiopia using sterile polythene bag. Soil samples were sieved (3–4 mm mesh), homogenized and stored at 4 ^{o}C for further use (Figure 3.1 and Table 3.1.)

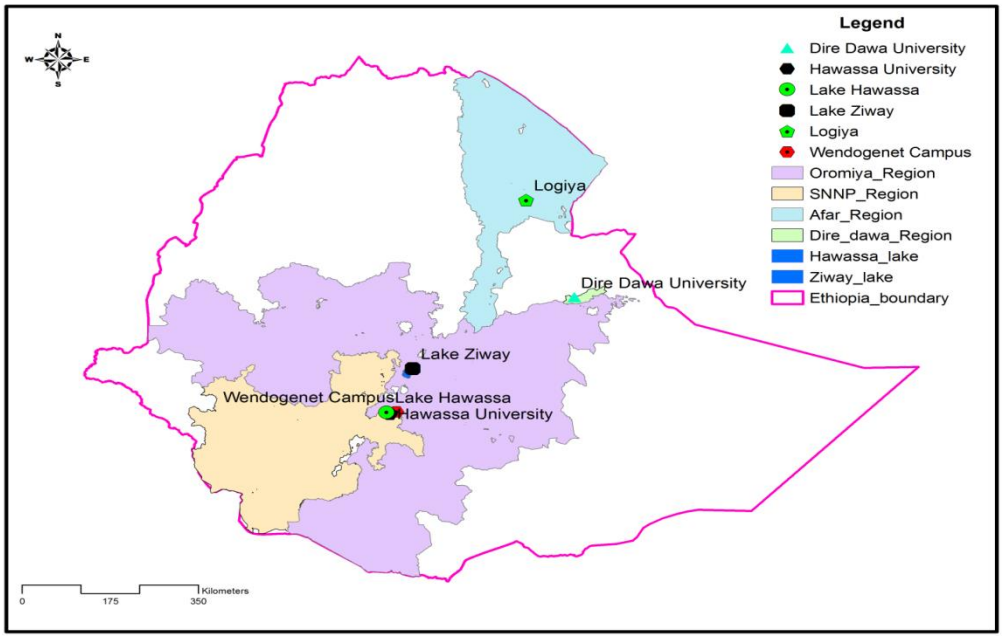


Figure 3.1: A map for sample collection sites

Table 3.1: Summary of samples and their collection sites

S.No	Site	Type of sample	Specific site	No of Samples
1.	Hawassa	Soil and dung	Dairy farm of Hawassa University and Lake Hawassa	20
2.	Wondogenet	Soil and Dung	Wondogenet College Dairy Farm and College forest	20
3.	Ziway	Soil	From around lake Ziway	6
4.	Semera	Soil	From Dairy farm area	11
5.	Dire Dawa	soil	From Dairy farm area	10
6.	Total	-		67

3.3. Isolation of microorganisms

3.3.1. Isolation of fungi

Ten grams of each soil sample was mixed with 90 mL sterile distilled water and homogenized by agitation for 20 min (Talantikite-Kellil *et al*., 2012). They were prepared to appropriate dilutions from which 0.1 mL of each sample suspension was plated on Potato Dextrose Agar (Oxoid, Hampshire, United Kingdom) plates containing chloramphenicol (0.05 g/L). They were incubated at 30 ^{o}C for 5-7 days to isolate distinctive colonies. Each colony was re-streaked on the same medium for purity and preserved at 4 ^{o}C.

3.3.2. Isolation of bacteria

Ten grams of each soil sample were mixed with 90 mL sterile distilled water, homogenizing by agitation for 20 min (Talantikite-Kellil *et al*., 2012). They were prepared to appropriate dilutions (10^5-10^6) from which, 0.1 mL was spread plated on nutrient agar media and incubated at 30 ^{o}C for 48 h. The bacteria were subcultured, purified and stored at 4 ^{o}C.

3.3.3. Preservation of cultures

The fungal and bacterial isolates were cultivated in Potato dextrose Agar (PDA) slants and Nutrient Agar (NA) slants at 30 ^{o}C for 3 days and at 30 ^{o}C for 48 h, respectively, stored at 4 ^{o}C and subcultured periodically (Baskar *et al*., 2014).

3.4. Primary Screening

3.4.1. Primary screening of milk-clotting protease (MCP) from fungi

Primary screening for MCP was tested using skim-milk agar (Nestle TM, Frankfurt, Germany) medium for the production of the clear zone (Saran *et al*., 2007). The detection medium (Skim-milk agar medium) was prepared using 20 g of skim milk, 20 g of agar-agar each dissolved in 200 mL distilled water and 600 mL of 0.2 M Phosphate buffer (K_2HPO4 and KH_2PO4 at pH 5.0). All the three media components were autoclaved separately to avoid coagulation and charring of milk due to the presence of buffer salts and later mixed under sterile conditions. The plates were then subsequently inoculated with previously purified fungal and bacterial isolates and incubated at 30 ^{0}C for 2 days. The plates were examined for the formation of the clearing zone by flooding them with a solution of 10% Trichloroacetic acid (TCA) or 10% tannic acid. The relative enzyme activity was calculated using the clear zone diameter and colony diameter (Khademi *et al*., 2013[a]).

$$\mathrm{REA} = \mathrm{CZD}/\mathrm{CD}$$

Where; REA: relative enzyme activity, CZD: Clear Zone Diameter, CD: Colony diameter

3.5. Secondary screening of milk-clotting protease

3.5.1. Solid-state and submerged fermentation for fungi

3.5.1.1. Inoculum preparation

Fungal isolates were grown on potato dextrose agar (PDA) and incubated at 30 ^{o}C for 5 days. They were scrapped using 10 mL of sterile distilled water to prepare spore suspension. 1 mL of spore solution (10^6 spores/mL) were used except for WGDFD 6 (10^5) and SMDFS 52 (10^4) as inoculum according to Sathya *et al.*, (2009).

3.5.1.2. Media and culture conditions of submerged fermentation

The medium contained 3% (w/v) glucose, 2% (w/v) peptone, 2% (w/v) casein and 1% (w/v) yeast extract, and adjusted to pH 6.0 before sterilization (Souza *et al.*, 2017). One mL of spore solution (10^6 spores/mL) was transferred into 250 mL Erlenmeyer flasks containing 50 mL of liquid medium. Then the flasks containing the fungi were incubated on a rotary shaker at 200 rpm at ambient temperature for 96 h.

3.5.1.3. Medium and culture conditions for solid-state fermentation (M1)

For solid-state fermentation, 1 mL of spore suspension (10^6 spores/mL) was transferred into 250 mL Erlenmeyer flasks containing wheat bran (10 g), skim milk (2.0 g) and 10 mL of salt solution (g/L: 2.0, KNO_3; 0.5, $MgSO_4 \cdot 7H_2O$; 1.0, K_2HPO_4; 0.439, $ZnSO_4 \cdot 7H_2O$; 1.116, $FeSO_4 \cdot 7H_2O$; 0.203, $MnSO_4 \cdot 7H_2O$ and pH 7) previously sterilized (Sathya *et al.*, 2009). The flasks were incubated at 30 ^{o}C for 5 days under static conditions.

3.5.1.4. Medium and culture conditions for solid-state fermentation for fungi (M2)

The solid-state substrate was also prepared in 250 mL Erlenmeyer flask containing 10 g of wheat bran (Durum wheat bran) moistened by adding 12 mL of (0.2M) HCl by mixing thoroughly. Then after the flasks were autoclaved at 121 ^{o}C for 30 min. Then 0.5 mL of ca. $0.5*10^{6}$ spore suspension was inoculated into SSF media and incubated at 30 ^{o}C for 6 days (Fernandez-Lahore *et al*., 1998).

3.5.1.5. Enzyme extraction

In all cases, enzyme extraction was undertaken according to Garcia *et al*., (2005). From submerged fermentation (SmF), samples were collected and filtered through a Whatman No 1 filter paper (90 mm) and subsequently centrifuged at 10000 rpm for 10 min. In the case of solid-state fermentation (SSF), samples were dispersed in 100 mL (1:10 ratio of Bran - solvent w/v) of distilled water, and vigorously shaken on a rotary shaker (MaxQ 2000 Open-Air Platform Shaker, Thermo Fisher Scientific, USA) at 240 rpm at room temperature for 40 min and filtered by cotton cloth. The filtrate was then centrifuged (Heraeus Pico17/21 centrifuge, Thermo Electron Led, Germany) at 10000 rpm for 10 min at 4 ^{o}C. The supernatant was used as a crude enzyme.

3.5.2. Secondary screening for milk-clotting protease enzyme using solid state and submerged fermentation

For submerged fermentation (SmF), the bacterial strains were grown overnight in Nutrient broth and 3% inoculum size was inoculated to the culture medium (50 mL) containing (g/L): glucose, 16.2; wheat bran, 30; NaCl, 5; $MgSO4{\cdot}7H_2O$, 5; KH_2PO_4,

2; and $CaCO_3$, 3 and pH 5.2 in Erlenmeyer flask (250 mL). They were incubated at 150 rpm for 96 h in an orbital shaker at ambient temperature (Ding *et al.*, 2012).

For solid-state fermentation (SSF), a 3% inoculum size was inoculated into a culture media in 250 mL Erlenmeyer flasks. The medium contained;10 g wheat bran moistened with 30 mL of phosphate buffer (0.01 M, pH 6.0; 0.25% w/v casein) (Dutt *et al*., 2009). The flasks were incubated at 35 ^{o}C for 72 h.

3.5.2.1. Enzyme extraction

Enzyme extraction was undertaken according to Garcia *et al*., (2005). Samples were collected and filtered through a Whatman No 1 filter paper and subsequently centrifuged at 10000 rpm for 10 min. In the case of Solid-state fermentation (SSF), samples were dispersed in 100 mL (1:10 ratio of Bran - solvent w/v) of distilled water, and vigorously shaken on a rotary shaker (MaxQ 2000 Open-Air Platform Shaker, Thermo fisher scientific, USA) at 240 rpm at a room temperature for 40 min and filtered by cotton cloth. The filtrate was then centrifuged (Heraeus Pico17/21 centrifuge, Thermo Electron Led, Germany) at 10000 rpm for 10 min at 4 ^{o}C. The supernatant was used as a crude enzyme.

3.6. Assay for milk-clotting activity

Milk-clotting activity (MCA) was determined according to the method of Arima *et al*., (1970) and expressed in terms of Soxhlet units (SU). One SU is defined as the amount of enzyme which clots 1 mL of a solution containing 0.1 g skim milk powder and 1.1 mg calcium chlorides in 40 min at 35 ^{o}C. In brief, 0.5 mL of tested materials were added to a test tube containing 5 mL of reconstituted skim milk solution (10 g dry skim-milk/100 mL, 0.01 M $CaCl_2$) pre-incubated at 35 ^{o}C for 10 min. The mixture was mixed well, and the clotting time T (s), the time period starting from the addition

of test material to the first appearance of clots of milk solution, was recorded and the clotting activity (CA) was calculated using the following formula:

$$SU = (2400 * 5 * D)/(T * 0.5)$$

Where T = clotting time (s) D= dilution of crude enzyme

3.7. Assay of protease activity

The proteolytic activity was assayed according to Arima *et al*., (1970). Thus, 0.5 ml of the enzyme extract was added to 2.5 mL of 1% (w/v) soluble casein in 20 mM potassium phosphate buffer at pH 6.5, and the mixture was incubated in a water bath at 35 ^{o}C for 10 min. After having added 2.5 mL of 0.44 M trichloroacetic acid to terminate the reaction, the mixture was filtered through Whatman No.1 (90mm) filter paper. The filtrate was then mixed with 1 mL volume of three times diluted 2 N Folin/phenol reagent and 2.5 mL of 0.55 M sodium carbonate solutions and incubated at 35 ^{o}C for 20 min to detect color development and measure optical density (OD) with spectrophotometer (UV-vis, Liantrinsat, and Model-CF728YW-UK) at 660 nm. One unit (1 U) of enzyme activity was defined as the amount of enzyme that liberated 1µg of tyrosine per 1 mL in 1 min.

$$\mathrm{PA\ (U/mL)} = \frac{\mu\mathrm{Tyr} * \mathrm{V_t}}{\mathrm{V_s} * \mathrm{T} * \mathrm{V_a}}$$

Whereas PA: Protease activity, µTry: µg of tyrosine equivalent released, Vt: Total volume of assay in mL (5 mL of substrate plus 1 ml of Enzyme plus 5 ml of TCA), Vs: Sample volume (ie. The volume of protease used for assay in mL), T: reaction time (i.e Time of incubation in minutes, 10 min), Va: Volume of assayed (i.e the final volume of the product used in calorimetric determination)

3.8. Identification of potential microorganisms

3.8.1. Phenotypic and molecular characterization of fungi

The cultural characteristics of the fungal isolates were determined by inoculating them on Czapek Dox agar (CYA), Potato Dextrose Agar (PDA) and Malt Extract Agar (MEA) (Atiqah and Zakaria, 2017). The morphological characters of the isolates were observed under the microscope (Motic Microscopes, B3-220ASC, and European Division) after having prepared them on a slide culture.

For the molecular characterization of fungi, the genomic DNA was isolated by the CTAB method. The gDNA was extracted from 3 days old cultures liquid broth (PDB, Potato Dextrose Broth). The broth medium was filtered with cotton cloth and fungal mass was crushed by pestle and mortar using liquid nitrogen. The gDNA was preciptated using lysis buffer and phenol: chloroform: Isoamyl alcohol (25:24:1). The purity was checked by Nanodrop (Thermo Fisher, Massachusetts, USA) and 1% garose gel electrophoresis (Li *et al*., 2011).

PCR amplification of fungal ITS region was carried out using Internal Transcribed Spacer Region primers (ITS); ITS86F and ITS 4R. ITS86F and ITS 4R primers amplified a 0.5 kb section of the ITS gene. The primers sequences were as follows: ITS86F (F): 5'- GTG AAT CAT CGA ATC TTT GA-3' and ITS4 (R): 5'- TCC TCC GCT TAT TGA TAT GC -3'. PCR amplification was performed in a 25 µL reaction containing 0.2 µL Phusion DNA polymerase, a 5* diluted 5 µL of Phusion buffer, 10 mM dNTPs (0.5 µL), 10 µM forward primer ITS86F (1.25 µL), 10 µM Reverse primer ITS4 (1.25µL), 1 µL (100 ng/µL) of template DNA and 15.75 µL distilled water. The PCR product was purified using a Nucleospin PCR purification kit according to the manufacturer's protocol (Macherey-Nagel, 2012). purification kit

following the manufacturer's instruction. The reaction conditions were as follows: initial denaturation at 98 ^{o}C for 30 sec, 36 amplification cycles, denaturation at 98 ^{o}C for 10 min, annealing at 59 ^{o}C for 30 sec, extension at 72 ^{o}C for 45 sec and a final extension at 72 ^{o}C for 7 min. PCR amplifications were carried out using a Thermo-Hybrid PCR thermal cycler (Thermo Fisher, Massachusetts, USA). Aliquots of the PCR products (5 µL) were analyzed in 1% (w/v) agarose gels by horizontal gel electrophoresis. DNA amplicons were visualized by UV excitation after staining with ethidium bromide (0.5 mg/L). The amplicons were then purified and submitted for Sanger sequencing at the Eurofins Genomics, Germany (Gontia-mishra *et al*., 2013).

The sequence was aligned with corresponding sequences from the database using BLAST (Gontia-mishra *et al*., 2013). Phylogenetic analysis of the sequences was performed using MEGA 7 software (Version 7.0.26, SUDHIR KUMAR). The phylogenetic trees were inferred using the neighbor-joining method and bootstrap (1000 replicates) analyses. The evolutionary distances were computed using the Maximum Composite Likelihood method (Gontia-mishra *et al*., 2013).

3.8.2. Characterization of bacteria

3.8.2.1. Phenotypic charcterization

The milk-clotting protease enzyme-producing bacteria were first characterized using morphological and biochemical characteristics; Gram's staining, catalase test, spore staining and API 50 CHB/E identification kits (Biomerieux, France) (Aruwa and Olatope, 2015).

3.8.2.2. Genome characterization

All bacterial isolates (14) were cultured in LB Broth with 1% glucose overnight at 37 ^{o}C and 200 RPM. They were harvested by centrifugation at 4000×g for 30 min and

the genomic DNA (gDNA) was extracted using Dneasy plant mini kit (50) QIAGEN. The DNA was finally suspended in 100 µl of elution buffer and quantified using NANO Drop (Thermo Fisher, Massachusetts, USA).. The total gDNA was kept at -20 ^{0}C before use (Guleria *et al.*, 2016).

PCR of 16S rRNA was carried out in 25 µl reaction containing 2 µl of template DNA, 1.25 µl of each primer FW fc1 (5′-GCAAGTCGAGCGGACAGATGGGAGC-3′) and reverse primer RV rc2 (5′-AACTCTCGTGGTGTGACGGGCGGTG-3′), 0.5 µl of 10 mM dNTPs and 0.25 µl Phusion polymerase (Gene Bangalore) in 5 µl of 5 × Phusion buffer. Reaction was cycled 36 times at 98 ^{o}C for 30 sec for initial denaturation, 98 ^{o}C for 10 sec for denaturation, 58 ^{o}C for 30 sec for annealing, 72 ^{o}C for 1.30 min for extension and at 72 ^{o}C for 7 min for final extension. The PCR products were analyzed on 1% agarose gel in 1 × TAE buffer, run at 100 V for 1 h (Guleria *et al.*, 2016).

Gels were stained with ethidium bromide and photographed. Amplified PCR products were purified using a Nucleospin PCR purification kit following the manufacturer's instruction and sequenced using Sanger sequencing at Eurofins Genomics, Germany. The sequence was aligned with corresponding sequences of 16S rDNA from the EZBioCloud database (Yoon *et al*., 2017). The phylogenetic tree was constructed by neighbor-joining method and bootstrap (1000 replicates) analyses with the help of MEGA7 software (version 7.0.26, SUDHIR KUMAR). The evolutionary distances were compute using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

3.9. Culture profile determination for selected fungal and bacterial species

The culture profiles of the selected milk-clotting and protease producing fungal and bacterial species were determined using standard methods under automated laboratory

(Downstream Processing Laboratory), Department of Life Sciences and Chemistry, Jacobs University, Bremen, Germany. The fungal strains were cultivated using Media 2 (10 g of Durum wheat bran moistened by 12 mL of (0.2 M) HCl) at 30 ^{o}C for 6 days. The bacterial species were determined using standard media ((g/L): glucose, 16.2; wheat bran, 30; NaCl, 5; $MgSO_4 \cdot 7H_2O$, 5; KH_2PO_4, 2; and $CaCO_3$, 3 and pH 5.2 in) at 35 ^{o}C and 150 rpm in incubator shaker for 96 h under automated laboratory (Downstream Processing Laboratory), Department of Life Sciences and Chemistry, Jacobs University, Bremen, Germany.

3.9.1. Determination of soluble carbohydrate and protein

The total protein of the crude extract was determined using Bicinchoninic acid (BCA) methods (Walker, 2002). The working reagent was prepared by mixing 24.5 mL of BCA (Thermo Fisher, Massachusetts, USA) reagent A with 0.5 mL BCA^{TM} Reagent B into 50 mL beaker. The protein content was calorimetrically determined (Eppendorf Biophotometer, Model # 61318, Marshal Scientific, Germany) at 562 nm against a standard curve prepared from known concentrations of bovine serum albumin (BSA).

Total soluble sugars of the crude extract were determined using the dinitro salicylic calorimetric (DNS) method. The absorbance was recorded using a spectrophotometer (Eppendorf Biophotometer, Model # 61318, Marshal Scientific, Germany) at 562 nm with glucose as standard (Gusakov *et al.*, 2011).

3.10. Determination of molecular weight

The approximate molecular weight of proteins in the crude enzyme extracted from potential fungi was estimated by 12% SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Protein bands were visualized by employing Coomassie brilliant blue R250 staining (Yegin *et al*., 2012).

3.11. Optimization of growth conditions and media composition for the production of milk-clotting protease from *Aspergillus oryzae* DRDFS13 under SSF

3.11.1. Raw materials

Four different agro-industrial substrates; wheat bran (WB), rice bran, pea bran, and grass pea bran were purchased from the local market and properly dried at 60 ^{o}C at Food Microbiology Labortory, Addis Ababa University.

3.11.2. Inoculum preparation

Aspergillus oryzae DRDFS13 was grown into 50 mL Falcon tubes containing 20 mL of Potato Dextrose Agar (PDA) and incubated at 30 ^{o}C for 5 days. Sporulated culture lawn was mixed well in sterilized distilled water. The spore suspension was diluted and adjusted to a concentration of 10^{6} spores/mL using a hemocytometer.

3.11.3. Experimental set-up for preliminary screening for production of milk-clotting protease (MCP) in SSF

The experimental set up for solid-state fermentation was according to Fernandez-Lahore *et al*., (1998) with slight modification. The inoculum (1 mL of 10^{6} spores/mL) was transferred into 250 mL Erlenmeyer flasks containing 10 g of wheat bran (WB) comprising; 12.43% crude protein, 3.77% crude fat, 47.23% carbohydrate, 18.77% fiber, 7.4% ash, and 10.40% moisture content, moistened with 12 mL of (0.2M) HCl. The flasks were incubated at 30 ^{o}C for six days.

3.11.3.1. Enzyme extraction

Enzyme extraction was done according to Garcia *et al*., (2005) and (Fernandez-Lahore *et al*., 1998). After the end of SSF a known quantity (10 g) of solid medium was dispersed in 100 mL (1:10 ratio of Bran-solvent w/v) of distilled water, distilled

water containing 0.1 M NaCl, a solution of Tween-80 (0.1 %) by shaking on a rotary shaker (MaxQ 2000 Open-Air Platform Shaker, Thermo Fisher Scientific, USA) (240 rpm, 40 min, at room temperature) and filtered using cotton cloth. The filtrate was centrifuged (Heraeus Pico17/21 centrifuge, Thermo Electron Led, Germany) at 10,000 x*g* for 10 min at 4 ^{o}C. The supernatant was used as crude enzyme source for milk-clotting and protease activities.

3.11.3.2. Effect of substrate on milk-clotting protease (MCP) production

Initial screening of the media composition for maximum milk-clotting protease (MCP) production was performed by a one-variable-at-a-time approach (Siala *et al*., 2012; Vishwanatha *et al*., 2010). Thus, 1mL of $1*10^{6}$ spore/mL from *Aspergillus oryzae* DRDFS13 was inoculated into ten grams of each substrate (SS media) in 250 Erlenmeyer flasks hydrated with 12 mL HCl (0.2 M) except 6 mL HCl (0.2 M) for rice bran and incubated at 30 ^{o}C for 144 h. The extraction was made as before, and the crude enzyme was assayed for milk-clotting and protease activity according to Arima *et al*., (1970). After having tested the effect of substrates on enzyme production, the highest enzyme-producing substrate was selected and tested for further optimization.

3.11.3.3. Effect of incubation time

After inoculation, the flasks were incubated at 30 ^{o}C for different time periods ranging from 24 h to 144 h and enzyme activity was monitored according to Arima *et al*., (1970).

3.11.3.4. Biomass determination

The Biomass was indirectly detected by the glucosamine (GlcN) released from the cell wall according to (Zamani *et al*., 2008).

Biomass (mg GlcN.gdfs^{-1}) = ([$e^{\wedge[(\Delta A650\text{-}b)/m]]} * DFsample$)/(0.1)

Where: ΔA650= A650 (sample)-A650 (sample blank)

$e^{\wedge[(\Delta A650\text{-}b)/m]}=mg_{GlcN}$

Standard curve values (logarithmic fit)= b&m

Sample's amount=0.1gdfs

Sample's dilution=DF_{sample}

3.11.3.5. Effect of incubation temperature

The fungal spores were inoculated into the SSF medium in 250 mL Erlenmeyer flask and incubated at 25, 30, 35 and 40 ^{o}C for 120 h to determine the optimum temperature for MCP production and the milk-clotting and protease activities were assayed according to Arima *et al.*, (1970).

3.11.3.6. Effect of inoculum size

The effect of inoculum size on MCP production was studied by inoculating 0.1mL ($1*10^5$ spores/mL), 0.5 mL ($1*10^6$ spores/mL), 1 mL ($1*10^6$ spores/mL), 2 mL ($2*10^6$ spores/mL), 3mL ($3*10^6$ spores/mL) and 4 mL ($4*10^6$ spores/mL) spore suspension in to SSF media and assayed for milk-clotting and protease activities according Arima *et al.*, (1970).

The following optimization study were undertaken by inoculating 0.5 mL of $1*10^6$ spore/mL using 10 g of wheat bran on SSF minimal medium in 250 mL Erlenmeyer flask and incubated at 30 ^{o}C for 120 h unless stated otherwise.

3.11.3.7. Effect of moisture content

The effect of initial moisture content on enzyme production was tested by moistening substrate using distilled water in different percentages of moisture content; 45%, 50%,

55%, 60%, 65%, and 70% to find out the best moisture content for MCP production (Bensmail *et al*., 2015). The milk-clotting and protease activities were assayed according to Arima *et al*. (1970).

3.11.3.8. Effect initial of medium pH

The effect of initial medium pH on milk-clotting protease production was studied by adjusting the SSF medium to pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0 using HCl or diluted NaOH. The milk-clotting and protease activities were assayed according to Arima *et al*. (1970).

3.11.3.9. Effect of supplementary carbon and nitrogen sources

The SSF production medium was supplemented with different C sources (glucose, galactose, fructose, sucrose, maltose, lactose, and starch), and N sources (casein, skim-milk, yeast extract, and urea) and inorganic nitrogen source (NH_4Cl, $(NH_4)_2SO_4$ and NH_4NO_3) at a level of 1% w/w of the SSF medium. Further, the amount of best nitrogen and carbon source (0.5%, 1%, 2%, 3%, 4%, and 5%) was optimized (Sathya *et al*., 2009). The milk-clotting and protease activities were assayed according to Arima *et al*., (1970).

3.11.3.10. Extraction efficiency of some solvents

The efficiency of some solvents were checked by extacting the milk-clotting protease using distilled water, 0.1M NaCl and 0.1% Tween 80 at 1:10 bran to solvsnt ratio. The crude enzyme extract was then assayed for milk-clotting activity and protease activity according Arima *et al*., (1970).

3.11.4. Optimization of major factors affecting milk-clotting protease production by response surface methodology (RSM)

3.11.4.1. Experimental design

The effects of factors that have been shown significant outcome on milk-clotting protease production during optimization by one-variable-at-a-time were further determined by response surface methodology (RSM). The RSM was used for studying the effects of interaction among pH, temperature and casein concentration on milk-clotting protease production. The optimized ranges for the selected variables were pH (4-7), incubation temperature (25-40 ^{o}C), casein concentration (0.5%-5%). RSM design was adopted to optimize the levels of the three factors, with three center points yielding a set of 20 experiments. The factors at three different levels (-1, 0, +1) with minimum and maximum range of values were presented in Table 3.2. The treatment schedule for the model is given in Table 5.4. The replicates (treatments 13-20 in Table 5.4) at the center of the design were used for estimation of the pure error sum of squares. The experiments were randomized to maximize the effects of unknown variability due to irrelevant factors in the observed responses (Vishwanatha *et al*., 2010).

Table 3.2: Experimental range and levels of the three independent variables used in RSM in terms of actual and coded factors

		Range and Levels		
S.No	Variables	-1	0	+1
1.	pH	4	5.5	7
2.	Temp	25	32.5	40
3.	Casein conc.	0.5	2.75	5

-1: minimum value; 0: average value ; +1: maximum value

3.11.5. Partial purification of Milk-clotting protease

3.11.5.1. Acetone precipitation

The crude enzyme extract was precipitated with chilled acetone (acetone or 75% acetone). Two volumes of chilled acetone were slowly added to the extract and the precipitate was then allowed to settle for 1 h at -18 ^{o}C to permit complete precipitation. The precipitated protein was separated by centrifuging (Heraeus Pico 17/21, Thermo Electron LED GmbH, Germany) at 10000 x*g* for 10 min at 4 ^{o}C. The pellet was then dried in the open air for 30 min and dissolved in 0.02 M phosphate buffer, pH 6.0 to remove trace amounts of acetone (Palpperumal *et al*., 2016).

3.11.5.2. Ammonium sulfate precipitation

Initially, 75.61 g of solid ammonium sulphate was added to 100 mL distilled water to get a final volume of 140.94 mL of 100% saturated ammonium sulphate solution. Then, 1200 µL (100%) saturated ammonium sulphate solution was added to 300 µL the crude enzymatic extract to get a final volume of 1500 µL (80% saturated) ammonium sulphate solution. This leads to precipitation of the protein. Then, the enzymatic solution was decanted for one night at 4 ^{o}C. Then centrifuged at 10000xg for 10 min at 4 ^{o}C and the pellet was suspended in the phosphate buffer (0.02 M; pH 6) (Nouani *et al*., 2011).

3.11.6. Inhibition study

The effects of some protease inhibitors on MCA of the crude extract were examined using cysteine protease inhibitor, iodoacetamide (10 mM); aspartic protease inhibitor, pepstatin A (1 mM); metalloprotease inhibitor, Ethylene-diamine-tetra-acetic acid (EDTA) (10 mM); serine protease inhibitor, phenyl-methane sulphonyl fluoride (PMSF) (10 mM). After the addition of inhibitors into the enzyme samples, the

mixtures were incubated at 35 ^{o}C for 30 min. After incubation, the residual milk-clotting activity was assayed. Residual MCA was defined as the percentage of the activity determined in the absence of inhibitors (Yegin *et al*., 2012).

3.12. Crude enzyme preparation (Dialysis)

The crude enzyme was produced from *A. oryzae* DRDFS13 under previously optimized conditions in SSF (Table 3.3). Then, the crude enzyme was subjected to dialysis against 20 mM phosphate buffer (pH 6.0) utilizing a 10 kDa cut-off membrane. After dialysis, a crude enzyme preparation was obtained by concentration and by contacting with carboxymethyl cellulose overnight (4 ^{o}C). The crude enzyme was resuspended in sodium phosphate buffer (20 mM, pH 6.0) (Ramachandran and Arutselvi, 2013).

Table 3.3: Optimized parameter used for cultivation of *Aspergillus oryzae* DRDFS13

Organism	Parameter	Measurement Unit
Aspergillus oryzae DRDFS13	Substrate	Wheat Bran
	Temperature	30 ^{o}C
	Inoculum size	0.5 mL (1*10^{6}) spore/mL
	Fermentation time	5 days
	Moisture content	55%
	Initial media pH	6.0
	Supplementary N-source	Casein (1%)
	Supplementary C-source	Glucose (0.5%)

N-source: Nitrogen source, C-source: carbon source

3.13. Enzyme purification

3.11.3. Enzyme purification by ion-exchange chromatography

The crude enzyme extract was purified using ion-exchange chromatography (IEC) on a DEAE Sepharose Fast Flow column (5 X 0.5 cm) with an ÄKTA purifier system (GE Healthcare, Munich, Germany). One hundred fifty mL of sample was applied to

the column using Buffer A (40 mM citrate-phosphate buffer, pH 6.0) as the equilibration and loading solution, while Buffer B (Buffer A containing 1M sodium chloride) was used as the elution buffer. The purification was run at a flow rate of 2.0 ml per minute and a linear gradient was applied for a period of 5 column volumes. The chromatography fractions (1 mL) were analyzed for enzyme activity and protein content. Fractions showing enzyme activity were pooled and concentrated (Yegin *et al*., 2012).

3.13.2. Enzyme purification by size-exclusion chromatography

Size-exclusion chromatography was performed with an ÄKTA purifier system (GE Healthcare, Munich, Germany). Hence the crude enzyme extract was 1st centrifuged for 20 min at 16,000 rpm and concentrated using vacuum concentrator. The SEC performed using 2 prepacked columns and the columns were equilibrated using 25 mL a 0.1 M solution of sodium phosphate buffer at pH 6.4 and 2.5 mL of the sample was injected into the column and eluted with 3.5 mL 0.1 M sodium phosphate buffer pH 6.4 (Yegin *et al*., 2012).

3.14. Molecular characterization of aspartic protease enzyme

Due to the low volume of the purified fractions, IEC A_8 was used only for molecular weight determination, inhibition study and deglycosylation assay.

3.14.1. Determination of molecular weight

The molecular weight of the enzyme was estimated using 12% SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) (Yegin *et al*., 2012). The purified enzyme was loaded on gel and electrophoresis was run at 300 V, 35 mA for 55 min. The protein bands were visualized using Coomassie Brilliant Blue solution (AppliChem^{TM}) staining overnight and destained overnight with destaining solution.

The molecular weight of the protein was determined using the prestained protein marker as a standard.

3.14.2. Deglycosylation studies

For deglycosylation assay, 1 mL of the IEC fraction was concentrated for 4 h in vacuum to which 20 µL of the dried sample and 2 µL of 10xGlyco Buffer (New England Biolabs^{TM}) were added into 2 mL Eppendorf tube and incubated in the thermomixer at 99 ^{0}C for 10 min. The content was mixed with 2 µL of denaturing buffer (New England Biolabs^{TM}) and 2 µL of endo-β-N-acetylglucosaminidase-H (Endo-H, EC 3.2.1.96) (New England Biolabs^{TM}) and incubated in the thermomixer at 37 ^{0}C for one h. Finally, SDS-PAGE analysis was conducted by loading the mixture of 20 µL the sample (pure sample (non-deglycosylated), deglycosylated sample and pure Endo H) with 20 µL of protein loading dye containing ß-mercaptoethanol. Protein bands were visualized from the gel by staining with Coomassie blue (Appl Chem TM) and destained with the destaining solution (Sigma Aldrich^{TM}) for one day. The molecular weight was determined from a linear semi-logarithmic plot of relative molecular weight versus the migration distance using standard molecular weight marker protein. The relative mobility (Rf) was also determined as follows.

Rf = migration distance of the protein/migration distance of the dye front

3.14.3. Inhibition study

The IEC fraction A_8 was subjected to inhibition studies using four protease inhibitors: iodoacetamide, cysteine protease inhibitors (1mM, 10mM); ethylenediaminetetraacetic acid (EDTA) metalloprotease inhibitors (5 mM, 10 mM); phenylmethyl sulphonyl fluoride (PMSF), serine protease inhibitors (1mM,10mM);

and pepstatin A, aspartic protease inhibitors (0.02mM, 0.04mM, 0.06mM and 0.08mM) using the method of Yegin *et al*., (2012). The mixtures were incubated at 35 ^{0}C for 30 min to determine the residual milk-clotting activity. Residual MCA was defined as the percentage of the activity determined in the absence of inhibitors. The milk-clotting activity and protease activity of the enzyme was also determined according to Arima *et al*., (1970).

3.14.4. Biochemical characterization of dialyzed enzyme

Since the purified enzyme (IEC fraction A_8) was very small, the dialyzed enzyme was used for further biochemical studies such as determination of optimum temperature and temperature stability, determination of optimum pH and pH stability, effect of cations, effect of substrate concentration and determination of KM and Vmax.

3.14.4.1. Determination of optimum pH and pH stability

The effect of pH on the milk-clotting activity of the enzyme was determined according to Yegin *et al*., (2012). Consequently, 0.01 M $CaCl_2$ was prepared in 20 mM citrate-buffer having the pH values (4.5-6.0) and 10 mM phosphate buffer having pH values (6.5-8.0). Ten grams of skim-milk powder dissolved in 100 mL of 0.01 M $CaCl_2$ solution that was prepared in 20 mM citrate buffer and 10 mM phosphate buffer and used for the milk-clotting assay. The pH stability was determined by incubating the enzyme sample diluted 10 times in 20 mM citrate buffer having different pH (4.5, 5.0, 5.5 and 6.0) and 10 mM potassium phosphate having pH (6.5, 7.0, 7.5 and 8.0) at 25 ^{o}C for 1 h. After incubation, the samples were examined for residual and/or relative milk-clotting activity using the standard procedures (Ageitos *et al*., 2007; Yegin *et al*., 2012).

3.14.4.2. Determination of optimum temperature and temperature stability

For the determination of the optimum temperature of the enzyme, the milk-clotting activity of the reaction mixture (10 % skim-milk (w/v) in 0.01 M $CaCl_2$) was assayed at different temperature ranging from 25-70 ^oC with 5 ^oC interval. Likewise, the thermal stability was determined by incubating the enzyme at a temperature ranging from 35- 60 ^oC with with 5 ^oC interval for 15 and 30 min. After incubation, the enzyme was cooled in an ice bath and residual milk-clotting activity was determined as described before (Yegin *et al*., 2012).

3.14.4.3. Effect of substrate concentration on the milk-clotting activity

To determine the effect of substrate concentrations on the milk-clotting activity of the enzyme, 25, 50, 100, 150 and 200 g/L of the skim-milk were taken and tested under the same reaction mixture and conditions as before (El-tanboly *et al*., 2013).

3.14.4.4. Effect of metal ions on the milk-clotting activity

The effect of varying monovalent and divalent metal ions on the milk-clotting activity in the forms of chloride and sulfate salts (Na, K, $Ca^{2+,}$ Mg^{2+} Mn^{2+}, Fe^{2+}, Zn^{2+}, Ni^{2+}, $Cu^{2+,}$ and $Co^{2+)}$ were added to the substrate at a final concentration of 10 mM and the milk-clotting assay was conducted after 30 min (Table 3.4). For comparison, the control (without the addition of $CaCl_2$ or any metal ion) was taken as 100% and the effect of various metal ions was expressed as residual or relative milk-clotting activity (Kumari *et al*., 2016).

Table 3.4: Metals ions used for milk-clotting assay

S.No	Type of Metal Ions	Concentrations
1.	Control (without adding any metal ion)	-
2.	NaCl	10 mM
3.	KCl	10 mM
4.	$CaCl_2$	10 mM
5.	$MgCl_2$	10 mM
6.	$FeCl_2$	10 mM
7.	$MnCl_2$	10 mM
8.	$NiCl_2$	10 mM
9.	$MgSO_4.7H_2O$	10 mM
10.	$MnSO_4.7H_2O$	10 mM
11.	$ZnSO_4.7H_2O$	10 mM
12.	$CuSO_4.7H_2O$	10 mM
13.	$FeSO_4.7H_2O$	10 mM
14.	$CoSO_4$	10 mM

3.14.4.5. Effect of $MnSO_4$ and $CaCl_2$ concentration on the milk-clotting activity

To study the effect of $MnSO_4$ and $CaCl_2$ on the clotting efficiency of the dialyzed enzyme, various concentrations of $MnSO_4$ and $CaCl_2$ (0.00 M, 0.005 M, 0.01M, 0.05M, 0.1M, and 0.2M) were incorporated in the reaction mixture (Table 3.5). Time taken for the appearance of the first clot was recorded and compared with the control sample (El-Tanboly *et al*., 2013).

Table 3.5: Concentration of $MnSO_4$ and $CaCl_2$ used for milk-clotting assay

S.No	Conc. Of $MnSO_4$ in mM	Conc. of $CaCl_2$ in mM
1	0.00	0.00
1.	5.00	5.00
2.	10.00	10.00
3.	50.00	50.00
4.	100.00	100.00
5.	200.00	200.00

3.14.4.6. Michaelis-Menten constant

The proteolytic activity was assayed on casein solutions at a concentration in the range of 0 - 2% in 20 mM potassium phosphate buffer (pH 6.5) (Otani *et al*., 1991). The kinetic parameter (Vmax and Km) of the enzyme was calculated from graphical

representations according to the Michaelis and Lineweaver-Burk methods (Hans Lineweaver, 1934).

3.15. Clonning and expression of aspartic protease enzyme from *A. oryzae* DRDFS13 into *Pichia pastoris*

3.15.1. Fungal and bacterial strain

The microorganism used as the source of the gene encoding aspartic protease enzyme was *Aspergillus oryzae* DRDFS13. The fungal strain was grown at 30 ^{o}C for 3 days in a liquid broth (Potato Dextrose Broth).

E. coli K-12 ER2738 was used to amplify the plasmids carrying the cloned gene. *E. coli* strains were grown overnight in Luria-Bertani medium (10 g L^{-1}, tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) at 37 ^{o}C, 220 rpm. *P. pastoris* X-33 was grown in YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} glucose) at 30 ^{o}C for 3 days with shaking at 250 rpm (Kangwa *et al*., 2018).

3.15.2. cDNA synthesis

First, total RNAs were extracted from the mycelia of *Aspergillus oryzae* DRDFS13 using a NucleoSpin® RNA extraction kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's standard protocol (Yegin and Fernandez-Lahore, 2013). Then, the first-strand cDNA was synthesized from RNA using the ProtoScript® II First Strand cDNA Synthesis Kit (#E6560S, New England Bio Labs Inc, Brüningstraße 50, 65929 Frankfurt am Main) according to the manufacturer's protocol. The reaction components were mixed and incubated at 42 ^{o}C for 1 h, followed by heat inactivation at 80 ^{o}C for 5 min. Products were then stored at -20 ^{o}C for further amplification of the aspartic protease gene (Antonio *et al*., 2013).

3.15.3. Aspartic protease gene amplification and sequencing

Amplification of the aspartic protease gene was done using forward primers APJM_Fw01 5'-CCT CGA GCA TGG TTA TCT TGA GCA AAG TCG C-3' and reverse primer APJM_Rw01 5'-GCG GCC GCC AAG CCT GGG CGG CGA AGC CGA G-3' (Yegin and Fernandez-Lahore, 2013).

Other PCR components were: 10 µl of 5x Phusion High Fidelity buffer, 1µl of 10 mM dNTPs solution mix, 0.5 µl of 2,000U Phusion High fidelity Polymerase, all purchased from New England Bio Labs Inc, Frankfurt am Main, 2.5 µl of 10 mM primer (forward and reverse) synthesized by Eurofins genomics, 2 µl of cDNA (50 ng/µl), and sterile distilled water was added to the final volume of 50 µl.

From the amplified DNA, 15 µl of sample was run on a 1% agarose gel (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) with 1X TPE buffer [stock concentration, 10X: 1 M Tris base, 20 mM EDTA, 225 mM phosphoric acid all products of Applichem GmbH, Darmstadt, Germany] at 90 V, maximum Amps for 55 min. The gel was stained in ethidium bromide solution and viewed using a Gel documentation system.

The remaining PCR product (gene) was purified using a Nucleospin Plasmid Kit according to the manufacturer's protocol as described by Macherey-Nagel (2012). The resulting samples were eluted in 30 µl of elution buffer; concentrations were measured using a Nanodrop-2000 Spectrophotometer. Samples were then sent to Eurofins Genomics for sequencing. The results were analyzed in comparison to the protein amino acid sequence of *Aspergillus oryzae* RIB140 aspartic protease sequences by BLAST (Antonio *et al*., 2013).

3.15.4. Cloning and expression of aspartic proteinase gene in *P. pastoris*

The aspartic protease gene was inserted into pGAPZαA using the restriction enzymes XhoI and NotI (both from New England Bio Labs Inc) thereby producing an expression vector pMK-AP with the 6xHis tag at the C-terminal and kanamycin for selection in bacteria, and while zeocin as for selection in yeast (Yegin and Fernandez-Lahore, 2013). Then the pMK-AP vector was transformed to *E.coli* K-12 ER2738 competent cells by electroporation at 1.8 kV for 5 milliseconds (Yegin and Fernandez-Lahore, 2013).

The tubes carrying competent cells were incubated at 37 ^{o}C with shaking for 60 min. Then two types of samples (concentrated and un-concentrated) were prepared for plating. For the un-concentrated samples, 100 µl was taken from the Eppendorf tube directly after incubation. While the preparation of the concentrated samples involved centrifugation for a minute (11000 rpm at 4 ^{o}C). Then 700 µl of the supernatant was discarded and the pellet was re-suspended in the remaining 200 µl. The cells were cultivated on LB agar plates supplemented with 25 µg/mL tetracycline and 25 µg/mL Kanamycin final concentrations (Carl Roth GmbH, Karlsruhe, Germany) and incubated overnight at 37 ^{o}C.

Positive colonies carrying the coding sequence of aspartic protease gene were identified by colony PCR by lysing a colony in 10µL sterile water heated to 100 ^{o}C for 10 min. One microliter of the lysed colony was further used in colony PCR using previously used primers and sequencing (Yegin and Fernandez-Lahore, 2013).

Plasmids carrying the AP coding sequence were extracted from positive colonies using a NucleoSpin® Plasmid Isolating Kit and further digested with AvrII restriction enzyme and inserted into *P. pastoris* X-33 using a heat shock method at 42 ^{o}C for 2

min and the cells were screened on YPD agar plates containing 40 µg mL^{-1} zeocin. Transformants carrying the AP coding sequence was identified by PCR amplification using the previous primers and verified by nucleotide sequencing (Yegin and Fernandez-Lahore, 2013).

3.15.5. Cultivation

Colonies from *P. pastoris* X-33 strain (control) and *P. pastoris* X-33 aspartic protease (X-33 AP) were cultivated on YPD broth at pH 5 and pH 7 and incubated overnight at 30 ^{0}C. For protein expression, 1 mL of *P. pastoris* X-33-AP was added into 3 flasks containing 75 mL of YPD (pH 5) media and 1 mL of X-33 was added in 1 flask with 75 mL of YPD (pH 5) media. The same was done for YPD media with pH 7. The flasks were incubated at 30 ^{0}C for 6 days in a shaker incubator at 225 rpm. Samples were collected on 2nd, 4th and 6th days and centrifuged at 4000 rpm and 4 ^{0}C for 30 min. Then, the supernatant was used as a crude enzyme (Yegin and Fernandez-Lahore, 2013).

3.15.6. Milk-clotting activity

The MCA of the enzyme was determined according to Arima *et al*., (1970).

3.15.7. Protein determination

Protein was determined according to the Bradford procedure utilizing bovine serum albumin as the standard (Yegin and Fernandez-Lahore, 2013).

3.15.8. Molecular weight determination

The molecular weight of the crude enzyme was estimated using 12% SDS–PAGE (Yegin *et al*., 2012).

3.16. Application of partially purified aspartic protease enzyme for Danbo cheese production

3.16.1. Microbial strains used for Aspartic protease enzyme production

The microorganisms used for the production of aspartic protease enzyme were *Aspergillus oryzae* DRDFS13 and *Bacillus subtilis* SMDFS 2B locally isolated from Ethiopian soil (Samara and Dire Dawa). The fungal enzyme was produced in SSF according to the method of Fernandez-Lahore *et al*., (1998). The bacterial enzyme was produced in SmF according to the method of Ding *et al*., (2012).

3.16.2. Detection of aflatoxins in fungal extract

Aflatoxins B1, B2, G1 and G2 were detected by methanol extraction methods (methanol /water 80/20 (v/v) + NaCl) at Eurofins Analytik GmbH, Neuländer Kamp 1, 21079 Hamburg, Germany. Then, the extract was diluted with Tween solution and applied on Immunoaffinity column, followed by washing the column and elution with methanol, and finally diluted with water. The measurement was taken using High Performance Liquid Chromatography- Fluorescence Detector (HPLC-FLD) with cobra cell post column derivatization.

3.16.3. Crude enzyme preparation

The crude enzyme was prepared according to the method of Ramachandran and Arutselvi, (2013).

3.16.4. Assay for MCA and PA

The MCA and PA was determined according to Arima *et al*., (1970).

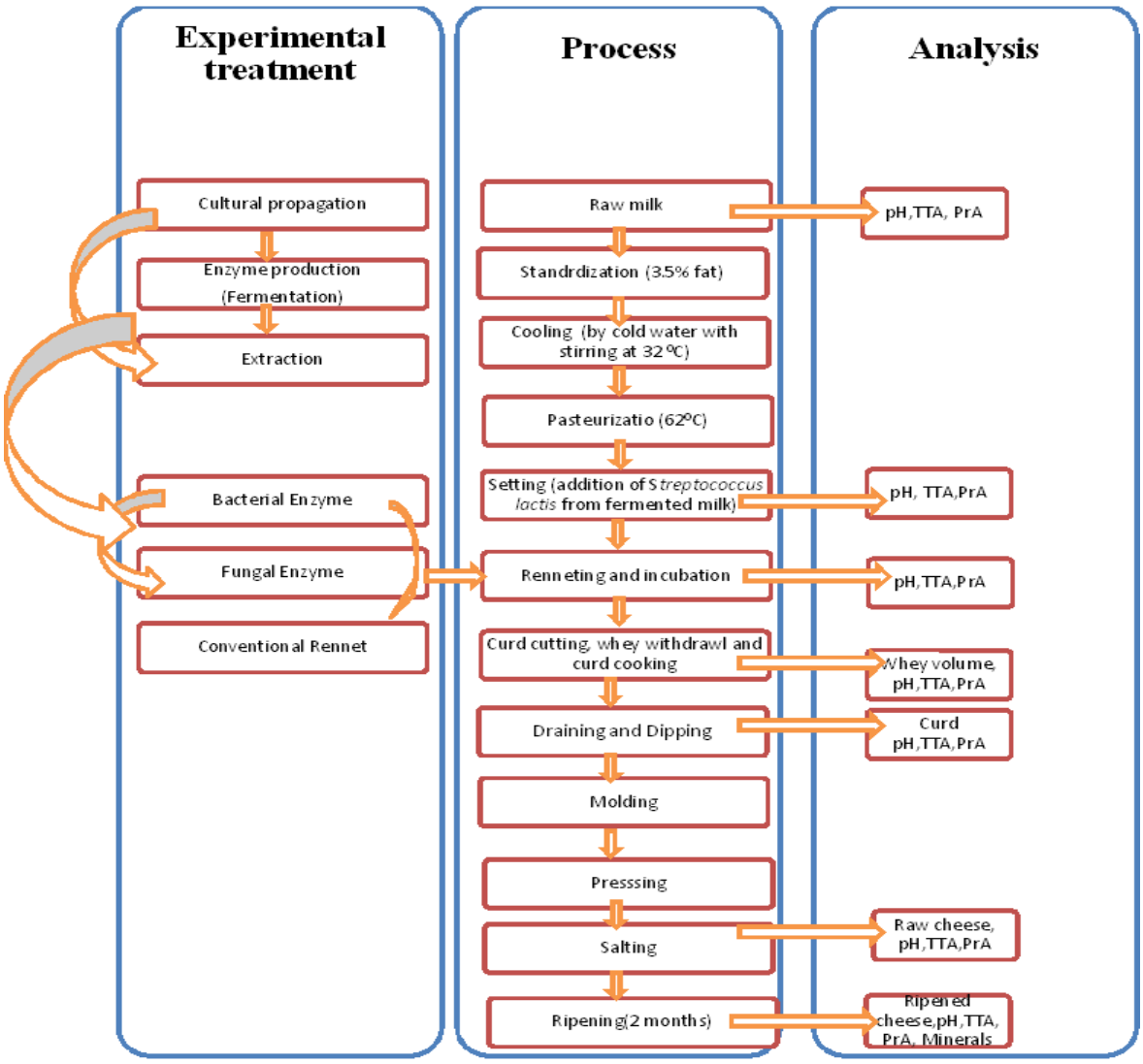
3.16.5. Protein determination

The protein in dialyzed enzyme was determined according to Kjedahl digestion, distillation and titration method of AOAC (2010).

3.16.6. Danbo cheese production

Danbo cheese was produced in a dairy pilot plant at the University of Nairobi, College of Agriculture and Veterinary Science, Food Science, Nutrition and Technology Department. The product was made according to the method described by (Wangoh, 2005). Cow milk (30 L) was standardized by skimming (fat removal), and pasteurizing by indirect heating at 62 ^{o}C for 10 min. A total of 30 L of cow milk divided into three equal portions (10 L each). All the three portions were inoculated with 0.6% fermented milk with mesophilic *Streptococcus lactis*. Then, the first portion was renneted using commercial rennet (rennin-coagulate (2 g/100 L) as a control (C). The second and third portions were renneted using the applicable ratio of dialyzed fungal and bacterial enzymes, 5.0% each and labeled as E1 and E2, respectively. After treatment with 40% $CaCl_2$ solution (10 mL/100 mL), all treatments were kept for 30 min for clotting. After which, the curd was cut using cheese knife at a size of 4-6 mm and stirred for 30-40 min in order to get better cheese grains. After removing the whey, the curd was cooked with hot water at (65-75 ^{o}C)-(39 ^{o}C) for 15-20 min, stirred for 30-40 min for precipitation. Then, the cheese was molded for 20-30 min and pressed for 2-3 h by using a metal weighing 30-35 kg/kg of cheese. Finally the pressed cheese samples were salted by dipping into 20% NaCl for 48 h and ripened at curing room for 2 months (Wangon, 2005). The whole production design is illustrated in flow chart in figure 3.2. The fresh Danbo cheese was weighed immediately and the yield was calculated as follows (Sulieman *et al*., 2012).

$$\text{Yield} = \frac{\text{weight of cheese}}{\text{Weight of milk}} * 100$$



PA: protease activity; PrA: proximate analysis; TTA: titrable acidity

Figure 3.2: The flow diagram for production and analysis design for Danbo cheese

3.16.6.1. Rating acceptance sensorial analysis of Danbo cheese

The Danbo cheese was subjected to sensory test using 13 panelists (8 men and 5 women) from dairy pilot plant and staff members of Food Science, Nutrition and Technology Department, University of Nairobi. Rating acceptance sensory test was scored for attributes of color, flavor, aroma, texture and overall acceptability on a 7-point hedonic scale where 7 being extremely like and 1 being extremely dislike. The order of product presentation to the panelists was randomized AOAC (2010). In brief,

sliced cheese (about 10 g) of each product was provided to the panelists randomly. Then after 3 min, they were provided with the next sample following the same procedure.

3.16.6.2. Proximate analysis of Danbo cheeses

3.16.6.2.1. Determination of Moisture, ash and fiber contents

The moisture content was determined according to AOAC (2010). Ash content was determined by burning 5 g of the sample in muffle furnace at 550 ^{0}C for 3 hours, as per the AOAC (2010) protocol.

3.16.6.2.2. Determination of pH and titrable acidity

About 10 g of the Danbo cheese sample was dispensed into conical flask to determine the pH using the pH meter (ABS accumet, Fisher Scientific, Singapore). The titrable acidity was determined by direct titration with 0.1 M NaOH. The results were converted to lactic acid concentration (Lemes *et al*., 2016).

3.16.6.2.3. Determination of crude protein and fat

The crude protein was determined according to Kjedahl digestion and titration method AOAC (2010). The protein content was calculated from the relationship:

$$\text{Total protein (\%)} = \frac{\text{Titre} * 6.25 * \text{Normality of NaOH (0.1N)} * 0.014}{1000 * \text{sample weight}} * 100$$

Protein conversion factor = 6.25 for milk

Protein (%) = % Nitrogen*6.25

Normality of acid (HCl) = 0.1 N

Sample weight = 1.0 g

Fat content was also determined by Soxhlet extraction method according to AOAC, (2010).

3.16.6.2.4. Determination of the carbohydrate contents and caloric value

Total carbohydrate content was determined by difference (i.e. subtracting the sum of the percentage moisture, ash, protein, and fat from 100%). Energy value was quantified indirectly considering the three groups of nutrients, which provide the body with energy carbohydrates, fats and proteins. One gram of carbohydrate (C), protein (P) and fat (F) will provide 4, 4 and 9 Kcal energy respectively. Therefore, the total caloric values are calculated as follows:

$$\text{Energy in Kcal}/100\text{ g of the sample} = (\text{P} * 4) + (\text{F} * 9) + (\text{C} * 4)$$

Where, P = Protein content (%), F = Fat content (%), C = Total carbohydrate (%)

3.16.6.3. Determination of mineral content

The concentration of minerals was determined using atomic absorption spectrophotometer (Analytic Jena Nov AA350, Germany) by the method of Osborne and Voogt (1978) (Singh *et al*., 2010). Briefly, 2.5 g of samples were transferred to a porcelain dish and heated at 120 ^{0}C for 4 h on a hot plate until the entire content had become carbonized. The samples were then heated in a furnace at 530 ^{0}C until free of carbon; the residue appeared grayish/white after 8 h. Then, the crude ash was dissolved in 5 mL of 6 M HCl on a hot plate for 2 h. Subsequently, 7 mL of 3 M HCl was added and heated on a hot plate until the solution boiled. The digested sample was cooled and filtered. Then, 5 mL of 3 M HCl was added to the dishes and heated the extract to dissolve the residue. For calcium determination, lanthanum chloride (10% w/v) was added to both standards and samples to suppress interference from phosphorus. Then, the concentration of the minerals sodium, potassium, magnesium, zinc, manganese, iron and calcium was analyzed using atomic absorption spectrophotometer against calibration curves prepared by plotting the absorption or

emission values against the metal concentrations in mg/100g using the following formula:

$$\text{Metal content (mg/100g)} = \frac{[(A-B)*V]}{10W}$$

Where,

W= weight of samples (g)

V=Volume of extract (mL)

A=Concentration of sample solution (µg/mL)

B=Concentration of blank solution (µg/mL)

3.17. Data analysis

Data analyses were performed using SAS software version 9 (Inc. Cary NC USA). The experiments were carried out in triplicate. Analysis of variance (ANOVA) and means comparisons were done by Duncan's multiple range tests.

Chapter 4

4. Isolation and Screening of Milk-clotting Protease (MCP) from Fungi and Bacteria

4.1.1. Isolation and Screening of Milk-clotting Protease (MCP) Producing Fungi from Ethiopian Soils

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Abstract

Milk-clotting enzymes are valuable in the cheese industry. Thus, the aim of the present study was to isolate and screen Ethiopian regional fungi species and select strains with a better milk-clotting activity that could further be used as a suitable alternative to commercial rennet enzyme during industrial cheese production. In this study, 67 soil and cow dung samples were collected from five sampling sites in Ethiopia. The fungi were isolated using potato dextrose agar (PDA) and screened for milk-clotting protease activity using a plate assay technique on skim-milk agar medium. The secondary screening was conducted by inducing the production of the enzyme with solid-state fermentation (SSF) and submerged fermentation (SmF). The enzyme was recovered as crude enzyme extract by filtration and centrifugation. Its milk-clotting and protease activities were determined. The isolates were identified to their respective taxa using phenotypic and molecular methods. A total of 188 fungal isolates were recovered of which 35 isolates showed a clear zone of casein hydrolysis on Skim-milk Agar. During the secondary screening, 17 isolates showed significant

MCA and were identified into the genera *Aspergillus*, *Fusarium*, and *Pleurotus* using Internal transcribed spacer ITS primers and phenotypically. Three strains; *Aspergillus oryzae* SMDFS19, *Aspergillus oryzae* DRDFS13, and *Pleurotus ostreatus* PENC were well grown on modified solid-substrate (media 2) with better MCA of 194.36 U/mL, 232.02 U/mL and 95.29 U/mL, respectively. A higher specific activity (49.47 U/mg) was also recorded from *Aspergillus oryzae* DRDFS13 at the fifth day of fermentation. Intense protein band was detected at the 32 KDa mark. The fungus *Aspergillus oryzae* DRDFS13 produced milk-clotting protease with enhanced milk-clotting activity and could be used as a potential candidate for large scale production of milk-clotting protease enzyme.

Keywords: *Aspergillus*, Fungi, Milk-clotting activity, Milk-clotting protease, Solid-state fermentation

4.1.2. Results

4.1.2.1. Primary screening of protease producing fungi by plate assay techniques

A total of 188 fungal isolates were recovered from different soil samples, of which, 35 isolates (19%) showed clearing zone on milk-clotting screening media (Table 4.1.1). The spore concentration of all the 35 isolates was determined using the Neubauer chamber and was in the range between $3X10^4$ and $1.01X10^8$ spores/mL. After secondary screening by solid-state and submerged fermentation, 17 fungal isolates were selected and subjected to phenotypic and genetic characteristics.

Table 4.1.1: Clear Zone diameter of fungi isolates on skim-milk agar (SMA)

No	Isolates	Spore conc/mL	Colony diameter Mean±SD	Clear Zone diameter Mean±SD	REA (CZD/CD)
1.	AWDFS5	$3.12X10^{6}$	15.00±1.00	$9.00±1.00^{defg}$	0.60
2.	AWDFS12A	$1.25X10^{7}$	18.00±2.00	$7.00±1.00^{ghi}$	0.39
3.	AWDFS13	$1.59X10^{6}$	15.00±1.00	$10.00±2.00^{cdef}$	0.67
4.	AWDFS17	$2.95X10^{7}$	16.00±2.00	$7.00±0.50^{ghi}$	0.44
5.	AWDFS18A	$6.06X10^{6}$	15.00±1.00	$10.00±1.00^{cdef}$	0.67
6.	AWDFD6	$3.80X10^{7}$	20.00±1.50	$10.00±2.00^{cdef}$	0.50
7.	AWDFD11	$7.08X10^{7}$	31.75±0.25	$3.75±0.25^{jk}$	0.12
8.	WGDFS5	$1.41X10^{7}$	12.00±1.00	$12.00±1.00^{bc}$	1.00
9.	WGDFD4	$1.01X10^{8}$	11.00±1.00	$13.25±0.75^{b}$	1.20
10.	WGDFD6	$2.83X10^{5}$	14.50±0.50	$6.50±0.50^{ghi}$	0.45
11.	WGDFD11	$2.32X10^{7}$	14.00±1.00	$5.75±0.25^{ij}$	0.41
12.	WGFRS4	$1.75X10^{7}$	17.50±0.50	$10.75±0.25^{bcde}$	0.61
13.	AWLS6	$1.26X10^{7}$	22.50±1.50	$6.25±0.25^{ghij}$	0.28
14.	DRDFS13	$3.57X10^{7}$	9.50±0.50	$11.50±0.50^{bcd}$	1.21
15.	DRDFS19	$3.00X10^{5}$	14.25±0.25	$11.50±0.50^{bcd}$	0.81
16.	DRDFS23	$3.09X10^{6}$	22.00±1.0	$6.50±0.50^{ghi}$	0.30
17.	SMDFS1	$1.27X10^{7}$	21.00±1.00	$8.00±1.00^{efghi}$	0.38
18.	SMDFS3	$1.24X10^{6}$	5.50±4.50	$6.50±0.50^{ghi}$	1.18
19.	SMDFS18	$1.85X10^{7}$	8.00±2.00	$17.00±0.50^{b}$	2.13
20.	SMDFS19	$2.00X10^{7}$	6.00±1.00	$9.00±0.50^{defg}$	1.50
21.	SMDFS29	$1.83X10^{7}$	6.00±0.00	$9.00±0.00^{defg}$	1.50
22.	SMDFS41	$7.73X10^{6}$	10.00±2.00	$2.50±1.50^{k}$	0.25
23.	SMDFS42	$6.88X10^{6}$	18.25±1.25	$5.75±0.25^{ij}$	0.32
24.	SMDFS44	$1.92X10^{7}$	23.00±2.00	$8.75±0.25^{defgh}$	0.38
25.	SMDFS46	$1.27X10^{7}$	28.00±2.00	$6.75±0.25^{ghi}$	0.24
26.	SMDFS47	$2.41X10^{7}$	24.00±1.00	$6.00±0.50^{hij}$	0.25
27.	SMDFS49	$2.48X10^{6}$	25.75±0.25	$11.00±0.50^{bcd}$	0.43
28.	SMDFS50	$1.19X10^{7}$	10.50±0.50	$5.25±0.25^{ij}$	0.50
29.	SMDFS51	$8.76X10^{7}$	22.00±1.00	$11.25±0.75^{bcd}$	0.51
30.	SMDFS52	$3.00X10^{4}$	14.00±1.00	$10.50±1.00^{cde}$	0.75
31.	SMDFS55	$4.25X10^{7}$	18.50±0.50	$5.50±0.50^{ij}$	0.30
32.	SMDFS57	$7.76X10^{7}$	16.50±0.50	$6.75±0.25^{ghi}$	0.41
33.	SMDFS58	$7.64X10^{7}$	13.25±0.75	$7.25±0.75^{fghi}$	0.55
34.	SMDFS59	$1.54X10^{7}$	17.00±1.00	$9.00±1.00^{defg}$	0.53
35.	PENC	$6.40X10^{6}$	12.00±1.00	$9.00±0.50^{defg}$	0.75

CZD: Clear zone diameter expressed in mm, CD: colony diameter expressed in mm, SD: standard deviation, REA: is the ratio of CZD//CD, Mean: is average of three measurements, Different letters (a, b, c, d, e, f, g) designate significantly different means as determined by Duncan multiple Mean comparison test ($P<0.05$)

4.1.2.2. Phenotypic characteristics of potential isolates

The fungal isolates (17 potential isolates) were identified phenotypically based on species descriptions (Appendix 15 and 16).

4.1.2.3. Molecular characterization of fungal isolates

All the 17 fungi isolates were successfully identified to the species level using ITS gene sequences and categorized into different groups using MEGA 7 (Figure 4.1.1). Thirteen were identified and categorized under genus *Aspergillus* whereas the rest four isolates were categorized under the genus *Pleurotus* (1 isolate) and genus *Fusarium* (3 isolates) (Table 4.1.2). Out of 13 fungal isolates categorized under genus *Aspergillus*, isolates DRDFS13 and SMDFS19 were shown >98% similarity with *A. oryzae*, isolates DRDFS19, AWDFS12A, AWDFS18A, SMDFS52, and SMDFS59 were shown >98% similarity *A. flavus*, isolates SMDFS 18, SMDFS50, SMDFS55, SMDFS57, and AWDFS5 were shown >98% similarity with *A. fumigatus*, isolate AWDFS1 was shown >98% similarity with *A. terreus* and isolate PENC was shown >97% similarity with *Pleurotus ostrateous*. The remaining 3 isolates such as SMDFS42, SMDFS49, and WGDFD6 were shown 100% similarity with *Fusarium cf.dimerum, Fusarium Sp.* and *Fusarium circinatum* respectively.

Table 4.1.2: ITS identity of selected mould isolates from soil and dung in Ethiopia.

Isolates	Accession Number	No of Isolates	Genera	Closest relatives	Similarity (%)
DRDFS 13	MN726447	2	*Aspergillus*	*A. oryzae* WM	98.86-99.85
SMDFS 19	MN726468			*A. oryzae* WM	
DRDFS 19	MN726455	5	*Aspergillus*	*A. flavus* aT3	98.40-100
AWDFS 12 A	MN726472			*A. flavus* F62	
AWDFS 18 A	MN726457			*A. flavus* O13	
SMDFS 52	MN726459			*A. flavus* O13	
SMDFS59	MN726464			*A. flavus* F	
SMDFS 18	MN726471	5	*Aspergillus*	*A. fumigatus* UMAGOD24	98.80-100
SMDFS 50	MN726458			*A. fumigatus* 21L05I2	
SMDFS 55	MN726460			*A. fumigatus* PF4-1	
AWDFS 5	MN726461			*A. fumigatus* EV32	
SMDFS 57	MN726463			*A. fumigatus* OnJ3	
AWDFS 17	MN726456	1	*Aspergillus*	*A. terreus* XJA8	98.37
PENC	-	1	*Pleurotus*	*P. ostreatus* CL-68	97.24
SMDFS 49	MN726473	3	*Fusarium*	*F.* sp.SX10	100.00
WGDFD 6	MN726462			*F. circinatum* UACH-128	
SMDFS 42	MN726454			*F. cf. dimerum* strain CSH_4	

SMDFS: Samara Dairy Farm Soil; DRDFS: Dire Dawa Dairy Farm Soil; AWDFS: Awasa Dairy Farm Soil; WGDFD: Wondogenet Dairy farm Dung

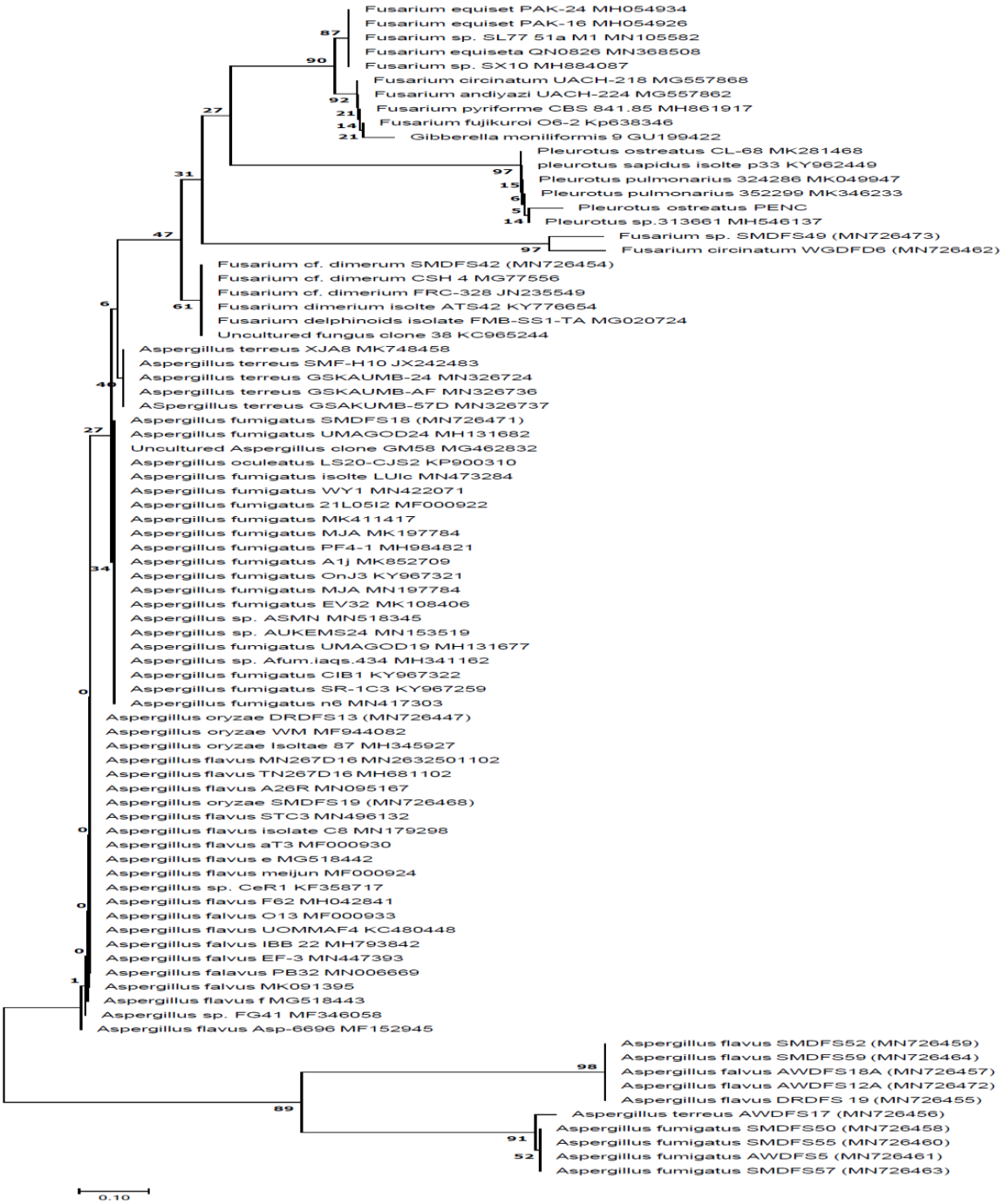

Figure 4.1.1: Phylogenetic tree of the 17 fungal strains (neighbor-joining method and bootstrap1000 replicates)

4.1.2.4. Secondary Screening of milk-clotting protease producing fungi by SmF and SSF

Thirty out thirty-five isolates that showed a relative enzyme activity (REA=CZD/CD) ≥0.3 were selected for further screening in solid-state and submerged fermentation. Out of the thirty isolates, 17 isolates showed a milk-clotting activity (MCA) and

protease activity (PA) under SSF, while none of the isolates showed MCA under SmF (Table 4.1.3). The highest MCA (100.18 U/mL) was recorded by *A. fumigatus* SMDFS18 and *P. ostreatus* PENC whereas the lowest MCA (18.33 U/mL) was noticed by *A. fumigatus* AWDFS5 under SSF. Regarding the protease activity, the highest protease activity (2577.62 U/mL) was recorded by *A. fumigatus* SMDFS18 whereas the least protease activity (497.24 U/mL) recorded by isolate *F. cf. dimerum* SMDFS42 in SSF. A MCA/PA ratio in the range of 0.02 to 0.08 was noticed from the crude enzyme extract in the present study. The highest MCA/PA ratio of 0.08 and 0.07 was recorded from *F. cf. dimerum* SMDFS 42 and *A.oryzae* DRDFS13 and *P. ostreatus* PENC, respectively (Table 4.1.3).

Table 4.1.3: MCA and PA of fungal isolates in SmF and SSF using Media 1

No.	Isolates	Spore conc/mL	SmF		SSF			
			MCA (U/mL)	PA(U/mL) Mean±SD	MCA (U/mL) Mean±SD	MCA (U/g)	PA(U/mL) Mean±SD	Ratio (MCA/PA)
1.	*A. fumigatus* AWDFS5	$3.12X10^6$	–	$682.98±44.41^{e}$	$18.33±0.42^{g}$	183.30	$1131.92±24.64^{gf}$	0.02
2.	*A. flavus* AWDFS12A	$1.25X10^6$	–	$807.78±9.72^{cd}$	$77.50±2.50^{bc}$	775.00	$2031.11±5.83^{c}$	0.03
3.	*A. terreus* AWDFS17	$2.95X10^6$	–	$702.10±0.65^{de}$	$30.77±0.39^{ef}$	307.70	$1788.65±71.96^{d}$	0.02
4.	*A. flavus* AWDFS18A	$6.06X10^6$	–	$781.20±12.32^{cde}$	$80.56±6.71^{b}$	805.60	$2327.38±5.19^{b}$	0.03
5.	*F. circinatum* WGDFD6	$2.83X10^5$	–	$886.54±3.57^{bc}$	$30.46±1.54^{ef}$	304.60	$1059.96±48.62^{gf}$	0.03
6.	*A. fumigatus* SMDFS18	$1.85X10^6$	–	$683.30±1.30^{e}$	$100.18±4.17^{a}$	1001.80	$1577.62±22.04^{a}$	0.06
7.	*A. oryzae* SMDFS19	$2.00X10^6$	–	$820.42±39.87^{bc}$	$66.68±0.93^{c}$	666.80	$1200.65±9.08^{f}$	0.06
8.	*A. oryzae* DRDFS13	$3.57X10^6$	–	$840.84±34.36^{bc}$	$66.78±2.78^{c}$	667.80	$1014.58±12.32^{gf}$	0.07
9.	*A. flavus* DRDFS19	$3.00X10^5$	–	$1031.11±48.30^{a}$	$80.36±5.36^{b}$	803.60	$2307.93±3.89^{b}$	0.03
10.	*P. ostreatus* PENC	$6.4X10^6$	–	$930.63±44.41^{ab}$	$100.18±4.17^{a}$	1001.80	$1526.74±70.66^{e}$	0.07
11.	*F.cf. dimerum* SMDFS42	$6.88X10^6$	–	$349.11±23.01^{f}$	$40.40±4.04^{de}$	404.00	$497.24±13.61^{j}$	0.08
12.	*F. sp.* SMDFS49	$2.48X10^6$	–	$429.17±25.28^{f}$	$40.18±2.68^{de}$	401.80	$775.69±24.31^{hi}$	0.05
13.	*A. fumigatus* SMDFS50	$1.19X10^6$	–	$123.50±21.72$	$50.35±4.20^{d}$	503.50	$1200.00±97.89^{f}$	0.04
14.	*A. flavus* SMDFS52	$3.00X10^4$	–	$153.97±10.05^{g}$	$40.01±0.67^{de}$	400.10	$705.67±0.32^{ij}$	0.06
15.	*A. fumigatus* SMDFS55	$4.25X10^6$	–	$178.28±1.94^{g}$	$50.02±0.83^{d}$	500.10	$970.82±91.57^{gh}$	0.05
16.	*A. fumigatus* SMDFS57	$7.76X10^6$	–	$177.63±6.48^{g}$	$25.01±0.52^{fg}$	250.10	$508.59±4.21^{j}$	0.05
17.	*A. flavus* SMDFS59	$1.54X10^6$	_	$101.13±26.58^{g}$	$44.69±3.31^{d}$	446.90	$1228.20±102.75^{f}$	0.04

MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL), Ratio: is the ratio of MCA/PA, SD: standard deviation, Mean: is average of three measurements, Different letters (a, b, c, d, e, f, g) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

4.1.2.5. Culture profile of potential fungal isolates

The culture profile of five potential fungal isolates was studied under SSF using medium 2. Three strains (*Aspergillus oryzae* DRDFS13, *Aspergillus oryzae* SMDFS19, and *Pleurotus ostreatus* PENC) grew on SSF medium showed better MCA. A better MCA/PA ratio was also recorded from these three strains. However, two fungi strains (*Fusarium cf. dimerum* SMDFS 42 and *A. fumigatus* SMDFS 18) did not grow on medium 2 under SSF. The maximum MCA of 232 U/mL 194 U/mL, and 95 U/mL were recorded from strain *A. oryzae* DRDFS13, *A. oryzae* SMDFS19, and *P. ostreatus* PENC at the 5^{th}, 4^{th} and 6^{th} days of fermentation, respectively. The highest protease activity for *A. oryzae* DRDFS13 (163 U/mL), *A. oryzae* SMDFS19 (227 U/mL) and *P. ostreatus* PENC (145 U/mL) was achieved at 6^{th}, 5^{th} and , 4^{th} days of cultivation, respectively (Table 4.1.4). The total protein content for isolates *A. oryzae* DRDFS13, *A. oryzae* SMDFS 19 and *P. ostreatus* PENC were found in the range from 4.66 to 9.17, 12.34 to 35.22 and 8.02 to 26.08 mg/mL respectively. The total soluble carbohydrate for isolates *A. oryzae* DRDFS 13, *A. oryzae* SMDFS 19 and *P. ostreatus* PENC were in the range of 5.62 to 37.92, 28.24 to 103.31 and 0.00 to 97.81mg/mL respectively (Table 4.1.4). A slight increase in both total protein and total soluble carbohydrates was observed during the first few days of incubation period and then start to decline.

4.1.2.6. Molecular weight determination of crude enzyme extract

The approximate molecular weight of the proteins from the crude enzyme extract of the three potential fungi strains (*A. oryzae* DRDFS13, *A. oryzae* SMDFS19, and *P. ostreatus* PENC) during 6 days of fermentation periods was determined by 12% SDS-PAGE analysis. Prominent protein band at around 32 kDa mark was observed from

the crude enzyme starting from the 3rd day of the incubation period (Figure 4.1.2). Additionally, several extra bands were also detected at varies marks from all the three isolates.

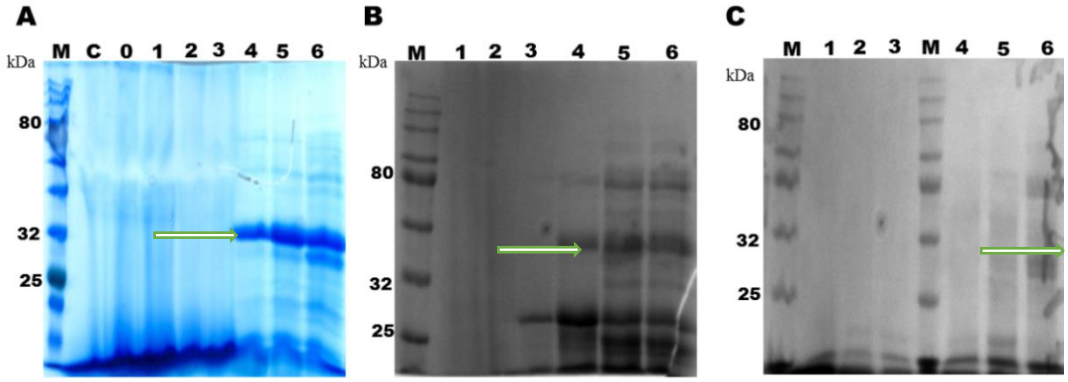

Figure 4.1.2: An SDS-PAGE gel Coomassie Blue stained of protein crude extract of A) *A. oryzae* DRDFS13, B) *A. oryzae* SMDFS19, and C) *P. ostreatus* PENC, Where M is the Color-Plus Pre-stained Protein marker; C controls, while 0, 1, 2, 3, 4, 5 and 6 indicate fermentation time in days)

Table 4.1.4: Culture profile of potential fungal isolates in SSF using medium 2 cultivated at 30 ^{o}C for 6 days.

Isolates	Day	pH	MCA (U/mL) mesn±SD	MCA (U/g)	Total protein (mg/mL)	Total Carbohyd.(mg/mL)	Specific Activity (U/mg)	PA (U/ml) mean±SD	Ratio MCA/PA
Blank	_	4.41	0.00	0.00	4.44±0.80	28.82±0.07	ND	40.88±4.38	ND
A. oryzae DRDFS13	1	4.41	0.00	0.00	8.38±0.32	32.46±0.36	ND	43.27±0.66	ND
	2	4.43	0.00	0.00	4.66±0.81	37.68±2.27	ND	45.13±1.46	ND
	3	4.39	30.36±5.90	303.60	5.22±1.17	37.92±1.33	5.82	56.41±3.98	0.54
	4	4.35	221.32±5.10	2213.20	4.73±0.60	27.20±1.25	46.79	114.08±3.19	1.94
	5	4.50	232.02±5.06	2320.20	4.69±1.00	17.62±0.06	49.47	101.76±36.77	2.28
	6	4.80	227.53±3.24	2275.30	9.17±1.29	5.62±0.22	40.49	162.73±5.44	1.40
Blank	_	4.49	0.00	0.00	10.60±0.42	21.17±0.24	ND	65.83±1.19	ND
A. oryzae SMDFS 19	1	4.24	0.00	0.00	12.34±3.98	28.24±1.48	ND	82.96±0.53	ND
	2	4.50	0.00	0.00	35.22±1.67	29.10±0.65	ND	75.79±0.27	ND
	3	4.52	0.00	0.00	20.35±4.85	97.32±0.77	ND	92.65±0.66	ND
	4	4.63	194.36±2.36	1943.60	28.71±4.44	103.3±2.33	6.77	170.43±1.19	1.14
	5	5.54	188.98±1.47	1889.80	28.64±4.05	46.57±1.10	6.60	226.84±1.86	0.83
	6	5.55	171.43±0.00	1714.30	24.26±2.67	36.57±0.41	7.07	220.47±1.86	0.78
P. ostreatus PENC	1	4.16	0.00	0.00	18.00±1.00	33.88±1.63	ND	83.09±1.19	ND
	2	4.47	0.00	0.00	19.57±4.67	69.70±1.18	ND	91.05±0.13	ND
	3	4.56	0.00	0.00	26.08±6.53	97.81±1.90	ND	134.32±0.40	ND
	4	4.64	40.62±0.62	406.20	17.39±1.75	83.44±6.33	2.34	145.08±2.12	0.28
	5	5.48	53.48±1.31	534.80	10.75±1.38	15.19±0.07	4.97	123.97±1.46	0.43
	6	6.33	95.29±4.72	952.90	8.02±0.15	0.00±0.00	11.88	139.90±1.99	0.68

ND = Not determined, MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL), Ratio: is the ratio of MCA/PA, specific activity: MCA (U/mL)/Total protein (mg/mL), SD: standard deviation, Mean: is average of three measurements

Table 4.1.5: Comparison of MCP produced from this study with other fungi strains

Organism	Ferm.	Substrate	Temp (°C)	Maximum MCA		PA	Ratio	References
				U/mL	U/g	U/mL	MCA/PA	
Aspergillus Oryzae DRDFS13	SSF	Wheat bran moistened with HCL(200mM) supplemented with glucose and casein	30	232.02	2320.2	101.76	2.28	This study
Aspergillus oryzae SMDFS 19	SSF	Wheat bran moistened with HCL (200mM) supplemented with glucose and casein	30	194.36	1943.60	170.43	1.14	This study
Pleurotus ostreatus PENC		Wheat bran moistened with HCL (200mM) supplemented with glucose and casein	30	95.29	959.90	139.90	0.68	This study
Mucor mucedo DSM 809	SmF	Submerged media	24	130.00	_	_	_	(Yegin *et al.*, 2010)
Aspergillus niger FFB1	SSF	Wheat bran, Czapeck dox, and mineral solution	30	166.00	830.00	195.29	4.25	(Bensmail *et al.*, 2015)
Aspergillus oryzae	SSF	Wheat bran and rice bran with a mineral solution	30	119.43	1194.30	-	_	(Pallavi *et al.*, 2012)
Aspergillus flavo furcates DPUA 1461	SmF	Acaia waste, rice bran, and mineral solution	30	116.19	_	_	2.86	(Alecrim *et al.*, 2015)
Aspergillus spp.	SSF	Wheat Rawa with fructose & chickpea meal moistened with mineral solution	32	26.00	130.00	_	_	(Radha *et al.*, 2012)
Rhizopus stolonifer	SSF	Wheat bran with galactose and peptone moistened by salt solution	28	120	1200	_	_	(Gais *et al.*, 2009)
Mucor spp.	SSF	Wheat bran moistened with HCl (200 mM)	24	710.00	7100.00	_	_	(Fernandz-Lahore *et al.*, 1999)

4.1.3. Discussion

In this atudy several fungal isolates were recoverd from soil and dung samples collected from different hot areas of Ethiopia to screen milk-clotting protease producing fungi. Thus, a total of 188 fungal isolates were screened for protease production by plate assay technique on skim-milk agar media, the results showed that 19% of the isolates had a significant clear-zone of hydrolysis and REA. Similar to the present study, substantial clear zone diameter has been reported for different

filamentous fungi (Choudhary and Jain, 2012), *Stenocarpella mydis* (Hernandez-Dominguez *et al.*, 2014), *Mucor* sp. (Ayana *et al*., 2015) and *A. niger* FFB1 (Bensmail *et al*., 2015) on agar reese media, agar media, skim-milk agar and agar media, respectively. However, a higher relative enzyme activity (2.29 to 3.00) than the current study was reported from *Aspergillus sp.*, *Penicillium* sp. and *Malbranchea* sp. in agar media (Choudhary and Jain, 2012). The slight variation in inhibition zone diameter and relative enzyme activity could be attributed to the low media pH used in the present study. The spore concentration of 35 fungal isolates in this study ranges between $3X10^4$ and $1.01X10^8$ spore/mL

Seventeen out of thirty isolates (57%) demonstrated milk-clotting activity (MCA) and protease activity (PA) in SSF. None of the isolates showed MCA in SmF. This could be due to the acid protease-encoding gene (PepA) of *A. oryzae* expressed only in solid-state fermentation (Kataoka *et al*., 2002). However, this gene was not expressed in submerged culture under acidic (pH 5.0), neutral (pH 7.0) and basic (pH 9.0) conditions (Kataoka *et al*., 2002). Filamentous fungi produce a variety of extracellular enzymes in a solid substrate (Vishwanatha *et al*., 2010). SSF is better than SmF in terms of enzyme yield, process simplicity, lower capital investment and energy requirement, low waste output and ease in product recovery (Fernandez-Lahore *et al.*, 1998).

The data obtained from MCA (333.3 U/g) of *A. oryzae* DRDFS13 under SSF was equivalent to the MCA reported by Pallavi *et al.*, (2012). Whereas, the milk-clotting activity of crude enzyme of *Mucor sp.* (710 U/mL) (Fernandz-Lahore *et al.*, 1999), *Rhizomucor nainitalensis* (8400 U/g) (Khademi *et al*., 2013) and *M. circinelloides* (7,059 U/g) (Sathya *et al.*, 2009) in SSF was higher than the MCA recorded from *A.*

fumigatus SMDFS18 and *P. ostreatus* PENC in the present study. This high variation in milk-clotting activity between this study and experimental results from other investigations could be caused by a difference in substrates and physical parameters used in solid-state fermentation.

The protease activity recorded from *Aspergillus* sp. (4-232 U/g), *Aspergillus niger* IHG9 (8.6 U/g), *A. oryzae* (low protease activity), *Stenocarpella maydis* (806 U/mL), mutant *A. oryzae* (115 U/mL) and *A. tamari* URM4634 (340 U/mL) under solid-state fermentation was lower than this study (Hernández-Domínguez *et al*., 2014; Mukhtar and Ikram-Ul-Haq, 2009; Murthya and Kusumoto, 2015; Pallavi *et al*., 2012; Radha *et al.*, 2012; Soares *et al*., 2016). The possible reason for the differences in protease activity might be the variation in fungal strains and/or in the substrate used for SSF.

In the present study, all the fungi isolates did not display milk-clotting activity (MCA) in SmF. Similarly, insignificant milk-clotting activity was reported for *A. niger* (0.9 U/mL) and *A. flavus* (1.06 U/mL) (Valarmathi *et al.*, 2016). However, the milk-clotting activity obtained from *M. mucedo* DSM 809 crude enzyme extract (130.7 SU), *M. miehei* NRRL 3420 crude enzyme extract (1200 SU) and *Termitomyces clypeatus* MTCC 5091 (333.33 U/mL) under submerged fermentation was higher than the present study (Lima *et al*., 2008; Majumder *et al*., 2015; Yegin *et al.*, 2010). The differences could be due to variation in strains, media and or/purification steps used.

All the 17 fungal isolates were also successfully identified to the species level using ITS region gene sequence. In comparison to the present study, the BLAST search using the genebank database of four fungal isolates showed that isolate TM1 shared 99% similarities with *Trichoderma harzianum*, isolate AM1 shared 99% similarity with *A. nomius*, isolate AM2 shared 99%–100% similarity with *A. tubingensis* and

isolate AM3 showed 100% similarity to *A. aculeatus* (Majid *et al*., 2015). The BLAST search of amplified ITS region gene sequences against the NCBI database for some fungi had also shown 97–99% similarity with 8 species of 8 fungi genera recorded in the bank (*Aspergillus, Penicillium, Cladosporium, Fusarium, Emericella, Thielavia, Scytalidium and Alternaria*) (Alwakeel, 2016). Molecular identification of 77 *Aspergillus* isolates recovered from corn grains using an ITS and β-tubulin sequences were also grouped into seven species such as *A. niger, A. flavus, A. oryzae, A. fumigatus, A. clavatus, A. terreus* and *A. tubingensis* (Atiqah and Zakaria, 2017).

The ratio of milk-clotting activity to protease activity is a very important criterion for the selection of a good milk coagulant. A higher ratio indicates a more desirable pattern of action during cheese making (Yegin *et al*., 2012). In the present study, the highest ratio of MCA to PA was recorded from isolates *F. cf. dimerum* SMDFS 42 (0.08), *A. oryzae* DRDFS13 (0.07) and *P. ostreatus* PENC (0.07) during SSF. This implies that the crude enzyme from these fungi strains has significant aspartic protease activity and requires further media optimization and enzyme purification to improve the ratio.

Five fungi strains with better MCA/PA ratio were selected for further production of milk-clotting protease under SSF using medium 2. However, only three strains (*A. oryzae* DRDFS13, *A. oryzae* SMDFS19, and *P. ostreatus* PENC) grew on media 2 under SSF. Two isolates (*A .fumigatus* SMDFS18 and *F. cf. dimerum* SMDFS 42) did not grow on media 2. This could be due to the low pH of the media developed by the addition of HCl. However, a better ratio (MCA/PA) and specific activity were obtained from the three strains that have been grown on media 2. Therefore medium 2 was used for further production of aspartic protease enzyme in this study.

The maximum milk-clotting activity that was obtained from *M. mucedo* DSM 809 (130.7 SU) (Yegin *et al.*, 2010), *Aspergillus niger* FFB1(166 U/mL) (Bensmail *et al*., 2015), *Aspergillus oryzae* (119.43 U/mL) (Pallavi *et al*., 2012), *Aspergillus flavo furcatis* DPUA 1461 (116.90 U/mL) (Alecrim *et al*., 2015), *Aspergillus spp.* (130 U/mL) (Radha *et al.*, 2012) and *Rhizopus stolonifer* (120 U/mL)(Gais *et al*., 2009) were comparable with the present study.

Whereas the milk-clotting activity (8575 U/g) (Sathya *et al.*, 2009) and (710 U/mL) (Fernandz-Lahore *et al*., 1999) obtained from *M. circinelloides* and *Mucor* sp., respectively were higher than the present study. The differences in milk-clotting activity could be due to variation in fungal strains and solid substrate used for fermentation.

The decline in total protein and soluble carbohydrate after first few days of incubation period may be attributed to the fact that microbes can use the protein and carbohydrate contents of the media for their biomass and enzyme synthesis. The total protein content from *M. mucedo* DSM 809 (31.82-39.91µg/mL is lower than the present study (Yegin *et al*., 2010). Similarly, the total soluble carbohydrate content from *M. mucedo* DSM 809 (0.3 -7.4 g/L) and *Mucor sp*. (2.4-11g/mL) is also less than this study (Fernandz-Lahore *et al*., 1999; Yegin *et al*., 2010). The deviations in total protein and total carbohydrate noticed could be due to the differences in fungi genera and types of fermentation used in the present study.

Significant protein bands were detected at around 32 kDa mark even if several extra bands were observed at varies protein marks. The formation of several extra bands at different protein marks could be due to the impurity in the crude enzyme extract. However, the band formed at around 32 kDa mark specifies the presence of aspartyl

proteinases in the crude enzyme extract as an aspartic protease with milk-clotting activity have a molecular weight between 32–45 kDa (Hsiao *et al*., 2014). Similar findings were also reported by Fernandz-Lahore *et al*., (1999) and (Yegin *et al*., 2012) from *Mucor sp.* (33kDa) and *M. mucedo* DSM 809 (32.7 kDa), respectively.

4.1.4. Conclusions

It is concluded from the present study, that Skim-milk Agar media was better for primary screening of milk-clotting protease, whereas solid-state fermentation was good for the production of milk-clotting protease from filamentous fungi. After several screening procedures, the filamentous fungi *Aspergillus oryzae* DRDFS13 were found to be good producers of milk-clotting protease under solid-state fermentation and could be found as a potential candidate for large scale production of milk-clotting protease.

4.2. Isolation and Screening for Milk-clotting Protease Producing Bacteria from Soil

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Abstract

The objective of the present work was to isolate potential bacterial strains endowed with amplified milk-clotting protease enzymes production. In this study, 49 bacterial isolates were isolated from soil and dung using nutrient agar media (NA). Primary screening of milk-clotting protease (MCP) producing bacteria was done by plate assay technique using skim-milk agar (SMA). The secondary screening was conducted by solid-state fermentation (SSF) and submerged fermentation (SmF). The enzyme was extracted from SSF and SmF using distilled water, centrifuged and the filtrate was used as a crude enzyme. The culture profile of six bacterial species was further studied under partially optimized conditions. The milk-clotting activity (MCA) and protease activity (PA) were tested by standard methods. The potential bacterial isolates were identified to the species level using a combination of biochemical and molecular methods. Fourteen bacterial isolates were screened for protease production with clear zone diameter between 5.25 mm and 21 mm. Further screening of the bacteria for MCP under SSF, indicated 11(78.57%) out of 14 isolates were produced a MCP with MCA between 8 U/mL and 100 U/mL except SMDFS 29 B that exhibited a MCA 382 U/mL. In SmF, all the 14 bacterial isolates produced a MCP with clotting activity between 100 U/mL and 480 U/mL. All the 14 bacterial isolates that exhibited

significant MCA under SmF were member of different species under genus *Bacillus*. Four isolates were shown >99% 16S rRNA gene sequence similarity with *Bacillus subtilis* NCIB 3610, seven isolates were shown 100% 16S rRNA gene sequence similarity with *B. tequilensis* KCTC 13622, while isolates SMDFS 5B, SMDFS 15B and SMDFS 29B were exhibited 100% 16S rRNA gene sequence similarity with *B. paramycoides* NH24A2, *B. siamensis* KCTC 13613 and *B. siamensis* KCTC 13613, respectively. The MCA of the selected bacterial species was significantly increased during culture profile determination, however, a maximum MCA (2533 U/mL) was recorded from *B. subtilis* SMDFS 2B. The total protein concentration of the crude enzyme was increased until optimum production is achieved while carbohydrates were drastically reduced upon the fermentation time. Thus, *B. subtilis* SMDFS2B produced a milk-clotting protease with improved milk-clotting activity and the cultivation of this bacterium under partially optimized conditions significantly increased the production of the MCP and its MCA.

Keywords: *Bacillus*, Milk-clotting activity, Milk-clotting protease, submerged fermentation

4.2.1. Results

4.2.1.1. Primary screening for protease producing bacteria by plate assay techniques

A total of 49 bacterial isolates were collected from different soil samples, of which, 14 isolates (29 %) showed clearing zone ranging from 5.25 up to 21mm (Table 4.2.1). All isolates were identified as Gram-positive, rod-shaped, catalase-positive and produced endospores. They displayed cream color, irregular, undulate, wrinkled colony characteristics with 0.5–1mm in diameter. Based on their biochemical and genetic characters, all the 14 bacterial isolates were classified under the genus *Bacillus*. The molecular characterization revealed that 7 isolates were shown 100% 16S rRNA sequence similarity with *Bacillus tequilensis,* 4 isolates were shown ≈100% 16S rRNA sequence similarity with *B. subtilis* and 2 isolates were shown 100% 16S rRNA sequence similarity with *B. siamensis* and 1 isolate was shown 100% 16S rRNA sequence similarity with *B. paramycoides* (Figure 4.2.1 and Table 4.1.2).

Table 4.2.1: Hydrolized clear zone diameter of bacterial isolates on SMA

No.	Isolates	Clear. Zone diameter (mm) Mean±SD
1.	SMDFS2B	11.00 ± 1.00^{bc}
2.	SMDFS3B	8.00 ± 1.00^{ced}
3.	SMDFS5B	7.00 ± 1.50^{ced}
4.	SMDFS6B	6.00 ± 1.00^{de}
5.	SMDFS7B	11.00 ± 2.00^{bc}
6.	SMDFS8B	6.00 ± 2.50^{de}
7.	SMDFS11B	10.00 ± 1.00^{bcd}
8.	SMDFS12B	5.25 ± 0.25^{e}
9.	SMDFS14B	9.00 ± 3.00^{ced}
10.	SMDFS15B	6.00 ± 1.00^{de}
11.	SMDFS16B	6.00 ± 0.00^{de}
12.	SMDFS18B	9.75 ± 1.75^{bcd}
13.	SMDFS29B	21.00 ± 1.00^{a}
14.	DRDFS13B	13.50 ± 0.50^{b}

SMA: Skim-milk Agar; CZD: clear zone diameter, SD: standard deviation, Mean: is average of three measurements, Different letters (a, b, c,d, e) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$)

Table 4.2.2: Identification of the milk-clotting protease producing bacteria using 16S rRNA gene

No	Isolates	Accession Number	Similarity (%)	Closest relatives
1	SMDFS 2B	MN715837	100	*Bacillus subtilis* NCIB 3610
2	SMDFS 3B	MN715838	100	*Bacillus tequilensis* KCTC 13622
3	SMDFS 6B	MN715840	100	*Bacillus subtilis* NCIB 3610
4	SMDFS 7B	MN715841	100	*Bacillus tequilensis* KCTC 13622
5	SMDFS 8B	MN715842	100	*Bacillus tequilensis* KCTC 13622
6	SMDFS 11B	MN715843	100	*Bacillus tequilensis* KCTC 13622
7	SMDFS 12B	MN715844	100	*Bacillus subtilis* NCIB 3610
8	DRDFS 13B	MN715845	100	*Bacillus tequilensis* KCTC 13622
9	SMDFS 14B	MN715846	99.89	*Bacillus subtilis* NCIB 3610
10	SMDFS 16B	MN715848	100	*Bacillus tequilensis* KCTC 13622
11	SMDFS 18B	MN715849	100	*Bacillus tequilensis* KCTC 13622
12	SMDFS 5B	MN715839	100	*Bacillus paramycoides* NH24A2
13	SMDFS 15B	MN715847	100	*Bacillus siamensis* KCTC 13613
14	SMDFS 29B	MN715850	100	*Bacillus siamensis* KCTC 13613

4.2.1.2. Secondary screening of MCP producing bacteria in SmF and SSF

The bacterial strains were further checked for milk-clotting activity (MCA) and protease activity (PA) under submerged (SmF) and solid-state (SSF) fermentations. All strains produced MCP under SmF, and all, but three strains; *B. subtilis* SMDFS 6B, *B. tequilensis* 3B and *B. paramycoides* SMDFS 5B showed MCA under SSF. They performed better in MCA on SmF fermentation systems ranging from 100 U/mL to 480 U/mL and PA ranging from 425 up to 1522 U/mL with the highest ratio of MCA/PA ratio (0.36) and the lowest ratio of MCA/PA (0.10). Although most strains produced protease enzymes under SSF, they showed the lowest MCA/PA activity of 0.01-0.09. However, *B. siamensis* 29B produced the highest MCA/PA activity of 0.4 under solid-state fermentation (Table 4.2.3).

The bacterial strains also showed significant differences in MCA amongst species in SmF ranging from the highest range of 338 U/mL up to 480 U/mL produced by *B subtilis,* followed by *B. tequilensis* with a range of 101 U/mL up to 437 U/mL.

However, *B. siamensis* and *B. paramycoides* produced a low quantity of the enzyme. They also showed a similar pattern of MCA/PA ratio ranging from 0.27-0.36 by *B subtilis* and 0.1- 0.36 by *B. tequilensis*.

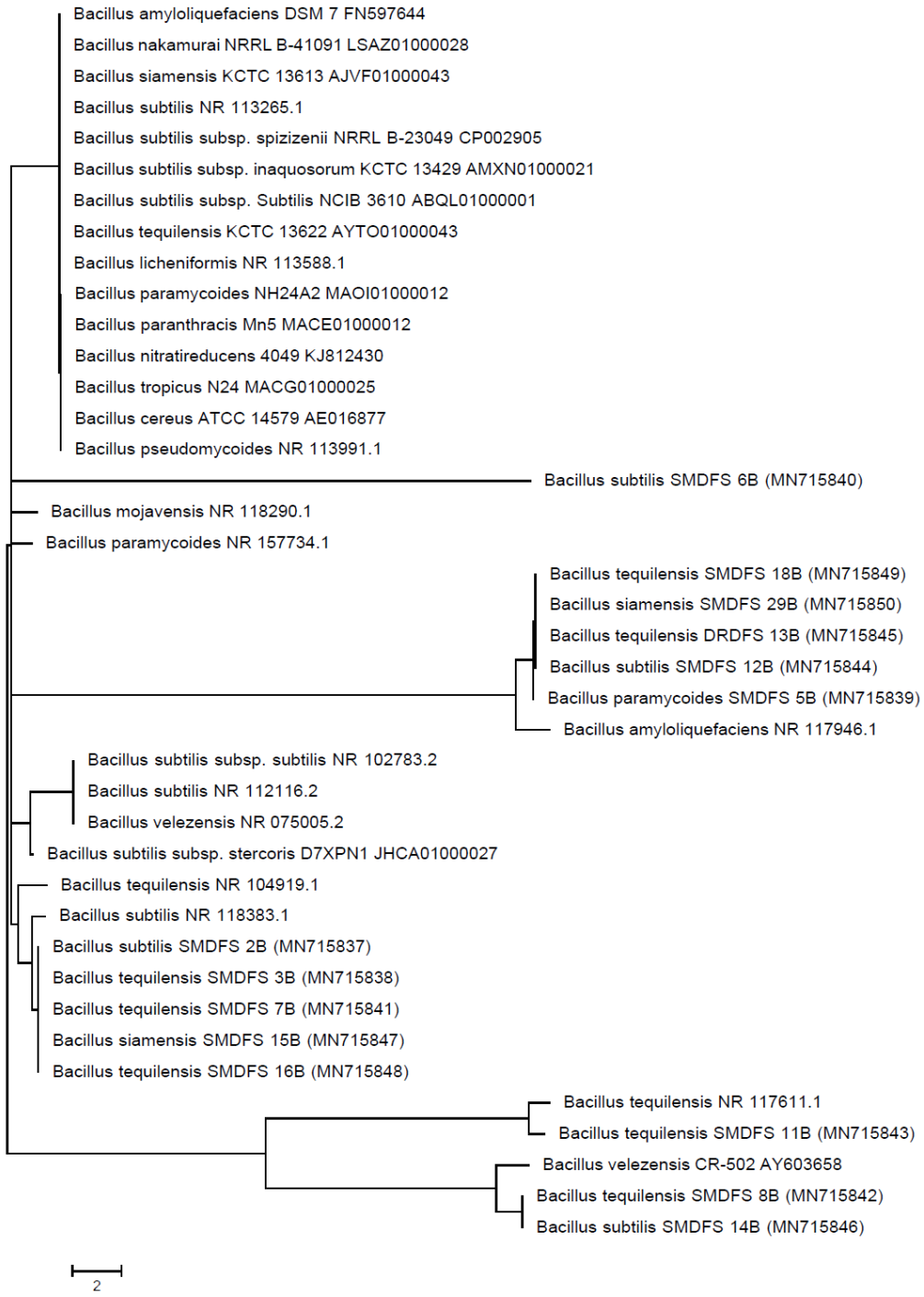

Figure 4.2.1: Phylogenetic tree of 14 bacterial strains (neighbor-joining method and bootstrap1000 replicates)

Table 4.2.3: MCA and protease activity of bacterial strain in SmF and SSF

	SmF			SSF		
Strain	MCA (U/mL) Mean ± SD	PA (U/mL) Mean ± SD	Ratio MCA/PA	MCA(U/mL) Mean SD	PA (U/mL) Mean ± SD	Ratio (MCA/PA)
B. subtilis 2B	480.77±19.23^{a}	1330.30±78.44^{ab}	0.36	57.14±0.00^{d}	974.07±8.75^{d}	0.06
B. subtilis 6B	429.81±23.03^{b}	1521.60±53.16^{a}	0.28	ND^{f}	1003.89±6.16^{d}	ND
B. subtilis 12B	338.30±9.53^{c}	1227.55±11.35^{b}	0.28	25.20±0.20^{e}	676.82±37.60^{e}	0.04
B. subtilis 14B	363.97±11.03^{c}	1347.16±35.01^{ab}	0.27	56.61±0.53^{d}	1341.32±72.61^{b}	0.04
B. tequilensis 3B	200.35±8.35^{e}	780.55±8.43^{c}	0.26	ND^{f}	229.50±25.28^{f}	ND
B. tequilensis 7B	436.51±7.94^{b}	1222.04±186.71^{b}	0.36	98.98±1.02^{b}	1779.25±4.86^{a}	0.06
B. tequilensis 8B	250.98±15.69^{d}	1317.66±33.39^{ab}	0.19	79.34±0.66^{c}	1247.00±244.73^{bc}	0.06
B. tequilensis 11B	344.62±24.62^{c}	1437.60±86.55^{ab}	0.24	7.99±0.10^{f}	593.19±12.32^{e}	0.01
B. tequilensis 13B	133.44±3.71^{f}	492.38±50.24^{d}	0.27	97.62±2.38^{b}	1085.25±35.01^{cd}	0.09
B. tequilensis 16B	358.93±16.07^{c}	1252.51±7.13^{b}	0.29	99.38±0.62^{b}	1604.86±25.61^{a}	0.06
B. tequilensis 18B	100.70±8.39^{g}	425.28±18.15^{d}	0.24	98.78±1.22^{b}	1057.05±63.21^{cd}	0.09
B. siamensis 15B	124.39±1.94f^{g}	1202.26±42.46^{b}	0.10	9.45±0.15^{f}	892.71.±9.08^{d}	0.01
B. siamensis 29B	201.40±16.78^{e}	888.81±90.76^{c}	0.23	381.82±18.18^{a}	952.02±21.07^{d}	0.40
B. paramycoides 5B	137.15±22.85^{f}	732.57±17.50^{c}	0.19	ND^{f}	561.75±3.57^{e}	0.00

ND: Not determined, MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL), Ratio: is the ratio of MCA/PA, SD: standard deviation, Mean: is average of three measurements, Different letters (a, b, c, d, e, f, g) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

4.2.1.3. Culture profile of potential bacterial species in submerged fermentation

All strains showed significantly higher milk-clotting activity of which *B. subtilis* SMDFS 2B displayed the highest milk-clotting activity of 2533 U/mL with a MCA/PA ratio of 14.05 upon the 3rd day of incubation. All other strains also showed an increase in MCA/PA ranging from 4.06 up to 10.26 after 4 days of incubation. This indicates a 10-40 fold increase in the milk-clotting per protease activity compared to the previous screening (Table 4.2.4). The strains also showed slight, but not significant variations on crude enzyme pH change upon fermentation compared to the blank. Thus, *B subtilis* SMDFS 2B reduced the crude enzyme pH from 5.93 to pH 5.34; whereas *B. subtilis* SMDFS 14B slightly raised the pH to 6.63. This shows that the optimum activity of the enzyme derived from the *Bacillus* sp. was within the acidic pH of 5.5 to 6.2. On the other hand, the soluble carbohydrates concentration showed up to five-fold decrease up on 4 days of fermentation. There is a sharp

increase (2-4 folds) in total protein and specific activity at their optimum incubation time.

Table 4.2.4: Culture profile of six potential bacterial isolates in submerged fermentation

Isolates	Day	pH of extract Mean±SD	Total Carbohyd. (mg/mL) Mean±SD	Total protein (mg/mL)	MCA (Uml) Mean±SD	PA (U/mL) Mean±SD	Sp. activity (U/mg)	Ratio MCA/PA
Blank		5.96±0.01	14.48±0.21	6.32±1.39	ND	47.05±2.72	-	-
B. subtilis SMDFS 2B	1	5.91±0.00	8.15±0.02	12.74±0.75	579.07±20.93	107.44±0.19	45.45	5.39
	2	5.66±0.01	7.28±0.00	23.85±1.80	2000.00±0.00	142.88±1.46	83.86	14.00
	3	5.47±0.00	6.56±0.09	28.68±0.30	2533.34±1.33	180.25±0.77	88.33	14.05
	4	5.34±0.00	5.56±1.01	17.67±0.33	873.02±15.88	140.80±6.43	49.41	6.20
B. subtilis SMDFS 6B	1	5.31±0.00	7.25±0.19	5.28±0.50	ND	65.21±0.74	ND	ND
	2	5.12±0.00	2.36±0.94	9.99±1.45	29.07±0.03	97.19±0.79	2.91	0.3
	3	6.11±0.01	1.78±0.06	11.51±0.02	46.56±0.27	249.81±4.01	4.05	0.19
	4	6.12±0.00	1.26±0.14	15.12±0.56	589.16±10.85	128.86±1.01	38.96	4.57
B. tequilensis SMDFS 7B	1	5.55±0.01	9.75±0.12	12.52±0.05	115.25±0.42	158.52±3.23	9.21	0.73
	2	5.61±0.03	5.55±0.32	13.74±2.09	202.53±0.00	238.10±34.6	14.74	0.85
	3	5.72±0.00	5.11±0.23	28.30±2.70	273.53±2.34	156.03±1.12	9.66	1.75
	4	5.69±0.00	2.07±0.03	31.07±3.38	285.76±3.41	70.33±0.59	9.19	4.06
Blank		5.99±0.00	16.34±0.28	7.13±0.99	ND	23.29±0.27	ND	ND
B. tequilensis SMDFS 12B	1	6.10±0.00	12.44±0.49	17.26±0.45	157.55±5.17	107.44±8.59	9.12	1.47
	2	5.51±0.00	7.20±0.08	18.44±0.76	461.93±13.33	204.69±7.26	25.05	2.26
	3	5.86±0.00	5.81±0.04	15.51±1.71	259.46±0.00	87.44±0.21	16.73	2.97
	4	5.48±0.01	3.85±0.05	11.27±1.01	270.44±2.28	96.73±0.24	23.99	2.80
B. subtilis SMDFS 14B	1	5.93±0.00	11.44±0.17	11.80±0.21	400.00±0.00	107.42±8.19	33.90	3.72
	2	6.29±0.00	11.27±0.26	15.01±1.26	545.53±6.20	294.35±3.20	36.34	1.85
	3	6.63±0.00	11.23±0.08	16.33±1.80	548.74±9.41	162.88±75.7	33.56	3.37
	4	6.16±0.00	7.70±0.39	13.66±0.09	658.03±18.03	64.12±0.89	48.17	10.26
B. tequilensis SMDFS 16B	1	6.12±0.00	8.77±0.06	6.19±0.60	13.56±0.23	157.60±9.44	2.19	0.09
	2	6.07±0.01	8.19±0.16	11.94±1.03	82.41±0.22	235.70±12.8	6.90	0.35
	3	5.86±0.01	6.02±0.01	15.82±4.86	202.54±0.86	48.12±2.40	12.80	4.21
	4	5.14±0.01	4.71±0.16	9.96±0.34	231.89±1.12	31.52±1.45	23.28	7.36

ND = Not determined, MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL), Ratio: is the ratio of MCA/PA, specific activity: MCA (U/mL)/Total protein (mg/mL), SD: standard deviation, Mean: is average of three measurements, Different letters (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

4.2.2. Discussion

In this atudy several bacterial isolates (49) were screened for milk-clotting protease producuction on the skim-milk agar medium and report almost 29% of the strains (14 strains) were capable of producing the enzymes. The selected strains also differ in their effectiveness to produce enzymes evident from the diameter of the clearing zone on the medium ranging from 5.25 up to 21 mm. Rupali (2015) also reported a similar pattern of clearing zone formation of 11-29 mm by protease producing bacteria isolated from soil samples in India.

Based upon phenotypic and molecular methods, our strains were as *Bacillus tequilensis, Bacillus subtilis, Bacillus paramycoides*, and *Bacillus siamensis* of which strains from *Bacillus tequilensis* were dominant followed by, *B subtilis*. Many bacteria, especially species belonging to *Bacillus*, are well known to produce a variety of extracellular enzymes, with a wide range of industrial applications including rennet (milk-clotting enzymes) (Wim, 2013). Some of the promising strains for rennet production belong to *Bacillus spp*; *B.coagulans, B. stearothermophilus, B. lichenformis, B. cereus, B. circulans B. laterosporus, B. pumilis, B. brevis,* and *B. sphaericus* and *B. macerans* were collected from soil samples of India (Sidra *et al*., 2006) and *B. amyloliquefaciens* (Guleria *et al*., 2016), *B. licheniformis, B. subtilis* and *B. subtilis* natto, (Akcan, 2012; Ding *et al.*, 2011). In this study, the Ethiopian species were different from other milk-clotting protease producing bacteria except for *B subtilis* indicting that the presence of rich microbial diversity for various biotechnological applications.

Although large clearing on the casein medium is a very good indicator of protease production, it does not necessarily corroborate with the performance of the

microorganisms under fermentation conditions (Singh and Bajaj, 2015). Thus, several medium components, physiological factors, type of fermentation and operational parameters influence the metabolic biochemical behavior of the microbial strain and subsequent metabolite production pattern. For this reason, process optimization has always been an instrumental factor for industrial enzyme production.

The milk-clotting activity recorded in submerged fermentation was higher than solid-state fermentation. This implies submerged fermentation could be better than SSF for the production of the milk-clotting enzyme from bacteria. Shieh *et al*., (2009) also showed that milk-clotting and proteolytic activities of *B. subtilis* (natto) strains were more than twice effective in MCA/PA ratio under SmF than the ones extracted from SSF. However, *B. siamensis* SMDFS 29B induced better MCA/PA activity with a ratio of 0.4 compared to the 0.36 ratio of all other species under solid fermentation. Although it is established that bacteria and fungi show higher activities under SmF and SSF, respectively due to various advantages, it is not common to find higher enzyme activity by bacteria under SSF, and by fungi under SmF, provided that the cultures are agitated to enhance the level of oxygen (Ding *et al*., 2011; Yegin *et al*., 2011).

The secondary screening with SmF and SSF and the enzyme profiling of the bacterial strains gave a better insight on the effectiveness of the operational parameters and the performance of the bacterial strains in relation to milk-clotting, for milk-clotting enzymes are invariably accompanied by producing other proteases. Thus, both milk-clotting activity (MCA) and total proteolytic activity (PA) were recorded to determine the MCA per PA activity. Under the circumstances, the bacterial strains induced up to 480.77 U/mL and 2533 U/mL MCA during the secondary screening and partly

controlled enzyme profiling, respectively. This was comparable and even higher than those obtained from *B. subtilis* (120.31U/mL), *B. amyloliquefaciens* SP1 (160 SU/mL) and *B. subtilis* isolated from marine sponge (581.8 U/mL) in submerged fermentation (Dutt *et al*., 2008; Guleria *et al*., 2016; Hala *et al*., 2016). However, the MCA per PA index of 0.36 in the preliminary screening and the 14 fold increase during enzyme profiling was much lower than the ones reported by the same authors MCA/PA of 219-592.

The ratio of MCA/PA is a very good indicator for the selection of milk-clotting proteases from the overall protease enzyme production. However, it is desirable to have more milk-clotting activity than proteolysis for cheese production. Low MCA/PA implies the presence of other non-specific proteolytic enzymes that are active after milk coagulation that further degrade casein fractions, resulting in lower cheese yield and bitter flavor due to extensive non-specific peptide bond attack (Crabbe, 2004; Yegin *et al.*, 2010).

A maximum specific activity 88.33 U/mg recorded from *B. subtilis* SMDFS 2B was higher than 21 U/mg from *Mucor mucedo* DSM 809 (Yegin *et al*., 2012). The total protein concentration was increased while soluble carbohydrate decreased upon fermentation time. This implies that enzyme production was supported by glucose metabolism, which was almost exhausted at the time of maximum enzyme production (Yegin *et al*., 2012).

The effect of fermentation time also had a profound effect on the production of milk-clotting protease, and all strains, except *B. subtilis* SMDFS 2B, showed maximum activity upon 4 days of fermentation. This strain also showed the maximum milk-clotting to protease (MCA/PA) activity of 14 which was 2-3 times higher than the

other strains. The strains also worked within pH 5.5.-6.6 similar to different *Bacillus* sp. characteristics of acidic proteases used for cheese production (Guleria *et al*., 2016). Several studies showed that one-variable-at-a-time optimization of the process of enzyme production by bacteria based on the type of media, fortification with additional carbon and nitrogen sources, under specific pH and temperature could enhance milk-clotting enzymes and reduce excess production of proteases. These, together with enzyme purification, could narrow down MCA/PA ratio (Ding *et al*., 2012; Hang *et al*., 2016; Shieh *et al*., 2009; Zhang *et al*., 2013).

4.2.3. Conclusion

The present study revealed that submerged fermentation best suits the bacterial species for better milk-clotting activity. The *B. subtilis* SMDFS 2B best performed under submerged fermentation and partially controlled experiments. *B. siamensis* 29B was also an interesting strain performing best under SSF. These two strains have a potential for commercial production provided that they are tested under different optimal nutrient, environmental and operational conditions.

Chapter 5

5. Optimization of media components and growth conditions for production milk-clotting protease (MCP) from *Aspergillus oryzae* DRDFS13 under SSF

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Abstract

The objective of this study was to investigate the effects of several physicochemical and environmental factors on MCP production by *A.oryzae* DRDFS 13 in order to select the optimal conditions that ensure the best milk-clotting activity. The production and optimization of milk-clotting protease obtained from *Aspergillus oryzae* DRDFS13 under solid-state fermentation (SSF) using different agro-industrial wastes as solid substrates were studied. The agro-industrial wastes used included wheat bran, rice bran, pea bran, and grass pea bran. The chemical composition of the best solid substrate was tested using standard methods. Others cultivation parameters were studied and the results showed that the optimum fermentation medium composed of wheat bran, casein (1% w/w) and glucose (0.5% w/w), and the conditions for maximum milk-clotting protease production were at the moisture content of 55.0%, inoculum of $0.5*10^6$ spores/mL, incubation temperature of 30 ^oC, pH of 6.0 and fermentation time of 5 days. The highest milk-clotting activity was obtained from the crude enzyme extracted using 0.1 M NaCl and partial purification of the crude enzyme using chilled acetone and 80% $(NH_4)_2SO_4$ increased the ratio of MCA/PA from 0.56 to 1.30 and 0.65, respectively. Moreover, the highest MCA (137.58 U/mL) was obtained at casein concentration of 0.5%, pH, 4.0 and 25 ^0C,

using RSM. Thus, results from the present study showed that the optimization of milk-clotting protease production from *A. oryzae* DRDFS 13 under SSF by both one variable at a time and RSM significantly increased the milk-clotting activity.

Keywords: *Aspergillus oryzae*, Milk-clotting activity, Optimization, Milk-clotting protease Solid-state fermentation

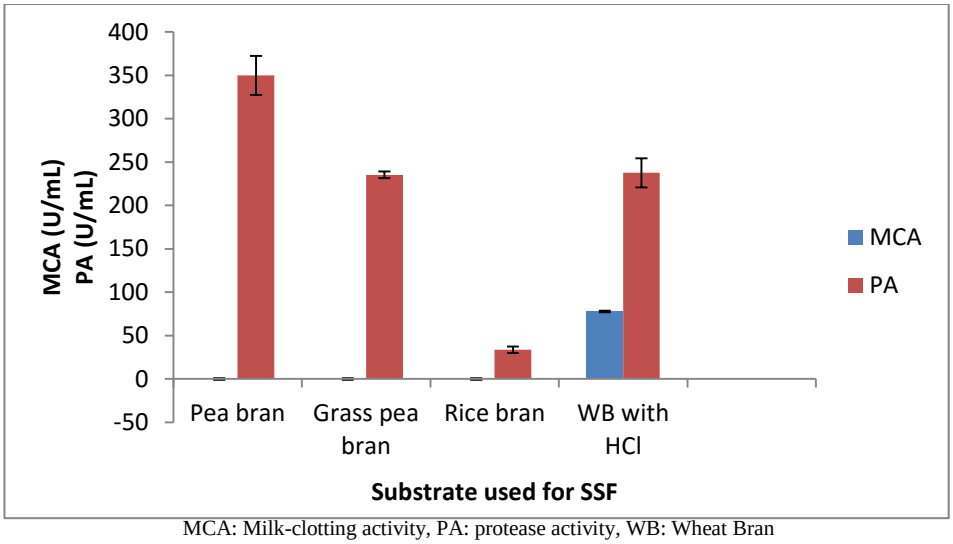
5.1.Results

5.1.1. Selection of solid substrate for production of milk-clotting protease (MCP) by *Aspergillus oryzae* DRDFS13

In this study, four agro-industrial substrates (wheat bran, rice bran, pea bran, and grass pea bran) were screened for the production of MCP from *A. oryzae* DRDFS13 under SSF. It was interesting to note that the fungus grew on all of the substrates (wheat bran, pea bran, grass pea bran, and rice bran) used in SSF, but did not show milk-clotting activity except in wheat bran. In the present study, a maximum milk-clotting activity of 77.74 U/mL was obtained from *A. oryzae* DRDFS13 using wheat bran moistened with HCl as a solid substrate (Fig. 5.1).

5.1.2. The effect of fermentation time on biomass and milk-clotting protease production by *Aspergillus oryzae* DRDFS13

The biomass of the organism (*A. oryzae* DRDFS13) was determined indirectly through releasing glucosamine (GlcN) by acid hydrolysis of chitin. The biomass of the filamentous fungi used in the present study increased gradually and reached the maximum at day 2 (19.77±8.20 mg GLcN/gdfs) and afterward start to decline slightly until the 6^{th} day of fermentation where the lowest biomass (10.96±0.71 mg GLcN/gdfs) was recorded (Table 5.1).



MCA: Milk-clotting activity, PA: protease activity, WB: Wheat Bran

Figure 5.1: Screening of various agro-industrial residues for MCP production by *A. oryzae* DRDFS13 in SSF

Table 5.1: Biomass, MCA, and PA of the crude enzyme from *A. oryzae* DRDFS13

Sample	Day	pH mean ±SD	Biomass in mg GlcN/gdfs	MCA(U/mL) mean ±SD	PA(U/mL) mean ±SD	Ratio MCA/PA
Blank	_	3.93±0.01^{a}	-	ND^{c}	0.00±0.00^{f}	ND
A. oryzae DRDFS13	0	3.91±0.01^{a}	10.15±0.11^{c}	ND^{c}	0.00±0.00^{f}	ND
	1	3.78±0.00^{a}	12.43±2.02^{c}	ND^{c}	0.00±0.00^{f}	ND
	2	3.90±0.09^{a}	19.77±8.20^{a}	ND^{c}	100.24±6.88^{e}	ND
	3	3.85±0.01^{a}	16.64±6.48^{b}	ND^{c}	129.07±1.64^{d}	ND
	4	3.90±0.02^{a}	11.43±0.18^{c}	63.76±6.62^{b}	196.39±3.12^{b}	0.32
	5	3.94±0.01^{a}	11.30±0.04^{c}	79.60±3.16^{a}	172.26±10.53^{c}	0.46
	6	4.02±0.20^{a}	10.96±0.71^{c}	70.20±1.20^{b}	231.24±1.01^{a}	0.30
Chymosin		-	-	2181.81±0.00	281.75±24.79	7.74

ND = Not determined (if milk did not clot within 40 min), MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL), Ratio: is the ratio of MCA/PA, SD: standard deviation, Mean: is average of three measurements, Different letters (a, b, c, d,e, f) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05).

5.1.3. The effect of incubation temperature on milk-clotting protease (MCP) production by *Aspergillus oryzae* DRDFS13

In this study, the solid-state fermentation carried out at 30 ^{o}C was the most suitable temperature for aspartic protease enzyme production with the milk-clotting activity of

71.99±3.21 U/mL. Whereas the highest protease activity was noticed at an incubation temperature of 35 ^{0}C (Fig 5.2).

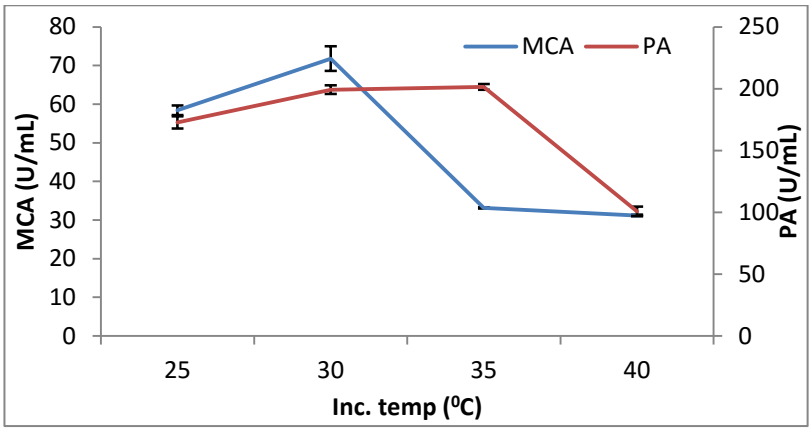
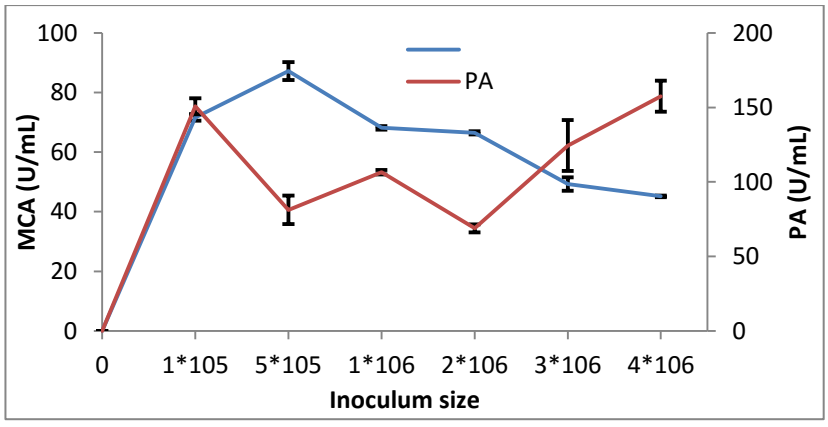


Figure 5.2: Effect of incubation temperature on MCP production by *A. oryzae* DRDFS13 under SSF

5.1.4. The effect of inoculum size on milk-clotting protease production by *Aspergillus oryzae* DRDFS13

The highest milk-clotting activity (87.22±35.12 U/mL) was recorded by the crude enzyme extract produced with an inoculum size of 5 mL (1*10^{5}) [2.27%] spores/mL. However, the highest protease activity was noticed at an inoculum size of 4 mL (1*10^{6} spores/mL) (Fig 5.3).

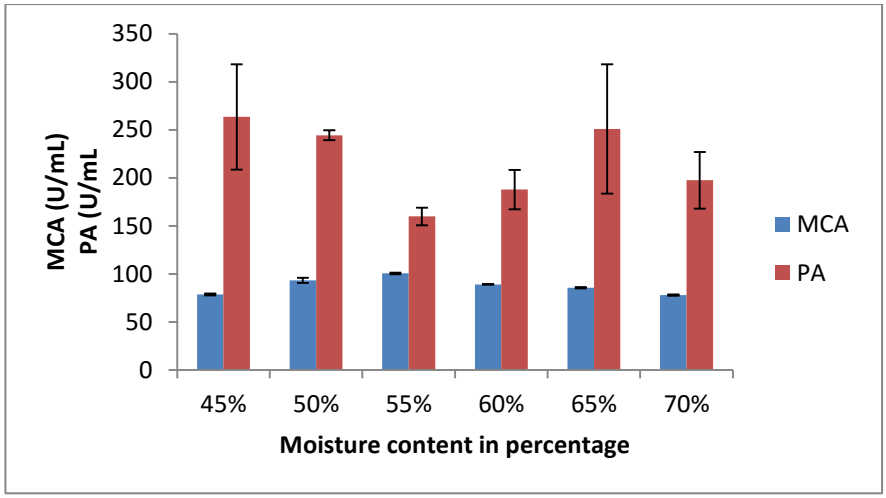


MCA: Milk-clotting activity, PA: protease activity

Figure 5.3: Effect of inoculum size on MCP production by *A.oryzae* DRDFS13 under SSF

5.1.5. The effect moisture content on milk-clotting protease production by *Aspergillus oryzae* DRDFS13

In the present study, the maximum milk-clotting activity of 100.43±0.84 U/mL was observed with 55% moisture content whereas significant milk-clotting activity was recorded at 45%-70% moisdture content (Fig 5.4).

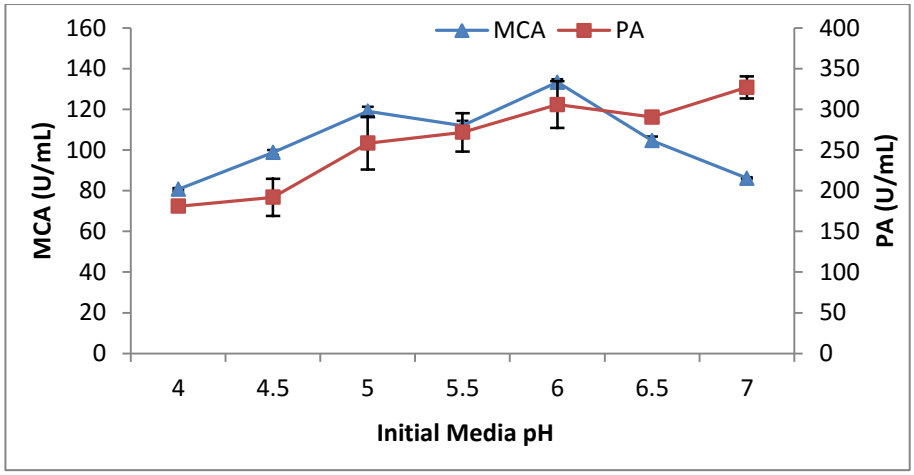


MCA: Milk-clotting activity, PA: protease activity

Figure 5.4: Effect of moisture content on MCP production by *A. oryzae* DRDFS13 in SSF

5.1.6. The effect initial media pH on milk-clotting protease production by *Aspergillus oryzae* DRDFS13

The effect of initial media pH on the production of MCP has been illustrated in Fig. 5.5. In the present study, the activity of milk-clotting protease gradually increased from pH 4.0 to pH 6 where the maximum milk-clotting activity of 133.35±1.48 U/mL was recorded at pH 6. Afterward, the enzyme activity was steadily decreased at all higher pH values than pH 6.

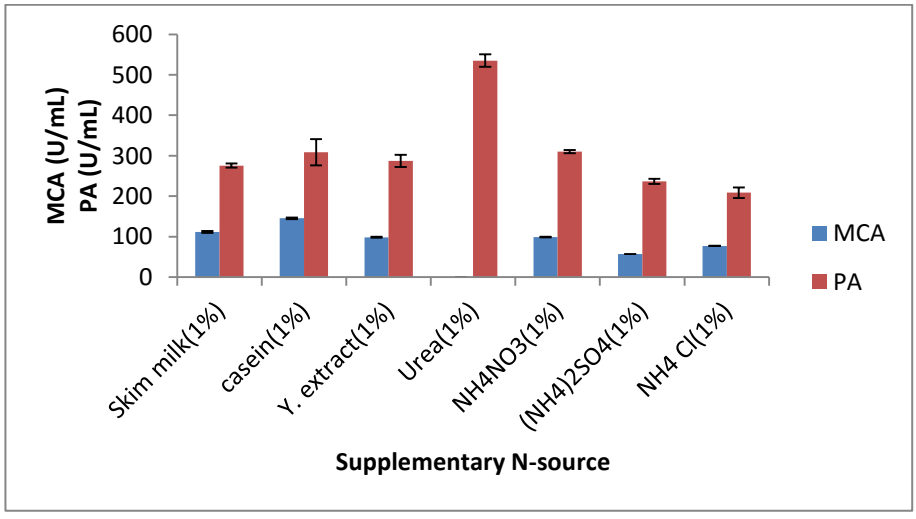


MCA: Milk-clotting activity, PA: protease activity

Figure 5.5: Effect of initial media pH on MCP production by *A. oryzae* DRDFS13 under SSF

5.1.7. The effect supplementary nitrogen source on milk clotting protease production by *Aspergillus oryzae* DRDFS13

Different nitrogen sources i.e., skim milk, casein, yeast extract, urea, NH_4NO_3, NH_4SO_4, and NH_4Cl were used in the SSF medium for the production of milk-clotting protease from *A. oryzae* DRDFS13. A maximum milk-clotting activity (145.48±1.76) was produced from *A. oryzae* DRDFS13 using casein as a supplementary N-source in SSF (Fig 5.6).

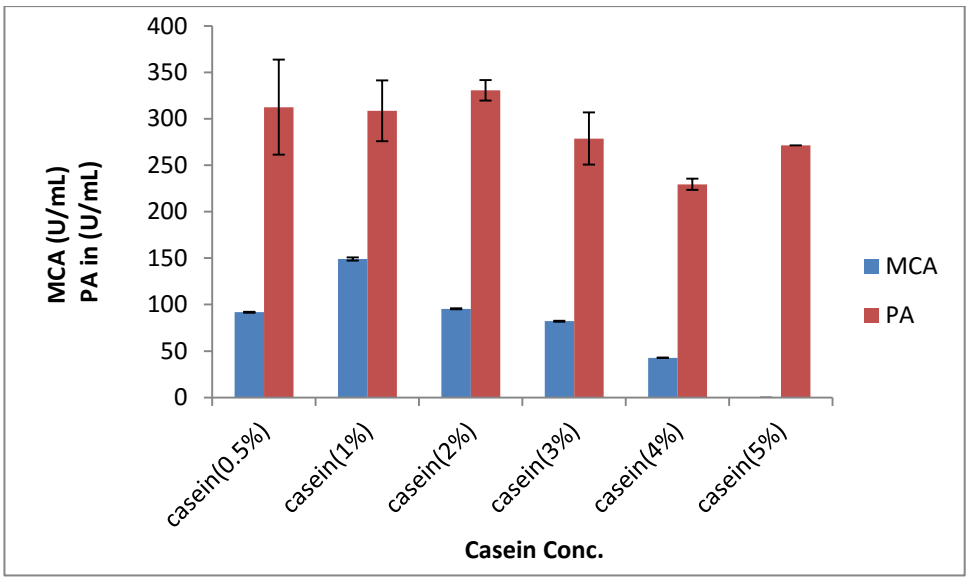


MCA: Milk-clotting activity, PA: protease activity

Figure 5.6: Effect of supplementary N-source on MCP production by *A. oryzae* DRDFS13

5.1.8. The effect of casein concentration on milk-clotting protease production by *Aspergillus oryzae* DRDFS13

Different quantity of casein (0.5%-5%) was added to media to determine the appropriate concentration used for milk-clotting protease production and maximum MCA (149.09 U/mL) was observed at 1% casein concentration (Fig. 5.7).

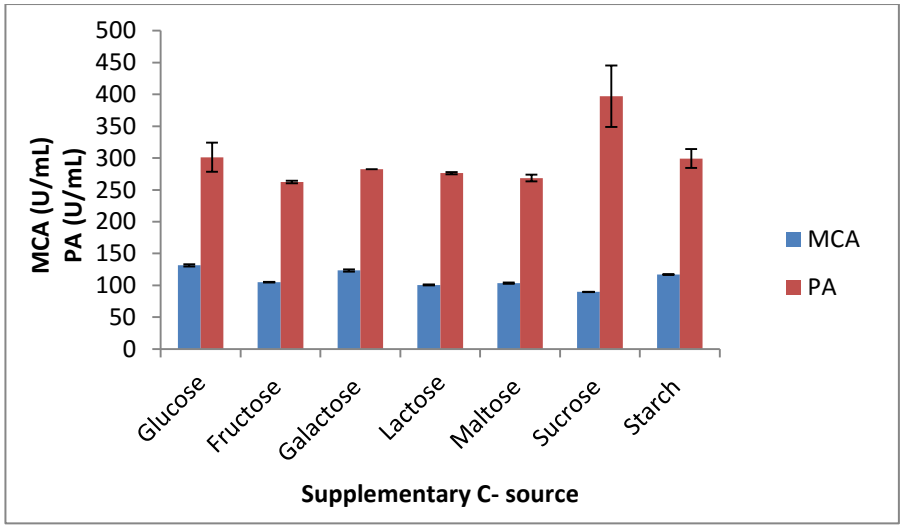


MCA: Milk-clotting activity, PA: protease activity

Figure 5.7: Effect of casein concentration on MCP production by *A. oryzae* DRDFS13 under SSF

5.1.9. The effect supplementary carbon source on milk-clotting protease production by *Aspergillus oryzae* DRDFS13

The effect of various additional carbon sources (glucose, fructose, galactose, maltose, lactose, fructose, and starch) on the production of MCP was studied under SSF. Supplementing the media with various carbohydrates sources enhanced the production of milk-clotting protease and the maximum milk-clotting activity (131.53 U/mL) was obtained using glucose (Fig 5. 8).



MCA: Milk-clotting activity, PA: protease activity

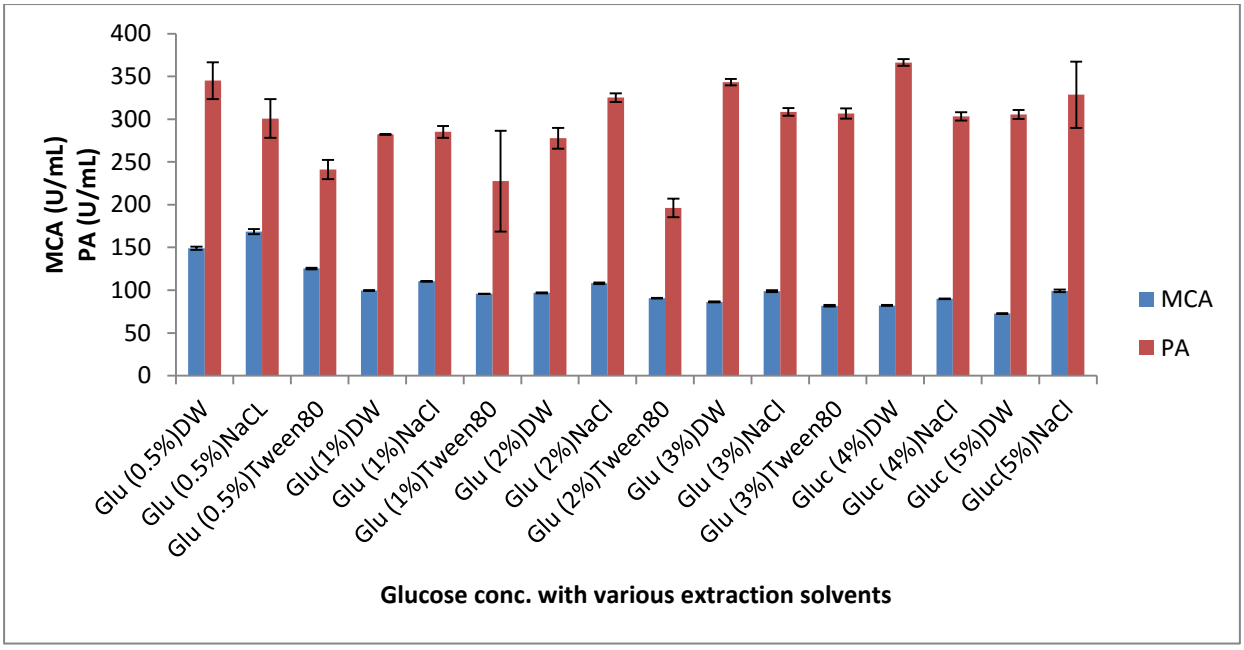
Figure 5.8: Effect of supplementary carbon source on MCP production by *A. oryzae* DRDFS13

5.1.10. The effect of glucose concentration on MCP production by *Aspergillus oryzae* DRDFS13

The effect of the best carbon-source concentration on the production of milk-clotting protease was studied by the addition of a different proportion of glucose (0.5%, 1%, 2%, 3%, 4%, and 5%) in the fermentation media. The highest milk-clotting activity (168.47±2.96 U/mL) and the ratio of MCA/PA (0.56) were obtained at 0.5% glucose concentration while the maximum protease activity (366.28 U/mL) was recorded at 4% glucose concentration (Figure 5.9).

5.1.11. The effect of extraction solvents on the activity of MCP produced by *A. oryzae* DRDFS13

Different extraction solvents (0.1M NaCl solution, 0.1% Tween 80 and distilled water) were used in this study (Figure 5.9). The highest milk-clotting activity (168.47 U/mL) was recorded from *A. oryzae* DRDFS13 using 0.1M NaCl while the MCA of 149.90 U/mL was recorded using distilled water as an extraction solvent for enzyme leaching in SSF.



MCA: Milk-clotting activity; PA: protease activity; DW: distilled water; NaCl: Sodium chloride; Glu: glucose

Figure 5.9: Effect glucose concentration and different extraction solvents on MCP production by *A. oryzae* DRDFS13

5.1.12. Partial purification of MCP from *A. oryzae* DRDFS13

Partial purification of the crude enzyme extract obtained from *A. oryzae* DRDFS13 using chilled acetone and 80% $(NH_4)_2SO_4$ was increased the ratio of MCA/PA while reducing the MCA and PA. The ratio of MCA/PA of the crude enzyme was increased from 0.56 to 0.65 and to 1.3 via 80% $(NH_4)_2SO_4$ and acetone, respectively (Table 5.2).

Table 5.2: The effect of acetone and ammonium sulfate precipitation on MCA/PA ratio of MCP from *A. oryzae* DRDFS13

Sample	Extraction solvent	Precipitating solvents	MCA (U/mL) Mean±SD	PA (U/mL) Mean±SD	Ratio (MCA/PA)
Crude enzyme (0.5% glucose)	0.1M NaCl	-	168.47±2.96^{a}	300.80±22.52^{a}	0.56
Crude enzyme (0.5% glucose)	0.1M NaCl	Acetone	84.96±1.06^{c}	65.28±3.07^{c}	1.30
Crude enzyme (0.5% glucose)	0.1M NaCl	80% $(NH_4)_2SO_4$	120.19±6.80^{b}	185.01±0.32^{b}	0.65
Chymosin	-	-	2533.33±188.56	99.76±5.36	25.39

MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL), Ratio: is the ratio of MCA/PA, SD: standard deviation, Mean: is average of three measurements, Different letters (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

5.1.13. Production of the milk-clotting enzyme (MCE) under SSF using Response Surface Methodology

Three factors, i.e., pH, casein concentrations and temperature were found to be the most significant factors affecting the production of MCP under SSF conditions, but their possible interactions were not evaluated. Thus, a statistical design, response surface methodology was employed here to study their possible interactions for its effect on milk-clotting enzyme production. Consequently, the data were fitted with the following regression equation which is an empirical relationship between the enzyme yields and test variables:

MCA (U/mL) = +323.85921-(14.75783* pH)-(4.90801 *Caesin conc.)-(4.99157 *Temp.)

The regression equation obtained from the ANOVA showed that the multiple correlation coefficient (R^2) was 0.8402. The "adjusted R^2" is 0.8102 and the predicted R^2 is 0.7161. The "adequate precision value" of the present model was 17.467. The model showed standard deviation, mean, and predicted R^2 values of 18.08, 66.97, and 0.7161, respectively (Table 5.3).

Table 5.3: ANOVA table for milk clotting activity in Response Surface Linear Model

Model terms	Value	Model terms	Value
Std. Dev.	18.08	R^2	0.8402
Mean	66.97	Adj R^2	0.8102
C.V. %	27.00	Pred R^2	0.7161
PRESS	9293.13	Adeq Precision	17.467
Model F-value	28.04	Lack of fit	4799.77

The results of the RSM experiments were presented in Table 5.4 as both predicted and experimental values. The highest MCA (137.58 U/mL) from *Aspergillus oryzae* DRDFS13 was recorded at a casein concentration of 0.5%, pH 4.0 and 25 ^{0}C.

Table 5.4: Experimental design used in the RSM studies of three independent variables with 3 center points for MCP production by *A. oryzae* DRDFS13 under SSF

Run order	A pH	B Casein conc.	C Temp.	MCA (U/mL) Actual value	MCA (U/mL) Predicted value
1.	4.00	0.50	25.00	134.85	137.58
2.	7.00	0.50	25.00	107.99	93.31
3.	4.00	5.00	25.00	131.18	115.50
4.	7.00	5.00	25.00	79.34	71.23
5.	4.00	0.50	40.00	83.05	62.72
6.	7.00	0.50	40.00	0.00	18.44
7.	4.00	5.00	40.00	15.95	40.62
8.	7.00	5.00	40.00	14.55	-3.65
9.	2.98	2.75	32.50	132.24	104.20
10.	8.02	2.75	32.50	49.49	29.74
11.	5.5	-1.03	32.50	69.78	85.54
12.	5.5	6.53	32.50	30.57	48.40
13.	5.5	2.75	19.89	101.95	129.93
14.	5.5	2.75	45.11	0.00	4.01
15.	5.5	2.75	32.5	56.65	66.97
16.	5.5	2.75	32.5	72.89	66.97
17.	5.5	2.75	32.5	59.54	66.97
18.	5.5	2.75	32.5	56.94	66.97
19.	5.5	2.75	32.5	79.18	66.97
20.	5.5	2.75	32.5	63.22	66.97

MCA: Milk-clotting activity (U/mL), casein conc.: casein concentration (%); Temp.: temperature in ^{0}C

The probability plot showed a satisfactory correlation between the experimental and predictive values (Fig. 5.10). In order to determine the optimal levels of each factor for maximum milk-clotting protease production, three-dimensional response surface plots were constructed. Figure 5.11 shows the response for the interactive factors; casein and pH when the temperature was at 32.5. The maximum milk-clotting activity in this condition was predicted to be 132.24 U/mL. The production of milk-clotting protease production varied considerably over the range tested from 30.57-132.24 U/mL. Figure 5.12. indicates the response for the interactive factors; casein and temperature when the pH was 5.5. The maximum milk-clotting activity in this condition was predicted to be 101.95 U/mL. The production of milk-clotting protease production varied considerably over the range tested from 0.00-101.95 U/mL.

Figure 5.13. shows the response for the interactive factors; pH and temperature when the casein concentration was 2.75. The maximum milk-clotting activity in this condition was predicted to be 45.11 U/mL. The production of milk-clotting protease production varied considerably over the range tested from 32.5-101.95 U/mL.

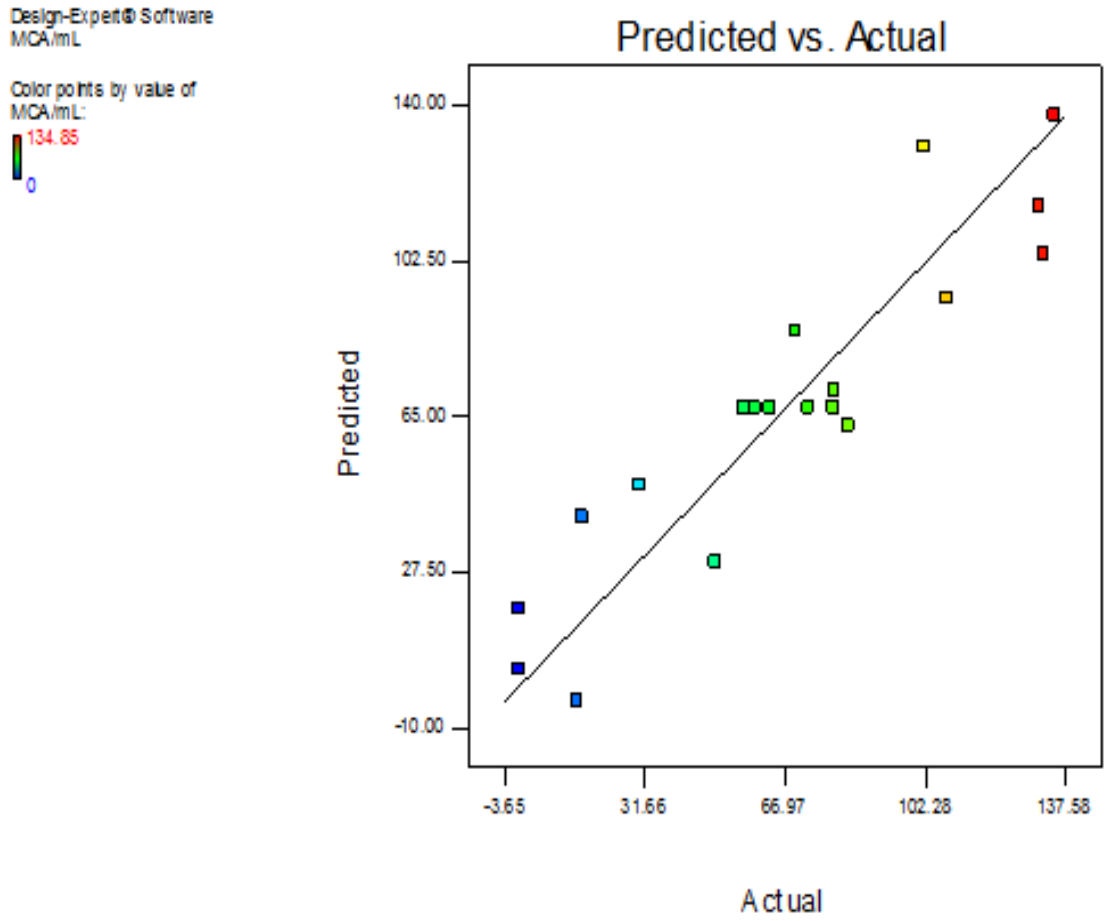


Figure 5.10: Comparative plot showing predicted vs. actual values of milk-clotting protease from *Aspergillus oryzae* DRDFS13

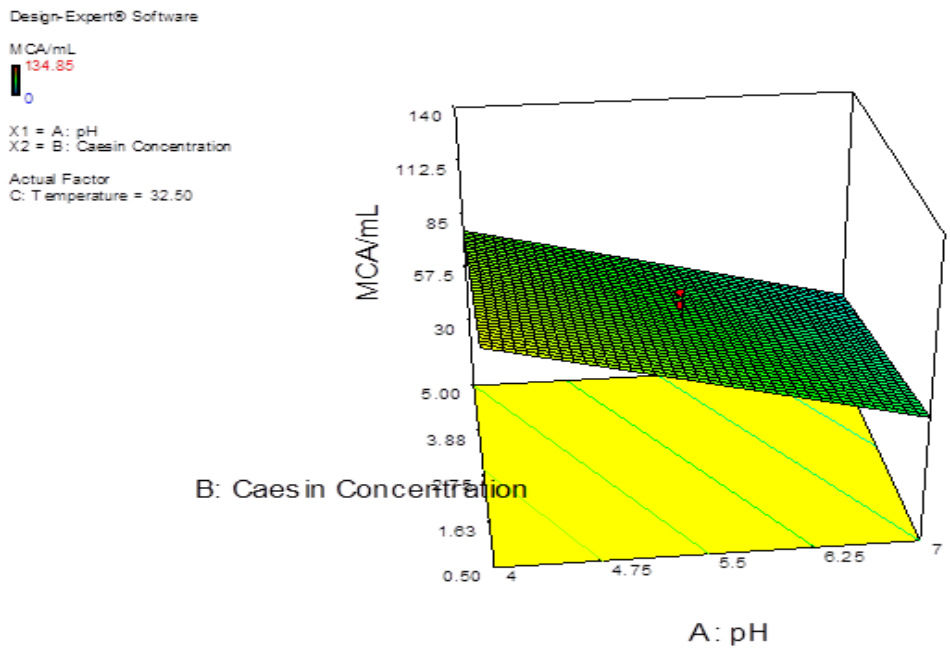


Figure 5.11: Response surface curves of milk-clotting protease production from *Aspergillus oryzae* DRDFS 13 showing the interaction between casein and pH

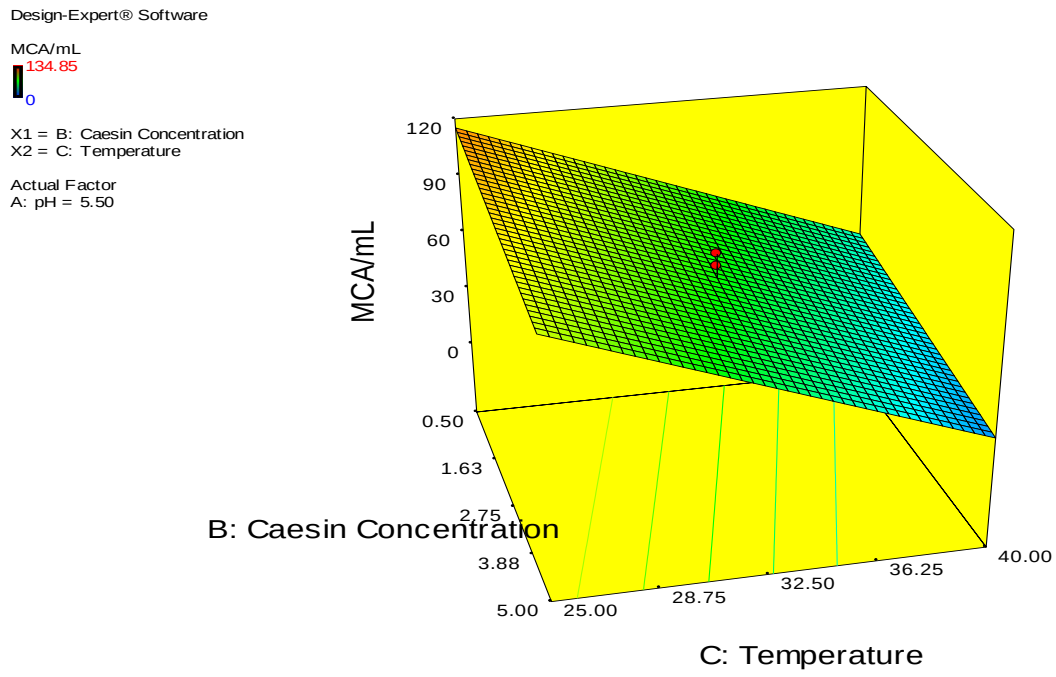


Figure 5.12: Response surface curves of milk-clotting protease production from *Aspergillus oryzae* DRDFS 13 showing the interaction between casein and Temperature

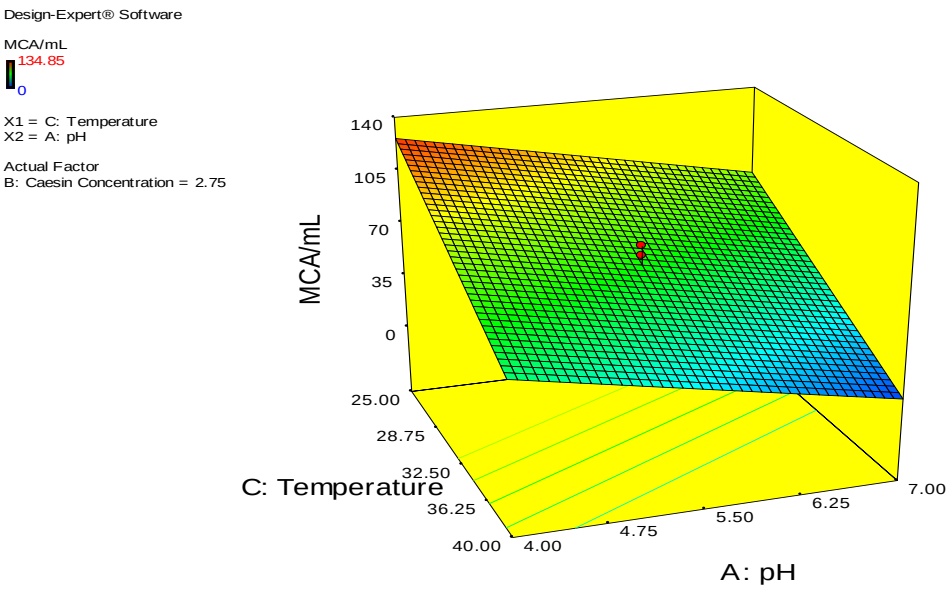


Figure 5.13: Response surface curves of milk-clotting protease production from *Aspergillus oryzae* DRDFS 13 showing the interaction between temperature and pH

5.1.14. Inhibition study

Incubation of crude enzyme with pepstatin-A completely inhibited the milk-clotting activity of the enzyme. On the other hand, more than 68% of residual MCA was recorded using Iodoacetamide, EDTA, and PMSF as protease inhibitors (Table 5.5).

Table 5.5: Inhibition study for the crude enzyme from *A. oryzae* DRDFS13

Sample	Fermentation Time (days)	MCA(U/mL) Mean±SD	Inhibition Study Residual MCA (%)			
			Pep A (1mM)	I.A (10mM)	EDTA (10mM)	PMSF (10mM)
Aspergillus oryzae DRDFS13	0	ND^{c}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
	1	ND^{c}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
	2	ND^{c}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
	3	ND^{c}	ND^{d}	ND^{d}	ND^{d}	ND^{d}
	4	63.76±6.62^{b}	0.00^{d}	97.14^{c}	94.56^{c}	95.78^{c}
	5	174.61±3.18^{a}	0.00^{d}	72.94^{b}	69.82^{b}	76.22^{b}
	6	170.22±1.21a	0.00^{d}	84.19^{ab}	89.52^{a}	74.80^{b}

ND: Not determined, MCA: Milk-clotting activity (U/mL), Pep A: Pepstatin A, I.A: Iodoacetamide, EDTA: ethylenediaminetetraacetic acid, PMSF: phenyl-methane sulphonyl fluoride, SD: standard deviation, Mean: is average of three measurements, different letters (a, b, c, for MCA (U/mL) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$), different letters (a, b, c,d for PepA, I.A, EDTA, and PMSF) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

Table 5.6: Comparison of MCP produced from *Aspergillus oryzae* DRDFS13 with other fungi

Organism	**Ferm**	**Substrate**	**Temp (^{0}C)**	**Media PH**	**Moist Cont. (%)**	**Inc. Time (h)**	**Inoculum**	**MCA**		**PA**	**Ratio**	References
								U/mL	**U/g**	**U/mL**	**MCA/PA**	
Aspergillus oryzae DRDFS13	SSF	Wheat bran moistened with HCl (200mM) supplemented with glucose and casein	30	6.0	55	120	$5*10^5$sp/ mL	168.47	1684.70	345.00	0.49	This study
Aspergillus niger FFB1	SSF	Wheatbran, Czapeck dox and mineral solution	30	4.0	39.2	72	10^6 sp/mL	166.00	830.00	195.29	4.25	(Bensmail *et al*., 2015)
Aspergillus oryzae	SSF	Wheat bran and rice bran with mineral solution	30	7.5	1:1 (50)	120	10^8 Sp/mL	119.43	1194.30	-	_	(Patil *et al.*, 2012)
Aspergillus flavo furcates DPUA 1461	SmF	Acaia waste, rice bran, and mineral solution	30	7.0	_	72	10^5 sp/mL	116.19	_	_	2.86	(Alecrim *et al*., 2015a)
Aspergillus spp.	SSF	Wheat Rawa with fructose & chickpea meal moistened with mineral soln	32	5.0	60	120	10%	26.00	130.00	_	_	(Radha *et al*., 2012)
Rhizopus stolonifer	SSF	Wheat bran with galactose and peptone moistened by salt solution	28	6.0	50-70	96	$3.6*10^6$ Sp/mL	120	1200	_	_	(Gais *et al*., 2009)
Aspergillus oryzae HG76	SSF	Wheat bran, flour, and water	31	-	57	86	$8.59*10^5$ sp/g	438.25	1753.0	_	_	(Li *et al*., 2014)
Mucor spp.	SSF	Wheat bran moistened with HCl (200 mM)	24	-	120	144	$5*10^5$ Sp/gm	710.00	7100.00	_	_	(Fernandz-Lahore *et al*., 1999)

5.2. Discussion

In this study, four agro-industrial substrates (wheat bran, rice bran, pea bran, and grass pea bran) were screened for the production of MCP from *A. oryzae* DRDFS13 under SSF. Although the fungus grew on all substrates (wheat bran, pea bran, grass pea, and rice bran), it did not show milk-clotting activity, except on wheat bran with milk-clotting activity (MCA) of 77.74 U/mL. Although the particle size of each substrate was not determined, the difference may be due to differences in particle size between wheat bran and the other three substrates which is partly related to porosity (Janser *et al*., 2014). Wheat bran is considered as the best substrate for production of acid protease from *A. oryzae* MTCC 5341 (Vishwanatha *et al*., 2010), other *Aspergillus* species (Bensmail *et al*., 2015; Pallavi *et al*., 2012) and *Mucor sp*. (Khademi *et al*., 2013a).

The growth and milk-clotting protease production in SSF was studied for 6 days under previously established optimal conditions. The fungus showed a steady growth starting from 10.15 mg GLcN/gdfs to 19.77±8.20 mg GLcN/gdfs implying a two-fold increase in biomass after two days of incubation. Similarly, the highest biomass levels of *M.mucedo* DSM 809 increase in biomass after two days of incubation. Similarly, the highest biomass levels of *M. mucedo* DSM 809 was achieved between 2-3 days (Yegin *et al*., 2012). This could be interpreted microbe undergo lag phase before active growth and start to decline in biomass when the nutrients depleted. Biomass production was supported by carbohydrates consumption, which was almost exhausted at the time of maximum enzyme production.

The milk-clotting activity was detected at 4^{th} day of incubation and the maximum value of MCA (79.6 U/mL) was recorded at a 120 h incubation period. Whereas, the

protease activity was noticed from the second day (100.24 U/mL) and the highest PA (231.24 U/mL) was recorded on the 6^{th} day of the incubation period. This might be due to the gene encoding aspartic protease enzyme that could be induced later than other types of protease. The maximum MCA recorded from *M. circinelloides, A. oryzae* MTCC and *Aspergillus sp*. on the 5^{th} day of fermentation time under SSF was comparable with the present study (Radha *et al*., 2012; Sathya *et al*., 2009; Vishwanatha *et al*., 2010). But, further incubation showed a reduction in enzyme production. The reduction in enzyme yield after the optimum period was probably due to the depletion of nutrients available to microbial growth (Sathya *et al.*, 2009). On the other hand, the highest milk-clotting activity obtained from *A. niger* FFB1 on the 3^{rd} day (Bensmail *et al*., 2015) and maximum acid protease activity noticed from *A. oryzae* HG76 at 80 h incubation time (Li *et al*., 2014) different from the present study.

The study indicated that the MCA activity started to steadily increase to 60-70 U/mL between 25 ^{0}C- 30 ^{o}C, and abruptly declined to 30-35 U/mL at 35-40 ^{0}C. Other studies also showed slight variations in the temperature optima for the production of protease enzymes by *Aspergillus sp.* from above 30 ^{o}C (32 ^{o}C) (Radha *et al*., 2012) and a decline at 35 ^{o}C from *A. oryzae* MTCC 5341 (Vishwanatha *et al*., 2010). Although the enzymes showed slight variation, no significant difference, in their temperature optimal activities, they were within the optimum temperature of 30 ^{o}C for the production of these enzymes with solid-state fermentation by other fungi.

The highest milk-clotting activity was recorded from the crude enzyme extract produced by *A. oryzae* DRDFS13 using an inoculum size of $0.5*10^{6}$ spores/mL followed by $0.1*10^{6}$ spores/mL. This was comparable to the highest enzyme activity

displayed by *A. niger* under SSF using an inoculum size of $1*10^6$ spores/mL (Bensmail *et al*., 2015). The acid protease activity observed from *A. oryzae* HG76 at an inoculum concentration of $0.75*10^6$ mL^{-1} under SSF was also similar to the present study (Li *et al*., 2014). As the level of inoculum size increased to $4*10^6$ spores/mL, the milk-clotting activity of the protease was decreased to lower activity (45.16 U/mL). Similarly, a milk-clotting activity of the protease lowered when an inoculum concentration of $4.5*10^6$ spores/mL was used in SSF (Bensmail *et al*., 2015). The decrease in MCA that has been observed with higher inoculum size could be due to the shortage of the nutrients available for the larger biomass and faster growth of the culture. Hence, a balance between the proliferating biomass and available material is vital for maximum enzyme production (Sathya *et al*., 2009).

A higher moisture content was reported to cause a decrease in porosity of the substrate, the loss of particle structure, the development of rigidity which causes the decrease in gas exchange (transfer of O_2 and CO_2) and increased development of aerial mycelium (Bensmail *et al*., 2015; Sathya *et al*., 2009). In the present study, the maximum milk-clotting activity of 100.43±0.84 U/mL was observed with 55% moisture content. Different studies showed that different SSF systems gave optimum protease activities at various moisture contents ranging from 40%-60% with various fungi *A. oryzae* HG76, *Rhizopus stolonifer*, *Rhizomucor miehei*, and *A. niger* FFB1 (Aljammas *et al*., 2018; Bensmail *et al*., 2015; Gais *et al*., 2009; Li *et al*., 2014). Sathya *et al*., (2009) reported maximum enzyme production from *M. circinelloides* at 20% moisture content in SSF. This may be related to the different types and porosity of substrates used for SSF.

The pH of the medium strongly affects many enzyme processes and transport of various compounds across the cell membrane. Thus, the effect of initial media pH on the production of MCP was determined. The MCA gradually increased from pH 4.0 to pH 6 where the maximum milk-clotting activity was recorded at pH 6. This could be due to Prolyl endopeptidase from *A. oryzae* was also produced at acidic medium pH (Eugster *et al*., 2015). Similarly, a maximum MCA from *A. oryzae* MTCC 5341 and *Rhizopus stolonifer* were recorded at pH 6.3 and pH 6, respectively (Gais *et al*., 2009; Vishwanatha *et al*., 2010). Other studies indicated optimum MCA production by *A. oryzae* and *Mucor circinelloides* at pH 7.0 (Pallevi *et al*., 2012; Sathya *et al*., 2009). The protease production by *A. niger* FFB1 and *A. oryzae* MTCC 5341 and other *Aspergillus sp.* was maximum between pH 4–5 (Bensmail *et al*., 2015; Radha *et al*., 2012; Vishwanatha *et al*., 2010) indicating the pH optima difference between acidophilic and neutrophilic fungi irrespective of their taxa.

Different nitrogen sources i.e., skim milk, casein, yeast extract, urea, NH_4NO_3, NH_4SO_4, and NH_4Cl were used in the SSF medium for the production of milk-clotting protease from *A. oryzae* DRDFS13. Supplementation of casein as a nitrogen source resulted in an increase in enzyme production, which was comparable to the highest MCA acquired from *A. oryzae* MTCC 5341 (Vishwanatha *et al.*, 2010) and *Rhizomucor nainitalensis* (Khademi *et al*., 2013b) on the same substrate. Although the effect of specific nitrogen supplement on milk-clotting enzyme production differs from organism to organism, the complex nitrogen sources such as casein and peptone are usually used for milk-clotting enzyme production (Pallavi *et al*., 2012). The maximum MCA was observed at 1% casein concentration. Similar to the present study, the highest MCA was recorded from *Rhizopus stolonifer* (Gais *et al*., 2009) and *Rhizomucor nainitalensis* (Khademi *et al*., 2013b) at 1% and 1.5% casein

concentration, respectively. Similarly, the addition of 2 g of casein into the solid substrate showed a significant increase in the production of microbial rennet from *M. miehei* (Araújo *et al*., 2015).

Among the various carbon sources tested, glucose at 0.5% was found to be the best source for milk-clotting enzyme production followed by galactose and starch. Although it is argued that simplest carbon sources like glucose in the media enhance protease production by *A. oryzae* (Dhurway *et al*., 2012), other studies also showed supplementation with lactose (Gais *et al*., 2009) led to maximum milk-clotting activity in the SSF media.

SSF is fermentation in the absence of free liquid, and recovery of the fermentation product requires its extraction from the solid fermented medium. The extraction efficiency is critical to the recovery of the enzyme from the fermented biomass; hence the selection of a suitable solvent is necessary (Ahmed *et al*., 2008). The highest MCA was recorded using 0.1M NaCl followed by distilled water as an extraction solvent for enzyme leaching in SSF. The acid protease enzyme extracted from *A. oryzae* MTCC 5341 using 0.1M NaCl solution that showed significant MCA is comparable with the present study (Vishwanatha *et al*., 2010). However, on the economic feasibility of fermentation, distilled water was chosen as a suitable solvent for further extraction of crude protease from fermented solids (Radha *et al*., 2012).

Partial purification of the crude enzyme extract obtained from *A. oryzae* DRDFS13 using chilled acetone and 80% $(NH_4)_2SO_4$ increased the ratio of MCA/PA. The ratio of MCA/PA of the crude enzyme increased by 2.3 fold (from 0.56 to 1.3) by acetone and by 1.2 fold (from 0.56 to 0.65) via 80% $(NH_4)_2SO_4$. Similarly, partial purification of protease from *A. oryzae* MTCC 5341, *A. terreus* and *P. aeruginosa* using chilled

acetone increased the purification by 5-fold, 2.6 fold, and 1.6 fold respectively (Niyonzima and More, 2015; Palpperumal *et al*., 2016; Vishwanatha *et al*., 2009).

The optimization of milk-clotting protease from *A. oryzae* DRDFS 13 was also conducted using RSM. The regression equation obtained from the ANOVA showed that the multiple correlation coefficient (R^2) was 0.8402 (a value >0.75 indicates the fitness of the model) (Dutt *et al*., 2009). The "adjusted R^2" is 0.8102 and predicted R^2 is 0.7161, indicating that the model is good as for a good statistical model, the R^2 should be in the range of 0–1.0, and the nearer to 1.0 the value is the more fit the model is deemed to be (Dutt *et al*., 2009). The "adequate precision value" of the present model was 17.467, suggesting that the model can be used to navigate the design space. The "precision value" is an index of the signal-to-noise ratio, and values of higher than 4 are prerequisites for a model to be a good fit (Dutt *et al*., 2009). The model showed standard deviation, mean, and predicted R^2 values of 18.08, 66.97, and 0.7161, respectively.

The optimization of the physicochemical and nutritional factors for the production of the MCE by RSM increased the MCA by ≈ 2.0-fold. Similar to the present study, Soares *et al*., (2010) reported the optimization of protease production from *Paecilomyces marquandii* in Central Composite Design (CCD) increased the enzyme activity by 2.57 fold. In the same way, the MCA of MCE from *B. subtilis* B1 was improved by 5.57-fold after optimization by response surface methodology (Ding *et al*., 2011). In another study, optimizing the production of MCE enzyme from *Mucor mucedo* KP736529 by the CCD led to a 6.12 fold increase in MCA compared with initial activity (Ayana *et al*., 2015). According to Leng and Xu (2011), the activity of

acid protease from a mixed culture of *A. oryzae* AS3042 and *A. niger* SL-09 was increased by 5-fold using response surface methodology (RSM) optimization.

The inhibition study of protease enzyme is very critical for determining the types of protease dominantly found in the crude enzyme extract. Therefore, incubation of the crude enzyme with pepstatin-A completely inhibited the milk-clotting activity of the enzyme indicating that the presence of aspatic protease in the crude enzyme extract. Similar results were reported by (Hsiao *et al*., 2014b; Merheb-Dini *et al*., 2010; Rodrigues *et al*., 2017; Souza *et al*., 2017; Yin *et al*., 2013).

In general, the MCA of the protease enzyme produced from *A. oryzae* DRDFS 13 in the present study was comparable with the MCA of protease reported by Bensmail *et al*., (2015). However, the MCA recorded for MCP in this study was higher than the MCA of proteases produced from *A. oryzae* (Pallavi *et al*., 2012), *A. flavo furcates* DPUA 1461(Alecrim *et al*., 2015), *Aspergillus spp* (Radha *et al*., 2012) and *Rhizopus stolonifer* (Gais *et al*., 2009). On the other hand, the activity of MCP from *A. oryzae* DRDFS 13 was lower than the milk-clotting activity of proteases produced from *Aspergillus oryzae* HG76 (Li *et al*., 2014) and *Mucor spp*. (Fernandz-Lahore *et al*., 1999). The variation recorded in MCA could be due to the difference of the substrate combination, mineral supplements, effectiveness of the fungus species, incubation time and/or moisture content used in SSF.

5.3. Conclusions

Optimization of the milk-clotting protease enzyme production form *A. oryzae* DRDFS 13 by both one-variable-at-a time and Response Surface Methodology (RSM) was significantly improved the milk-clotting activity by two-fold. However, a better milk-clotting activity was recorded from a one-variable-at-a time approach than RSM.

Chapter 6

6. Purification and Characterization Aspartic protease Enzyme from Fungi and Bacteria

6.1. Purification and Characterization of Aspartic Protease Produced from *Aspergillus oryzae* DRDFS13 under Solid-State Fermentation

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Abstract

Aspartic proteases (E.C.3.4.23.) are endopeptidases with molecular masses ranging between 30–45 kDa. They depend on aspartic acid residues for their catalytic activity and show maximal activity at low pH. Thus the main objective of the present study was to purify and characterize aspartic protease from locally identified fungi by solid-state fermentation. The aspartic protease in the current study was obtained from *A. oryzae* DRDFS13 under SSF. The crude enzyme extract was purified by size-exclusion (SEC) and ion-exchange (IEC) chromatography. The protein contents of crude enzyme and IEC fractions were determined by BCA methods while the presence of N-glycosylation was checked using Endo-H. Inhibition studies were conducted using protease inhibitors. The milk-clotting activity (MCA), protease activity (PA); molecular weight and enzyme kinetics were determined using standard methods. Optimum temperature and stability, optimum pH and stability, and the effect of cations on MCA were assessed using standard methods. The maximum

MCA (477.11 U/mL) was recorded from IEC fraction A_8. The highest specific activity (183.50 U/mg), purification fold (6.20) and yield (9.2%) were also obtained from the same fraction (IEC A_8). The molecular weight of 40 kDa was assigned for the purified enzyme (IEC A_8). However, its molecular weight was decreased to 30 KDa upon deglycosylation assay which infers that the protein is glycosylated. Incubation of the pure enzyme (IEC A_8) with pepstatin A caused a 94 % inhibition on MCA. The dialyzed enzyme showed a Km and Vmax values of 17.50 mM and 1369 U, respectively. The enzyme showed maximum MCA at 60 ^{o}C and pH 5.0 with stability at pH 4.5-6.5 and temperature 35-45 ^{o}C. Most cat-ions stimulate the activity of the enzyme; moreover, the highest MCA was detected at 50 mM of $MnSO_4$. Furthermore, the results obtained in the present study confirmed that the aspartic protease enzyme produced from *A. oryzae* DRDFS13 and purified in ion-exchange chromatography could be used as a substitute source of rennet enzyme for cheese production.

Keywords: Aspartic protease, *Aspergillus oryzae*, Ion-exchange chromatography, Milk-clotting activity, Purification

6.1.1. Results

6.1.1.1. Cultural profile used for enzyme production

Figure 6.1.1 shows the cultivation profile of aspartic protease produced by *Aspergillus oryzae* DRDFS13 under SSF for 6 days using WB media. The fungus was able to grow in stationary conditions and secrete the enzyme to the culture media starting from the 4th fermentation day with the highest milk-clotting activity (175 U/mL) and protease activity (231 U/mL) recorded on the 5th and 6th days of fermentation time, respectively. It can also be seen that after the 5th fermentation day, the milk-clotting

activity decreased slightly. Since, the highest ratio for MCA/PA was detected on the 5th fermentation day, the crude enzyme extract from the 5th fermentation day was used for enzyme purification.

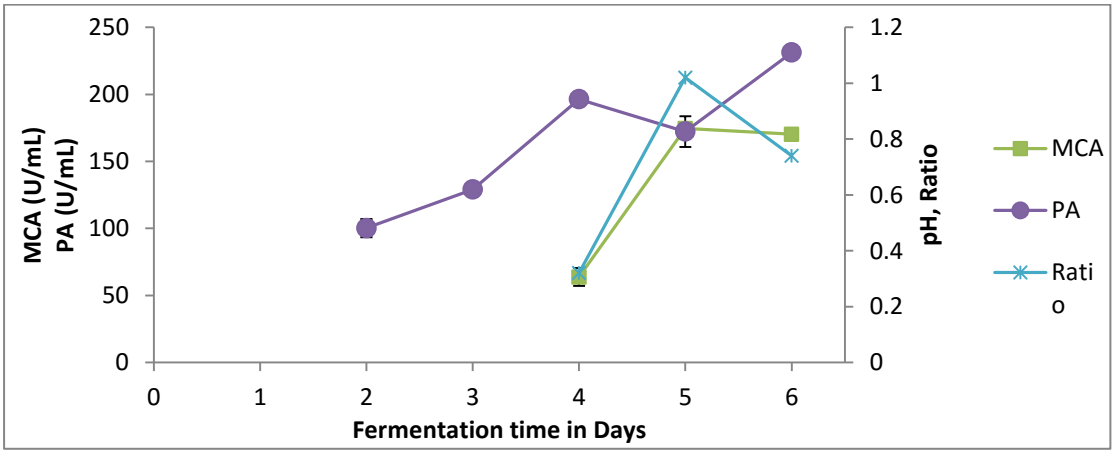

Figure 6.1.1: pH, MCA, PA and ratio (MCA/PA) of the crude enzyme extract from *A. oryzae* DRDFS13

6.1.1.2. Recovery and purification of aspartic protease

The crude enzyme extract from *A. oryzae* DRDFS13 exhibiting a milk-clotting activity (174.61 U/mL) was separated by chromatography using DEAE-sepharose column (IEC) and sepharose 12 pre-packed column (SEC) and eluted with stepwise NaCl gradient. The chromatographic profile of IEC is shown in fig. 6.1.2. There are several major protein peaks; however, the prominent peak observed at about 155 min and 310 retention volumes were shown significant milk-clotting activity. Further fractionation of the IEC were shown fractions A_{7-10} contained the target enzyme activity (Fig 6.1.3.) whereas the highest MCA (477.11 U/mL) was obtained from the active fraction A_8. After IEC, active fraction A_8 was purified 6.2-fold from a crude enzyme, with a final yield of 9.2% and an observed specific activity of 183.5 U/mg. The MCA/PA of active fraction A_8 was also increased by 3.3 fold. However, significant MCA were also detected from SEC (186.86 U/mL), IEC A_7 (283.01

U/mL), $A_{9,}$ (73.89 U/mL) and A_{10} (9.38 U/mL) (Fig. 6.1.3 and Table 6.1.1).

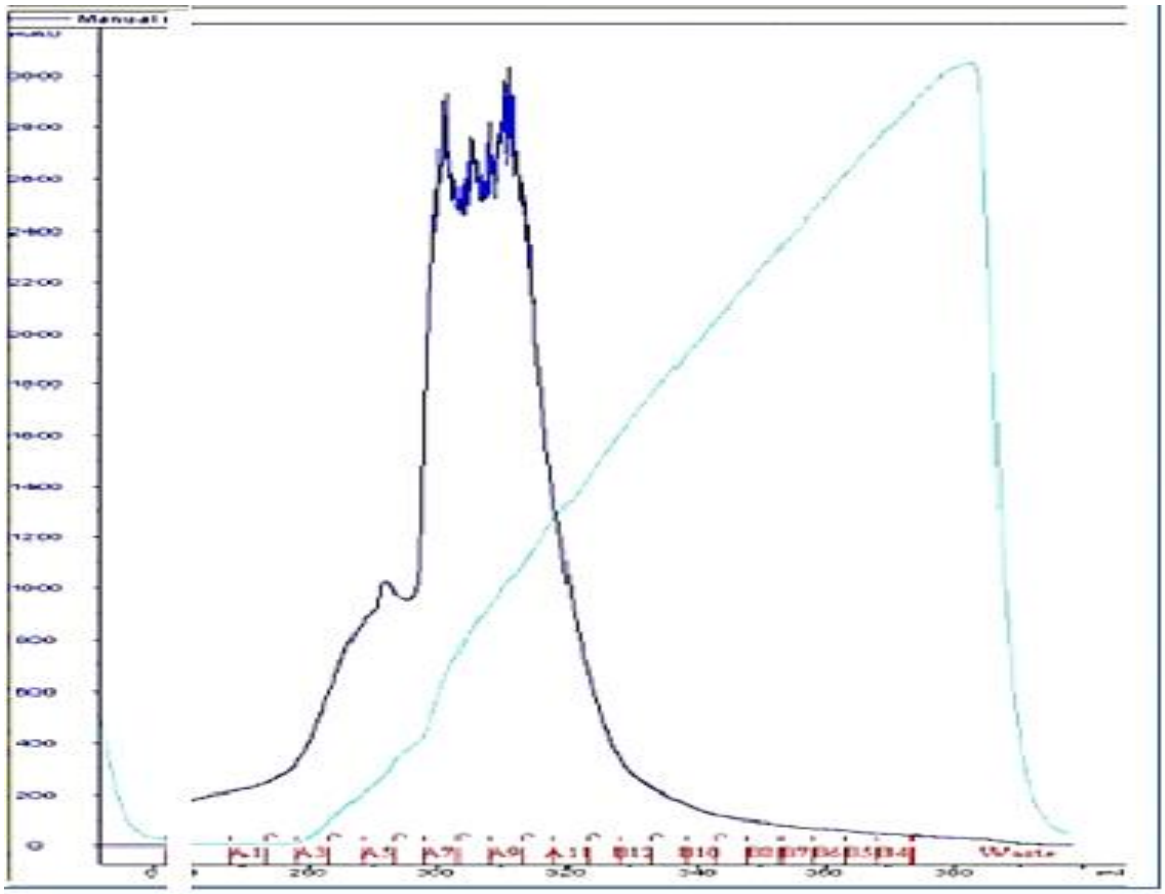

Figure 6.1.2: Peaks of ion exchange chromatography obtained after three hours. The x-axis represents retention volume in mL; the Y-axis represents the mAU

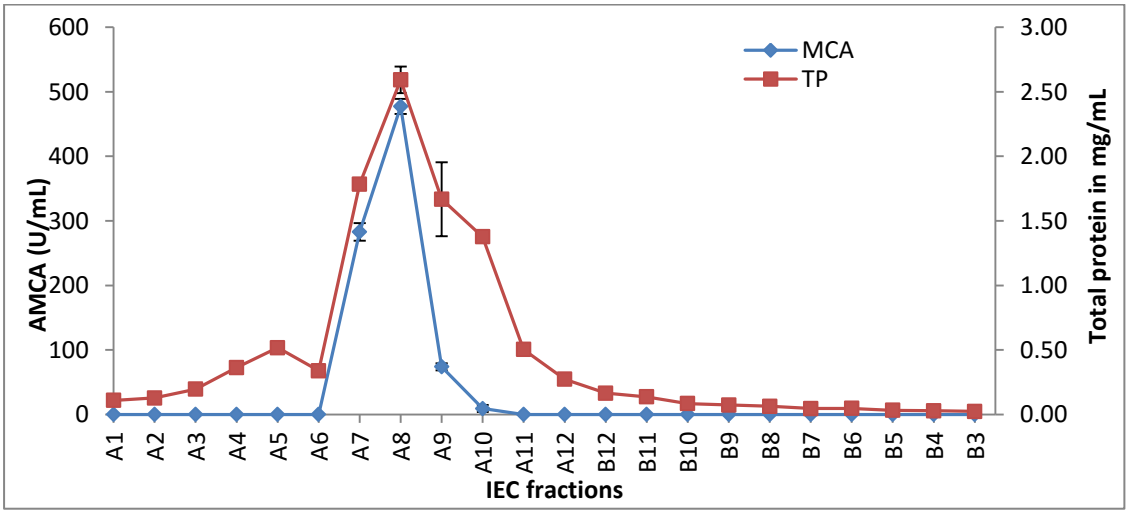

Figure 6.1.3: Line graph for MCA and TP of IEC Fractionate from *A. oryzae* DRDFS13 (A_1-B_3 are ion-exchange chromatography fractions)

Table 6.1.1: MCA, specific activity and purification fold of aspartic protease from *A. oryzae* DRDFS13 purified by an-ion exchange, size exclusion chromatography

Samples	Fractions	Volume (mL)	MCA(U/mL) Mean ±STD	Protease Activity (U/mL)	Ratio (MCA/PA)	Total MCA (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Recovery(%) Yield (%)
Crude Extract	-	150	174.61±3.10	172.26±10.53	1.01	26191.50	883.50	29.65	1	100
IEC fractions (DEAE - Sepharose Fast Flow column)	A_7	5	283.01±13.61	149.00±1.24	1.90	1415.05	8.90	158.99	5.36	5.35
	A_8	5	477.11±11.72	141.93±1.59	3.36	2385.55	13.00	183.50	6.20	9.20
	A_9	5	73.89±5.50	91.79±5.56	0.81	369.45	8.35	53.54	1.80	1.41
	A_{10}	5	9.38±5.69	23.34±2.12	0.40	46.90	6.90	6.80	0.23	0.18
SEC fraction	-	5	186.86± 2.34	155.97±4.25	1.19	934.30	11.75	79.51	2.68	3.56

A7, A8, A9 and A10: Ion exchange fractions, MCA: Milk-clotting activity (U/mL), specific activity: MCA (U/mL)/Total protein (mg/mL), SD: standard deviation, Mean: is average of three measurements, Different letters (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

6.1.1.3.Molecular characterization of purified aspartic protease

6.1.1.3.1. Molecular weight determination

Enzyme purity was confirmed by SDS-PAGE and the active IEC fraction A_8 migrated as a single band with an apparent molecular weight of 40 KDa (Fig 6.1.4B, Fig 6.1.5A and Table 6.1.2). Whereas three bands (one clear band at about 32 kDa mark and 2 small bands at around 11KDa) were obtained from SEC (Fig 6.1.4C). Similarly, three bands (one at about 32 kDa marks, the second band at slightly above the 56 kDa marks and the third (two small bands) around the 11 kDa marks) were visualized from crude enzyme on SDS-PAGE gel (Fig 6.1.4A).

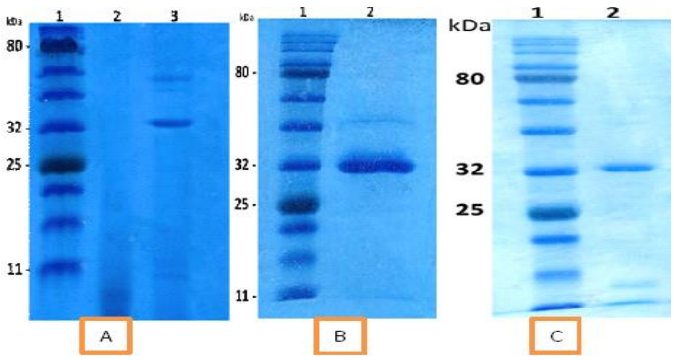

Figure 6.1.4: (A) depicts the SDS-PAGE for the crude enzyme extract, well-1: protein marker with pre-stained color plus, well-2: negative control, well-3: crude enzyme extract. (B) depicts the SDS-PAGE for IEC fraction A8: well-1: protein marker, well-2: IEC fraction A8 (C) depicts the SDS-PAGE for pure enzyme after size exclusion chromatography: well-1: protein marker, well-2: SEC fraction.

6.1.1.3.2. Protein deglycosylation

In this study, de-glycosylation of the aspartic proteinase (IEC A_8) from *A. oryzae* DRDFS13 was performed by using Endo H. The evidence from SDS-PAGE showed the presence of N-linked protein glycosylation, since a change in molecular weight was observed after treatment with Endo-H indicating the removal of glycan from enzymes (Fig. 6.1.5A, B). It can be seen that the glycosylated, deglycosylated and Endo-H have a MW of 40, 30 and 27 KDa, respectively (Table 6.1.2).

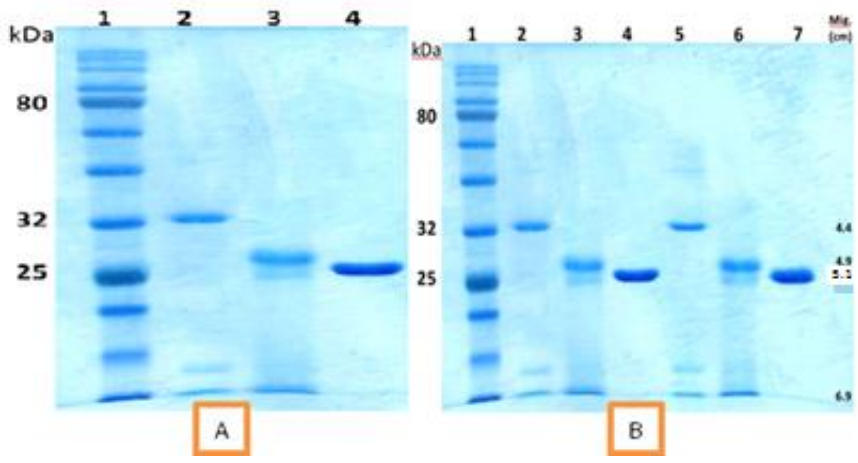

Figure 6.1.5: (A) depicts SDS-PAGE for the purified enzyme (A8) before and after protein de-glycosylation and Endo-H. Well 1 contains the protein marker, 2 contain the pure enzyme (non-deglycosylated), and 3 contain the protein after de-glycosylation and well 4 contain the pure Endo H (B): depicts the migration distances of the bands in the SDS-PAGE gel Non-de-glycosylated enzyme, glycosylated enzyme, and Eno-H. Well, 1 contains the Protein marker, Wells 2 and 5 contain the pure enzyme, and well 3 and 6 contain the protein after the deglycosylation assay. Lastly, wells 4 and 7 show the pure Endo H enzyme used for the assay.

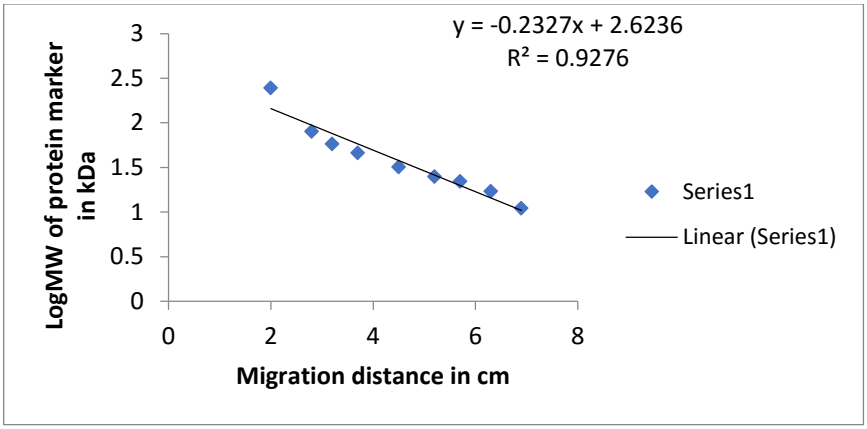

Figure 6.1.6: depicts the log MW (y-axis) versus the migration distance in cm (x-axis) of the bands on the marker protein.

Table 6.1.2: The particular molecular weight assigned for the bands on the marker protein with their respective R_f values

Sample	MW(kDa)	Rf value
Non-deglycosilated enzyme (2 and 5)	40	0.63
The deglycosylated enzyme (3 and 6)	30	0.71
Endo-H (4 and 7)	27	0.74

MW: molecular weight, Rf: relative mobility

6.1.1.3.3. Inhibition study

The purified proteinase from *A. oryzae* (IEC A_8) was subjected to inhibition studies with several class-specific agents (Table 6.1.3). While iodoacetamide, PMSF and EDTA showed slight effect on milk-clotting activity at concentrations up to 10 mM, pepstatin A caused a substantial inhibition (94 %) on milk-clotting activity of the enzyme.

Table 6.1.3: Inhibition study of IEC fraction A_8 from *A. oryzae* DRDFS13

Inhibitors	Conc	MCA (U/mL) mean±SD	Residual MCA (%)
Pepistatin A	0.02 mM	116.24±1.41^{f}	24.34
	0.04 mM	55.17±0.13^{g}	11.55
	0.06 mM	46.88±0.09^{g}	9.81
	0.08 mM	33.30±2.05^{g}	6.97
	0.1 mM	28.69±1.57^{g}	6.01
Iodoacetamide	1 mM	417.68±10.90^{b}	87.45
	10 mM	396.94±9.84^{bc}	83.11
EDTA	5mM	384.22±9.22^{cd}	80.44
	10mM	376.47±23.53^{cd}	78.82
PMSF	1mM	360.22±26.88^{d}	75.42
	10mM	318.01±6.32^{e}	66.58
Control (IEC fraction A_8)	_	477.62±2.38^{a}	100.00

MCA: Milk-clotting activity (U/mL), IEC: Ion-exchange chromatography; pep A: Pepstatin A; EDTA: Ethylene diamine tetraacetic acid; PMSF: Phenylmethyl fluoride sulphonyl, ND = Not determined, SD: standard deviation, Mean: is average of three measurements, Different letters (a, b, c, d, e, f, g) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

6.1.1.4. Biochemical characterization of aspartic protease

Due to the shortage of purified enzyme (IEC A_8), the dialyzed protease was used for further biochemical enzyme characterization such as determination of optimum temperature and temperature stability, determination of optimum pH and pH stability, the effect of cations, effect substrate concentration, and Km and Vmax. Dialysis of the crude enzyme using membrane tube was purified the enzyme 2.2 fold, with a final yield of 5.63% and a detected specific activity of 65.3 U/mg (Table 6.1.4).

Table 6.1.4: MCA, Specific activity, purification fold, and % yield of aspartic protease enzyme dialyzed and purified by an-Ion exchange chromatography

Sample	Volume (mL)	Protein conc. (mg/mL)	MCA (U/mL)	Protease activity (U/mL)	Ratio (MCA/PA)	Total MCA	Specific activity	Purification fold	% Yield
Crude Extract	150	5.89	174.61±3.10^{c}	172.26±10.53	1.01	26191.50	29.24	1	100
Dialyzed Enzyme	5	4.46	290.92±1.44^{b}	151.75±1.54	1.92	1454.68	65.25	2.2	5.63
IEC A_8	5	2.6	477.11±11.72^{a}	141.93±1.59	3.36	2385.55	183.50	6.2	9.2

IECA8: Ion exchange chromatography fraction A8, MCA: Milk-clotting activity (U/mL), specific activity: MCA (U/mL)/Total protein (mg/mL), Mean: is average of three measurements, Different letters (a, b, c,) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

6.1.1.4.1. Km and Vmax

The catalytic activity of the dialyzed enzyme from *A. oryzae* DRDFS13 was determined at 35 ^{o}C and pH 6.5 at varying casein concentrations (0-20 mg/mL). The Km and Vmax of the enzyme was 17.50 mM and 1369 U (Fig 6.1.7). The enzyme also showed a good correlation fit of R^2=0.9862.

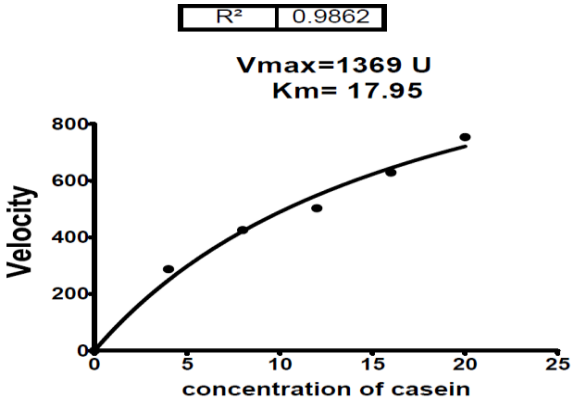

Figure 6.1.7: Km and Vmax for the dialyzed enzyme from *A. oryzae* DRDFS13

6.1.1.4.2. Effects of pH on enzyme activity and stability

The relative milk-clotting activity (%) was studied as a function of the pH of the skim-milk substrate at 35 ^{o}C (Fig. 6.1.8). The dialyzed enzyme was most active between pH

values 4.5-6.5 with maximum activity at pH 5.0. Its activity decreased by more than 50% at a pH 7.0 and was insignificant at pH 8.0.

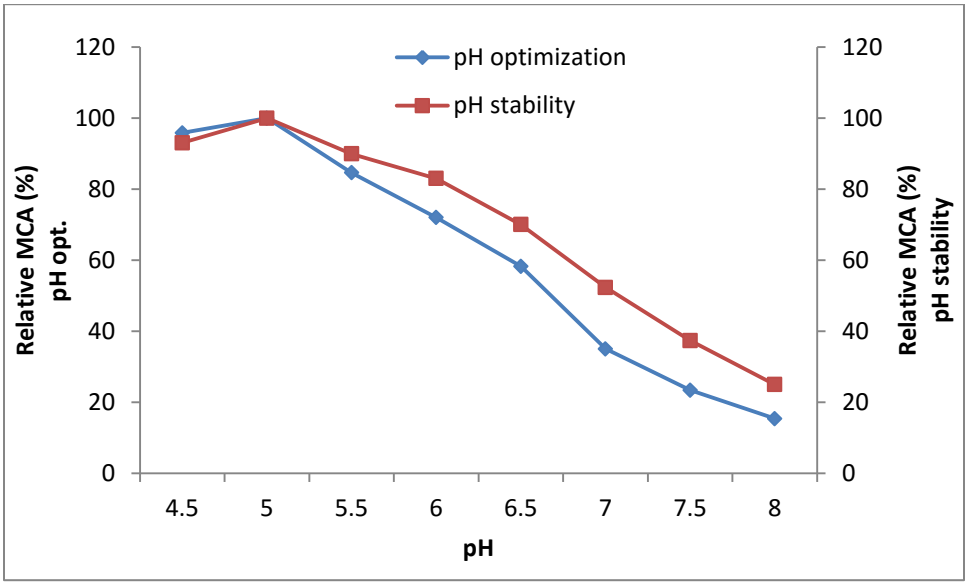

Figure 6.1.8: pH optimization and stability test for the dialyzed enzyme from *A. oryzae* DRDFS13

6.1.1.4.3. Effects of temperature on enzyme activity and stability

As shown in Fig. 6.1.9, the milk-clotting activity of the dialyzed protease from *Aspergillus oryzae* DRDFS13 increased with increasing temperature and reaching a maximum at 60 ^{o}C. Beyond 60 ^{o}C, the milk-clotting activity of the protease was started to decline sharply.The enzyme retained more than 85% of its MCA at a temperature between 35 to 45 ^{o}C (Fig. 6.1.10).

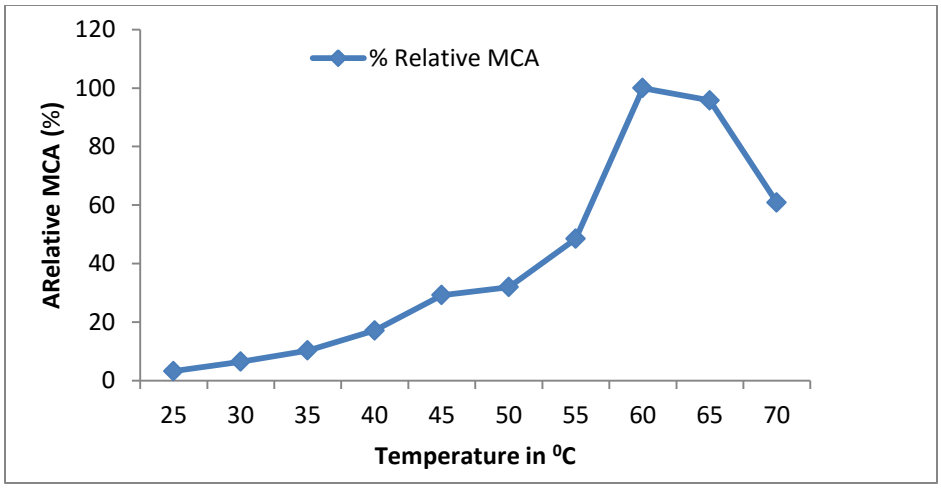

Figure 6.1.9: Temperature optimization test for a dialyzed extract from *A. oryzae* DRDFS13

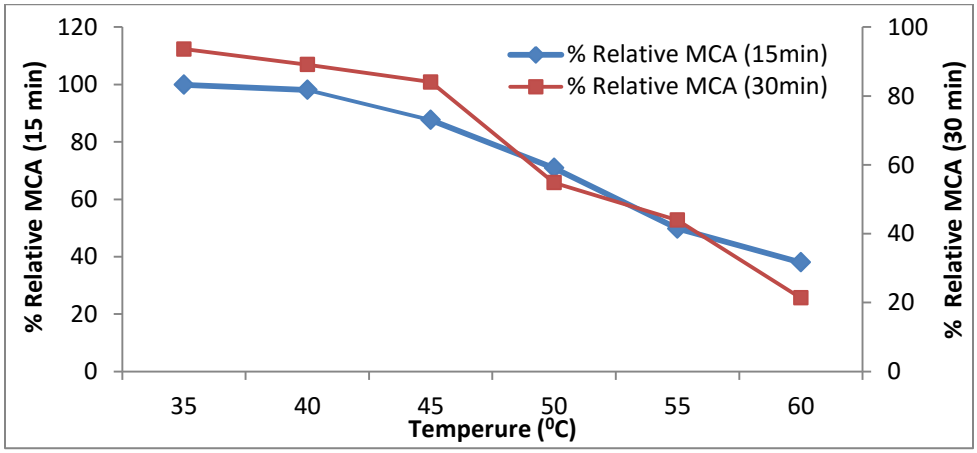

Figure 6.1.10: Temperature stability test for a dialyzed extract from *A. oryzae* DRDFS13

6.1.1.4.4. Effects of cations on milk-clotting activity

The effect of cations (10 mM) on milk-clotting activity were examined at 35 ^{o}C using skim-milk as a substrate (Table 6.1.5). Accordingly, most cations gave a significant effect on the enzyme activity except $NiCl_2$ and $ZnSO_4$ that inhibited the MCA as compared to the control. However, the highest MCA (649 U/mL) was obtained from $MnSO_4$. Further optimization of $MnSO_4$ concentration, showed the highest MCA (1550.00±50.00 U/mL) at 50 mM (Fig. 6.1.11).

Table 6.1.5: The effect metal ions on MCA of the dialyzed enzyme from *A. oryzae* DRDFS13

Metals	Conc. (mM)	MCA (U/mL) Mean ±STD	Relative MCA (%)
cont	-	9.96 ± 0.05^{ij}	1.53
NaCl	10	92.84 ± 0.18^{g}	14.30
KCl	10	88.73 ± 0.16^{g}	13.67
$CaCl_2$	10	280.71 ± 1.64	43.24
MgCl2	10	592.69 ± 7.32^{b}	91.31
FeCl2	10	466.01 ± 4.52^{c}	71.80
NiCl2	10	0 ± 0.00^{j}	0.00
MnCl2	10	417.42 ± 3.63^{d}	64.31
MgSO4	10	298.150 ± 1.85^{f}	45.93
MnSO4	10	649.13 ± 17.54^{a}	100.00
ZnSO4	10	0 ± 0.00^{j}	0.00
CuSO4	10	19.53 ± 0.48^{i}	3.01
FeSO4	10	396.72 ± 3.28^{e}	61.12
COSO4	10	38.07 ± 0.03^{h}	5.86

Cont: skim milk only NaCl: skim milk with Nacl.., Mean: is average of three measurements, Different letters (a, b, c, d, e, f, g) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05).

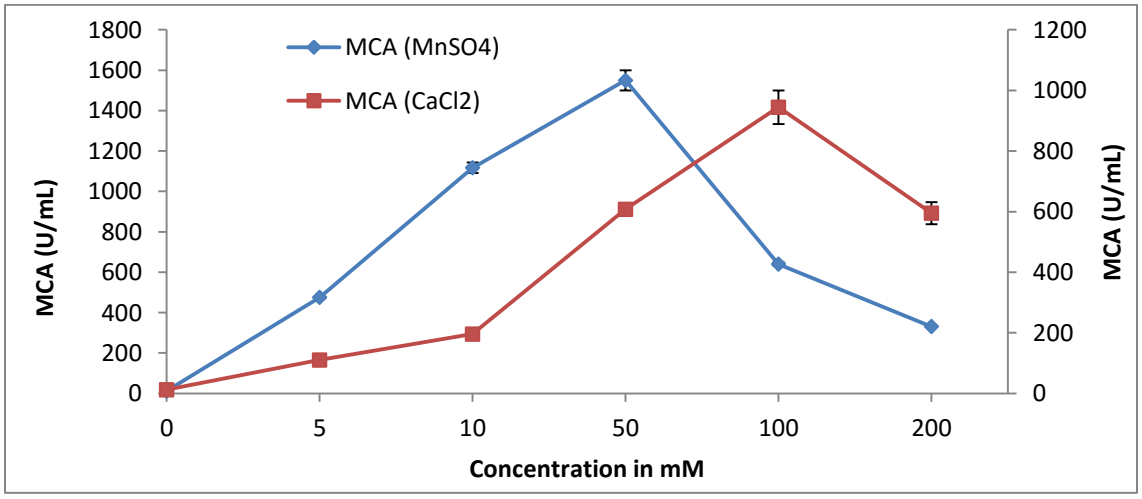

Figure 6.1.11: The effect of $MnSO_4$ and $Cacl_2$ concentration on MCA of the enzyme from *A. oryzae* DRDFS13

6.1.1.4.5. The effect substrate concentration on the milk-clotting activity

The present data also showed substrate concentration had an effect on the milk-clotting activity of the dialyzed enzyme in that the highest MCA (905.98±17.09 U/mL) was obtained at 200 g/L skim-milk concentration (Table 6.1.6).

Table 6.1.6: The effect of skim milk conc. on MCA of the enzyme from *A. oryzae* DRDFS13

Conc. of Skim milk (g/L)	MCA (U/mL) Mean ±STD	Relative MCA (%)
25	107.41±1.68^{e}	11.86
50	161.63±1.63^{d}	17.84
100	246.32±6.32^{c}	27.19
150	539.39±6.06^{b}	59.54
200	905.98±17.09^{a}	100.00

MCA: Milk-clotting activity (U/mL), SD: standard deviation, Mean: is an average of three measurements, Different letters (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

6.1.2. Discussion

In this study, we purified an extracellular aspartic protease from *Aspergillus oryzae* DRDFS13 by means ion exchange and size-exclusion chromatography and investigate its biochemical characteristics using 10% solution of skim-milk.

The highest milk-clotting activity (175 U/mL) and MCA/PA ratio (1.02) of the crude enzyme from *A. oryzae* DRDFS13 were observed at 120 h of incubation time at 30 ^{o}C under SSF. However, the biomass levels reached maximum (19.77±8.20 mg/gdfs) at 48 h and decreased afterwards. This implies that the fungus secrete the enzyme after its maximum growth is attained by utilizing various carbon and nitrogen sources in solid-substrate. Similar to the present study, a milk-clotting activity between 46.4 to 160.3 U/mL (Merheb-Dini *et al*., 2010), 60.5 CU/mL (Silva *et al.*, 2014) and 165 U/mL (Mukhtar, 2015) were obtained from the crude enzyme extracted from *Thermomucor indicae-seudaticae* N31, *Thermomucor indicae-seudaticae* N3 and *M. pusillus* IHS6, respectively.

Purification of the enzyme by dialysis, SEC and IEC revealed that, the active fractions A_8 obtained after IEC was purified 6.2-fold from a crude enzyme, with a yield of 9.2%, milk-clotting activity of 477 U/mL and an observed specific activity of 183.5 U/mg. The MCA/PA of the same IEC fraction was also increased by 3.3 fold. The present finding suggests that ion-exchange chromatography is an appropriate purification step in improving the activity of fungal aspartic protease as compared to simple dialysis and size-exclusion chromatography.

Similar to the present study, aspartic proteases purified from *A. oryzae* BCRC 30118 (Yin *et al*., 2013) and *Phanerochaete chrysosporium* (Rodrigues *et al*., 2017) showed high enzyme activity with a specific activity of about 117.62 U/mg and 224.7 U/mg, respectively. The purification fold of 10.0 (Ao *et al*., 2018) and 9.0 (Fazouane-Naimi *et al*., 2010) gained for the neutral protease from *A. oryzae* Y1 after DEAE-Sepharose Fast Flow chromatography and for aspartic protease enzyme from *A. niger* FFB1after gel filtration was comparable with the present study. However, the specific activity of 1.2 U/mg (Nouani *et al*., 2011) and (248.1 U/g) (Souza *et al*., 2017) obtained for an aspartic protease from *M. pusillus* and *A. foetidus*, respectively after gel filtration were smaller than this study. The differences recorded in the specific activity and purification fold from this study and others could be due to variation in microbes and/or enzyme purification methods used.

Enzyme purity was also confirmed by SDS-PAGE and the active IEC fraction A_8 migrated as a single band with a molecular weight of 40 KDa. This value was in the range of molecular weights normally determined for aspartyl proteinases (32-45 kDa) (Hsiao *et al*., 2014). The molecular weight of the purified enzyme was similar with MW

reported for aspartic protease from *Rhizopus oryzae* (≈39 KDa) (Hsiao *et al*., 2014), *Phanerochaete chrysosporium* (38 kDa) (Rodrigues *et al*., 2017), *A. oryzae* BCRC 30118 (41 KDa) (Yin *et al*., 2013). However, the molecular weight of the purified enzyme was higher than reported for aspartic protease from *M. mucedo* DSM 809 (33 kDa) (Yegin *et al*., 2012) and *Mucor species* (33.5 KDa) (Fernandez-Lahore *et al*., 1999), *A. oryzae* (32 kDa)(Dhurway *et al*., 2012). The difference in MW could be due to varies type of aspartic protease can be produced from different microorganisms.

The data from de-glycosylation assay exhibited the reduction of molecular weight of the purified protein from 40 KDa to 30 KDa. This indicates the presence of N-linked protein glycosylation as the change in the molecular mass was observed after treatment with Endo-H. This post-translational modification of the aspartic protease enzyme increased its thermal stability and resistant to proteolysis (Goettig, 2016; Wang *et al*., 2008). Similar to this study, two potential N-glycosylation sites were identified for the amino acid sequence of purified recombinant aspartic protease from *Pichia pastori*s (Sun *et al*., 2018). The purified enzyme produced in this study was better in thermal stability and resistance to proteolysis than the aspartyl proteinase from *M. mucedo* that lacks of N-linked protein glycosylation (Yegin *et al*., 2012).

The purified aspartic protease (IEC A_8) from *A. oryzae* DRDFS 13 was strongly inhibited by pepstatin A, with nearly complete inactivation happening at an inhibitor concentration of 0.1 mM. Similar results were reported by Hsiao *et al*., (2014); Merheb-Dini *et al*., (2010); Rodrigues *et al*., (2017); Souza *et al*., (2017); Yin *et al*., (2013). In contrast, other inhibitors (Iodoacetamide, PMSF, and EDTA), did not show significant inhibition even at higher concentration of 10 mM. The nearly complete inhibition could be due to

specific and irreversible binding of pepstatin A within the active site of aspartate and hence it protects the enzyme-substrate (skim-milk) binding. Strong inhibition by pepstatin A (aspartic protease inhibitor), suggests that the purified protein (IEC A_8) is classified as an aspartic protease (Hsiao *et al*., 2014).

The results of the dialyzed enzyme from *Aspergillus oryzae* DRDFS13 showed an apparent Km and Vmax value of 17.95 mg/mL (1.80%) and 1369 (U/mL) µmol/mL/min($22.82*10^{-3}$mmol/mL/sec), respectively. The catalytic result from the present study suggested a wide specificity of the enzyme towards casein substrates and require further determination of the Km and Vmax using purified enzyme to increase the enzyme affinity towards substrate and increase its reaction velocity. The Km and Vmax value estimated for protease from *A. oryzae* (4.9 mg/mL and 5446.3 U/g) (Janser *et al*., 2014) and acid protease from *Onopordum acanthium* (12.25 mM and 1329.6 U/mL) (Benkahoul *et al*., 2016) using casein substrate was similar to the present study. The Km (19.5 mg/mL) value recorded for the purified MCE from *M. pusillus* QM 436 using casein as a substrate was also comparable with this study (El-Tanboly *et al*., 2013).

On the other hand, the Km and Vmax value estimated for purified milk-clotting enzyme from *A. candidus* (0.059 mg/mL and $8.59*10^{-3}$ mmol/ml/sec) (Baskar *et al*., 2014), for acidic protease from *A. oryzae* using hemoglobin as a substrate (0.12 mM, 14.29 µmol/min) (Yin *et al.*, 2013) and for acid protease from *A. foetidus* for azocasein substrate (1.92 mg/mL; 0.8 mM, 357.14 U/mL) (Souza *et al*., 2015) were different from the present study. The observed variation in Km and Vmax value between the present and previous study could be due to a dialyzed enzyme (not a purified enzyme) and/or different substrates used for the determination of catalytic activity.

The dialyzed enzyme from *A. oryzae* DRDFS13 was shown best milk-clotting activity between pH values 4.5-6.5 with highest activity at pH 5.0. This implies that the enzyme is active at acidic pH and appropriate for cheese production (Kumari *et al*., 2016). Similarly, the optimal milk-clotting activity for aspartyl proteinase from *Mucor mucedo* DSM 809 (Yegin *et al*., 2012), milk-clotting enzyme from *Rhizomucor miehei* (Moghaddaml *et al*., 2008), acid protease from *A. niger* FFB1 (Fazouane-Naimi *et al*., 2010), milk-clotting protease from *Thermomucor indicae-seudaticae* N31 (Silva *et al*., 2014) and milk-clotting enzyme from *Thermomucor indicae-seudaticae* N31 (Merheb-Dini *et al*., 2010) were observed at pH 5.0–5.6, 5.3, 5.5, 5.6 and 5.7, respectively. On the other hand the highest milk-clotting activity for milk-clotting enzyme from *A. oryzae* MTCC 5341(Vishwanatha *et al*., 2010) and milk-clotting enzymes from *A. flavo furcatis* (Alecrim *et al*., 2015) were detected at pH 6-6.3 and 7, respectively.

Regarding pH stability, the dialyzed enzyme retained (83-100%) of milk-clotting activity during 1h incubation at pH range from 4.5-6.0. However, its activity decreased by more than 50% at pH 7.0 and was insignificant at pH 8.0. Therefore, the stability of the enzyme in MCA in the present study corresponds with a milk pH (5.0 to 6.5) at the time of enzyme addition that is appropriate for cheese production (Kumari *et al*., 2016). The instability of the enzyme at alkaline pH's suggested an irreversible inactivation of enzyme (Kumari *et al*., 2016) or alkaline pH's may cause a protein denaturation in the dialyzed enzyme (Fazouane-Naimi *et al*., 2010). Similar stability values were also found for proteinase from *M. muced*o (pH 5.0–5.5) (Yegin *et al*., (2012), milk-clotting protease from *Thermomucor indicae-seudaticae* N31(pH 3.5-6.0) (Merheb-Dini *et al*., 2010), rennet like enzyme from *A. niger* (pH 3.0–5.5) (Fazouane-Naimi *et al*., 2010) and milk-

clotting enzyme from *A. flavo furcates* (pH 4.0-6.0) (Alecrim *et al*., 2015). In contrast, the stability values noticed at pH 3.5-4.5 for the milk-clotting protease from *Thermomucor indicae-seudaticae* N31 (Silva *et al*., 2014) and at pH 5.0–8.0 for milk-clotting enzyme from *A. oryzae* MTCC 5341 (Vishwanatha *et al.*, 2010) were different from this study.

The maximum milk-clotting activity of the dialyzed enzyme was obtained at 60 ^{o}C. The result from this study corresponds with that of Kumari *et al*., (2016) that optimum temperature of majority of MCEs lies in the range of 30-75 ^{o}C. Similarly, the optimum temperature observed for the milk-clotting/acid protease activities recorded for milk-clotting protease from *Thermomucor indicae-seudaticae* (65 ^{o}C)(Silva *et al.*, 2014), aspartic peptidase from *Phanerochaete chrysosporium* (60-65 ^{o}C) (Rodrigues *et al*., 2017), milk-clotting enzyme from *Rhizomucor miehei* (65 ^{o}C) (Moghaddaml *et al*., 2008), milk-clotting enzyme from *A. oryzae* MTCC 5341 (55 ^{o}C) (Vishwanatha *et al*., 2010) and acid protease enzyme from *A. awmari* (55 ^{o}C) (Souza *et al*., 2015) were equivalent to the present study. However, the optimum temperature noticed for milk-clotting/acid protease activities for milk-clotting enzyme from *A.candidus* (35-50 ^{o}C) (Baskar *et al*., 2014), milk-clotting protease from *A. niger* FFB1 (45 ^{o}C) (Fazouane-Naimi *et al*., 2010), aspartyl proteinase from *M. mucedo* DSM 809 (35-40 ^{o}C)(Yegin *et al*., 2012) and milk-clotting enzyme from *A. flavor furcates* (40 ^{o}C) (Alecrim *et al*., 2015) were lower than this study.

The thermostability profile of dialyzed enzyme from *A. oryzae* DRDFS13 showed that the enzyme was stable in the temperature range from 35-45 ^{o}C by retaining ≥85% of its

MCA activity upon 15 min and 30 min incubation. Further incubation of the enzyme at 55 ^{o}C for 15 min and 30 min resulted in 50% and 66% loss of its MCA, respectively. Only 21.5% of the MCA of the enzyme was retained after 30 min incubation at 60 ^{o}C. This result suggests that aspartic proteases from *A.oryzae* DRDFS13 seem to be stable at temperatures below 45 ^{o}C and susceptible to inactivation above this temperature. The nearly complete inactivation of the enzyme activity upon incubation at 55 ^{o}C for 30 min may be a technological advantage since no bitterness development can occur during cheese ripening due to the inactivated proteolytic action of the enzyme after the cooking process of the curd (Sousa *et al*., 2001). Similar to this study, the rennet like enzyme from *A. niger* FFB1 (Fazouane-Naimi *et al*., 2010), milk-clotting enzyme from *Aspergillus oryzae* MTCC 5341 (Vishwanatha *et al*., 2010), milk-clotting enzyme from *Thermomucor indicae-seudaticae* N31 (Silva *et al*., 2014) and aspartyl proteinase from *M. mucedo* DSM 809 (Yegin *et al*., 2012) completely lost their milk-clotting activity at 60 ^{o}C, 65 ^{o}C, 62 ^{o}C, 60 ^{o}C and 55 ^{o}C upon incubation for 30 min, 15 min, 60 min and 10 min, respectively.

The addition of 10 mM of $MgCl_2$, $FeCl_2$, $MnCl_2$, $MnSO_4$, $FeSO_4$, and $CaCl_2$ showed a pronounced effect on the milk-clotting activity of the enzyme reflecting significant effect on second phase of milk clot formation. However, the highest MCA was obtained from $MnSO_4$. Monovalent salts such as KCl and NaCl were also shown slight stimulatory effect on milk-clotting activity. On the other hand, $NiCl_2$ and $ZnSO_4$ showed an inhibitory effect on the milk-clotting activity as compared to the control (Skim-milk only). Similarly, Ca^{2+} (El-Tanboly *et al*., 2013), Na^{+}, K^{+}, Mn^{2+} and Ca^{2+} (Liu and Huang, 2015) and Ca^{2+}, Mg^{2+}, Mn^{2+} (Kumari *et al*., 2016) and Mn^{2+}, Ca^{2} (Ahmed *et al*., 2016) were

shown a noticeable effect on enhancing the the activity of milk-clotting enzyme from *M. pusillus* QM 436, acidic protease from *Rhizopus stolonifer*, MCE from *B. subtilis* MTCC 10422 and MCE from *B. stearothermophilus*, respectively. Whereas the addition of Co^{2+}, Cd^{2+} and Zn^{2+} were found to have an inhibitory effect on the activity of MCE from *B. subtilis* MTCC 10422 (Kumari *et al*., 2016). Generally, low pH, higher temperature and increased levels of calcium improved the MCA of the enzyme perhaps due to it promotes the better enzyme-substrate reaction and hence reduce the clotting time (Alecrim *et al*., 2015; El-Tanboly *et al*., 2013).

The highest MCA was observed at 50 mM $MnSO_4$ concentrations. In contrast, the optimal MCA for the rennet-like enzyme from *A.niger* FFB1 were obtained at 0.01 M $CaCl_2$(Fazouane-Naimi *et al*., 2010). However, acidic protease from *A. oryzae* BCRC 30118 was not affected by any metals at a concentration of 1.0 mM (Yin *et al*., 2013). The Milk-clotting activity of aspartic protease from *A. oryzae* DRDFS13 was increased uniformly as the concentration of skim-milk increased from 25 to 200 g/L. The highest MCA was achieved at 200 g/L skim-milk concentration.

6.1.3. Conclusions

The milk-clotting activity, specific activity, purification fold, biochemical characteristics, inhibition study and results from deglycosylation assay obtained from ion-exchange chromatography fraction A_8 in the present study confirmed that the enzyme is aspartic protease and could be used as a possible candidate to substitute rennet enzyme for cheese production.

6.2. Production and partial characterization of a milk-clotting proteinase produced by *Bacillus subtilis* SMDFS-2B in submerged cultures

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Abstract

This study focused on the production and partial characterization of a milk-clotting protease produced by *Bacillus subtilis* SMDFS 2B in submerged cultures, under partially optimized conditions. The crude enzyme was recovered in the culture supernatant and concentrate was produced after cell removal and subsequent dialysis. Inhibition studies were conducted employing four distinct protease inhibitors: Pepstatin-A, Phenylmethane-sulphonyl-fluoride (PMSF), Ethylenediaminetetraacetic acid (EDTA), and iodoacetamide (IA). The effect of temperature, pH, metal ions and substrate concentration on milk-clotting activity were also evaluated. The thermal stability of the enzyme was determined by incubating the crude enzyme at a temperature value ranging from 35 ^{o}C to 60 ^{o}C. Similarly, pH stability was determined at pH values ranging between 4.5 and 8.0. The highest milk-clotting activity was observed at a temperature of 55 ^{o}C and pH 5.5. The crude enzyme preparation remained stable on incubation at 35 ^{o}C and 40 ^{o}C for 15 min and at pH 5.5. The enzyme also showed the lowest residual milk-clotting activity in the presence of EDTA (7.94%) and Pepstatin-A (26.71%). The addition of Mg^{2+} and Mn^{2+} significantly increased milk-clotting activity. The enzyme also showed an elevation in its apparent milk-clotting activity upon increasing the substrate (skim-milk) concentration. Thus, the milk-clotting protease

produced by *B. subtilis* SMDFS 2B by submerged fermentation revealed some interesting milk-clotting characteristics. This may open the way for applications in the food and dairy industries.

Keywords: *Bacillus subtilis*, Characterization, Inhibition, Milk-clotting activity.

6.2.1. Results

6.2.1.1. Crude enzyme production and partial purification

Bacillus subtilis SMDFS 2B was cultivated in shaking flasks under submerged fermentation conditions utilizing a simple media containing wheat bran, under partially optimized conditions (Table 6.2.1). The maximum milk-clotting activity (312 U/mL) was recorded in the culture supernatant after 72 h of fermentation. The culture supernatant was dialyzed and concentrated to yield a crude enzyme preparation. The milk-clotting activity was increased 2.0-fold after dialysis (Table 6.2.2).

Table 6.2.1: Production of crude enzyme from *B. subtilis* SMDFS2B under SmF

Parameters	Measurement Unit
Initial media pH	5.5
Inoculum size	4% of ($2.5*10^8$ Cells/mL)
Fermentation time	72 h
rpm	150
MCA	311.74±4.05 U/mL
PA	231.04±5.18 U/mL

rpm: revolution per minute, MCA: Milk-clotting activity (U/mL), PA: protease activity (U/mL)

Table 6.2.2: MCA, specific activity and concentration fold of dialyzed protease from *B.subtilis* SMDFS 2B

Sample	MCA (U/mL) Mean ±SD	Protein conc. (mg/mL)	Specific activity (U/mg)	Purification Fold
Crude enzyme	$311.74±4.05^b$	17.67	17.64	1
Dialyzed enzyme	$406.90±6.90^a$	11.13	36.56	2.07

MCA: Milk-clotting activity (U/mL), specific activity: MCA (U/mL)/Total protein (mg/mL), SD: standard deviation, Mean: is average of three measurements, Different letters (a, b,) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

6.2.1.2. Inhibition study

The type of milk-clotting proteinase produced by *B. subtilis* SMDFS 2B was determined by conducting inhibition experiments using four protease inhibitors. Incubation of the enzyme with individual protease inhibitors showed a significant reduction in milk-clotting activity. Residual milk-clotting activity values of 27%, 43%, 7.9% and 37% were found when using Pepstatin A (0.1 mM), Iodoacetamide (10 mM), EDTA (10 mM) and PMSF (10 mM), respectively (Table 6.2.3).

Table 6.2.3: Inhibition study of the dialyzed enzyme from *B.subtilis* SMDFS 2B

Inhibitors	Conc. in mM	MCA (U/ml) mean±SD	Residual MCA (%)
Pepstatin A	0.02 mM	279.11 ± 3.25^{b}	68.59
	0.04 mM	193.30 ± 10.09^{d}	47.51
	0.06 mM	160.58 ± 2.69^{fg}	39.46
	0.08 mM	147.13 ± 10.77^{g}	36.16
	0.1 mM	108.68 ± 2.95^{h}	26.71
Iodoacetamide	1 mM	214.30 ± 1.91^{c}	52.67
	10 mM	175.19 ± 1.28^{e}	43.06
EDTA	5 mM	177.12 ± 0.65^{e}	43.53
	10 mM	32.32 ± 0.02^{i}	7.94
PMSF	1 mM	161.54 ± 8.67^{f}	39.70
	10 mM	150.47 ± 0.47^{fg}	36.98
Control (dialyzed Enzyme)	-	406.90 ± 6.90^{a}	100.00

ND: Not determined, MCA: Milk-clotting activity (U/mL), Pep A: Pepstatin A, I.A: Iodoacetamide, EDTA: ethylenediaminetetraacetic acid, PMSF: phenyl-methane sulphonyl fluoride, SD: standard deviation, Mean: is average of three measurements, different letters (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test ($P<0.05$).

6.2.1.3. Effects of temperature on enzyme activity and stability

In determination of the optimum temperature for the dialyzed enzyme, the milk-clotting activity was assayed at the temperature range from 25 ^{o}C to 70 ^{o}C using skim-milk at pH 6.2. The highest milk-clotting activity (2833 U/mL) for the dialyzed milk-clotting protease from *B. subtilis* SMDFS-2B was obtained at 55 ^{o}C. Thereafter, the milk-clotting activity of the enzyme drastically decreased and insignificant activity was observed at 70 ^{o}C (Fig 6.2.1). Thermal inactivation experiments were conducted at a temperature ranges from 35 ^{o}C to 60 ^{o}C using skim-milk at pH 6.2. The dialyzed enzyme from *B. subtilis* SMDFS-2B showed stability on incubation at 35 ^{o}C and 40 ^{o}C for 15 min by exhibiting a residual activity of 81% and 74 %, respectively. However; the residual activity of the enzyme preparation slightly decreased to 55% after incubating at 60 ^{o}C for 15 min. Further increase in incubation time to 30 min drastically reduced the milk-clotting ability of the enzyme (Fig. 6.2.2).

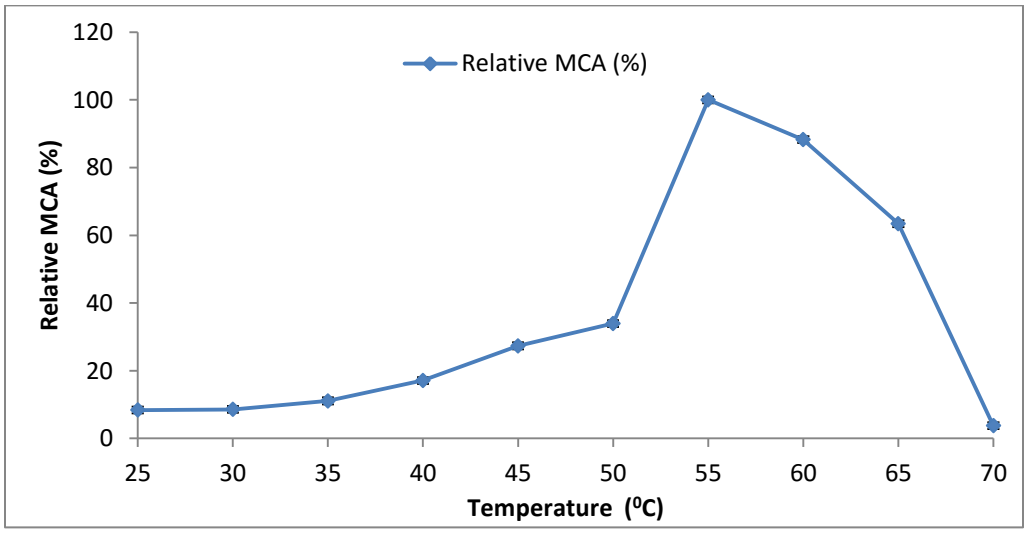

Figure 6.2.1: The effect of temperature on the activity of milk-clotting protease produced by *B. subtilis* SMDFS 2

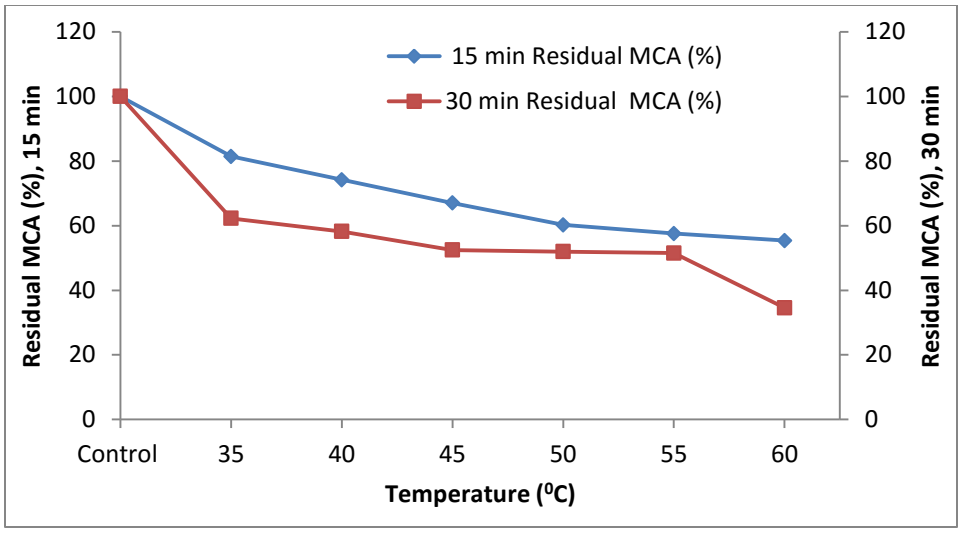

Figure 6.2.2: The effect of temperature on the stability of milk-clotting protease produced by *B. subtilis* SMDFS2B

6.2.1.4. Effect of pH on enzyme activity and stability

The effect of pH on the milk-clotting activity of the dialyzed enzyme was studied at pH ranges from 4.0 to 8.0 at 35 ^{o}C. The enzyme exhibited the maximal rate of reaction at a pH range (4.0-6.0) as shown in Fig. 6.2.3. However, the highest milk-clotting activity (375 ± 6 U/mL) was obtained at pH 5.5. The stability of the dialyzed enzyme at different pH values is shown in Fig. 6.2.3. The enzyme showed maximum stability at pH 5.5. As the pH becomes extreme to either side, the activity of the enzyme decreased significantly.

6.2.1.5.Effect of skim-milk concentration on enzyme activity

The effect of skim-milk concentration (25 g/L – 200 g/L) on the milk-clotting activity of the dialyzed enzyme is shown in Fig.6.2.4. The enzyme showed an elevation in its milk-clotting activity with increasing skim-milk concentration (Figure 6.2.4). The MCA reached a maximum when the skim-milk concentration was increased to 200 g/L.

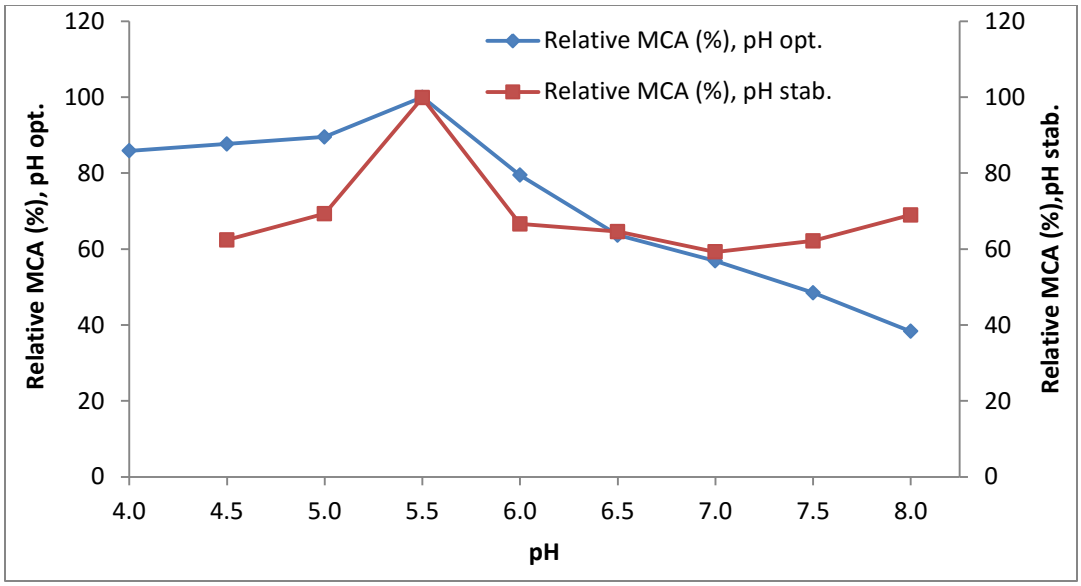

Figure 6.2.3: The effect of pH on the activity and stability of milk-clotting protease produced by *B. subtilis* SMDFS 2B

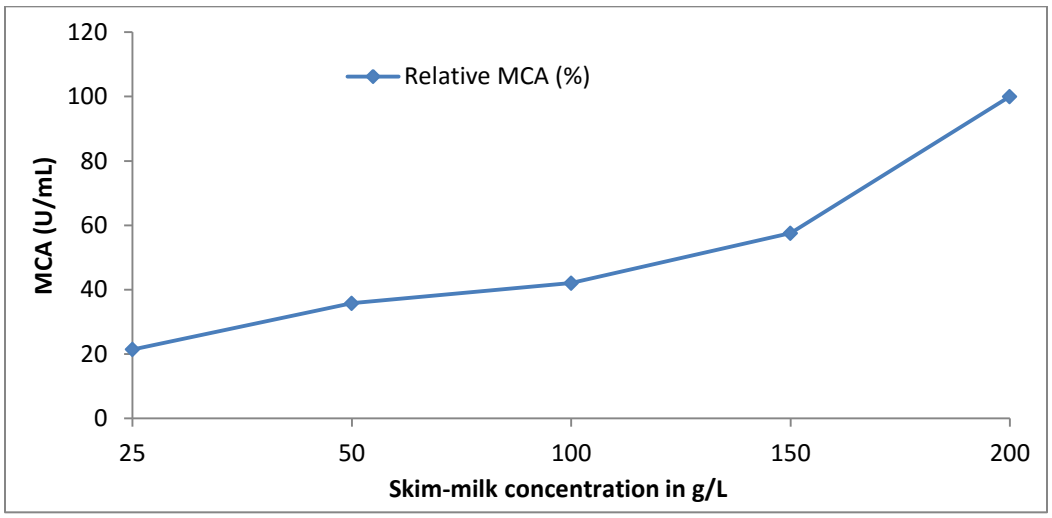

Figure 6.2.4: The effect of skim-milk concentration on the activity of milk-clotting protease from *B.subtilis* SMDFS 2B

6.2.1.6.Effect of additives on enzyme activity

The effect of various monovalent and divalent ions on the milk-clotting activity of the dialyzed enzyme from *B. subtilis* SMDFS-2B was studied at 10 mM concentration (Fig. 5.2.5). Na^{+}, K^{+}, and Zn^{2+} had a slight stimulatory effect on milk-clotting activity, whereas

$Cu^{2+,}$ Co^{2+}, and Ni^{2+} significantly inhibited the milk-clotting activity. In contrast, Mg^{2+} and Mn^{2+} had a significant stimulatory effect on the milk-clotting activity of the crude enzyme from *B. subtilis* SMDFS-2B.

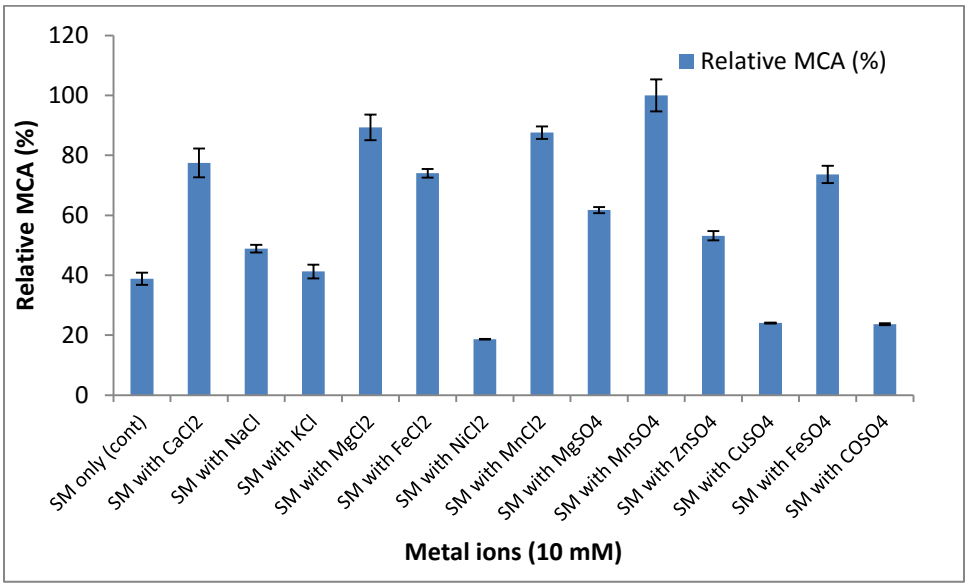

Figure 6.2.5: The effect of metal-ions on activity of milk-clotting protease from *B.subtilis* SMDFS 2B

6.2.1.7. Effect of $CaCl_2$ and $MnSO_4$ concentration on enzyme activity

The effect of $CaCl_2$ and $MnSO_4$ concentration on the milk-clotting activity of the dialyzed enzyme from *B. subtilis* SMDFS-2B was studied in the range from 5 mM to 200 mM. The highest milk-clotting activity was obtained at 10 mM and 50 mM concentration of $MnSO_4$ and $CaCl_2$, respectively (Fig. 6.2.6). The clotting time increased with increasing concentration of Ca^{2+} and reached the maximum at 50mM and thereafter declined severely. The milk clotting activity of the crude enzyme also increased with increasing concentration of Mn^{2+} and reached highest at 10 mM and reduced sharply afterward.

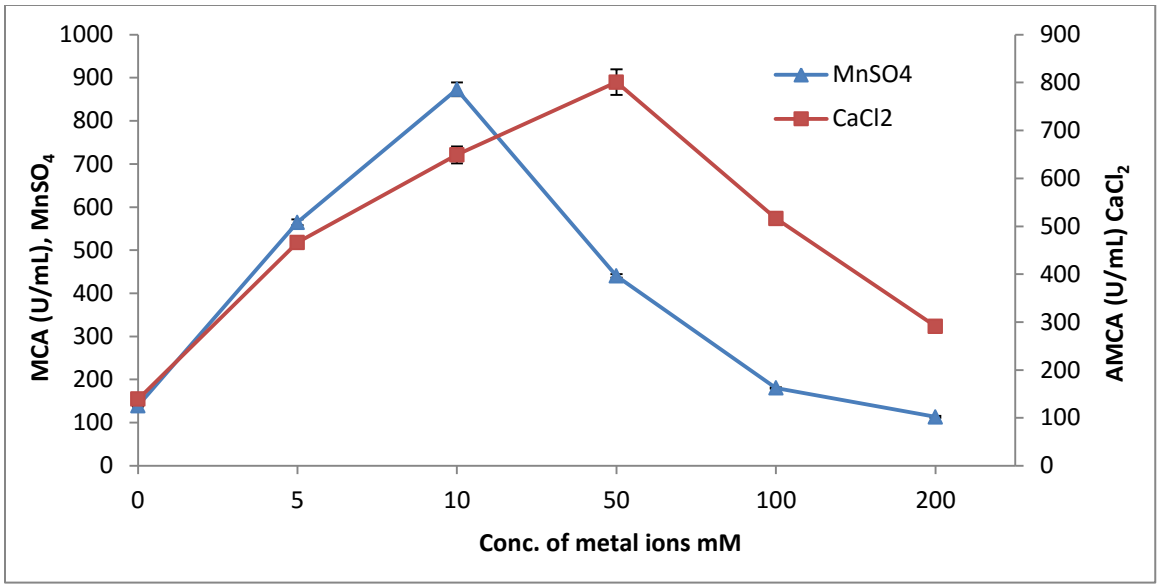

Figure 5.2.6: The effect of $MnSO_4$ and $CaCl_2$ concentration on the activity of milk-clotting protease produced by *B.subtilis* SMDFS 2B

6.2.2. Discussion

The milk-clotting activity of the crude enzyme preparation (312 ± 4 U/mL) obtained from *B. subtilis* SMDFS-2B in the present study was higher when compared to the activity recorded for crude milk-clotting protease from *Thermomucor indicae-seudaticae* N31 (160 ±12 U/mL) (Merheb-Dini *et al*., 2010) and from *Bacillus amyloliquefaciens* SP1(130 U/mL) (Guleria *et al*., 2016). However, a higher activity (1190 U/mL) was obtained from an indigenous *Bacillus subtilis* strain after the production media was optimized using response surface methodology (RSM) (Dutt *et al*., 2009). The variation recorded in milk-clotting activity could be due to the differences in microbial strain and/or media used for the enzyme production.

Dialyzing the crude enzyme resulted in an increase in the milk-clotting activity of the preparation by 2-fold. This may be due to the removal of low-molecular-weight

interfering or inhibitory compounds. Similarly, partial purification of a milk-clotting protease from *M. pusillus* (Nouani *et al*., 2011), a serine alkaline protease from *B. mojavensis* (Beg and Gupta, 2003) and mixed proteases from *B. amyloliquefaciens* (Cho *et al*., 2002) using 70-85% saturated ammonium sulfate increased their activity values by 3-fold, 6-fold, and 2.5-fold, respectively. However, partial purification of a novel protease from *B. licheniformis* strain K7A by $(NH4)_2SO_4$ fractionation (40-70%) plus dialysis increased the enzyme activity by 12-fold (Hadjidj *et al*., 2018).

The activity of the dialyzed enzyme was strongly inhibited in the presence of pepstatin (0.1 mM), iodoacetamide (10 mM), EDTA (10 mM) and PMSF (10 mM). The results of the inhibition study suggested that the enzyme preparation could contain all the four types of protease classes (Aspartic, Cysteine, and Serine and Metallo-proteases). Further purification studies or analytical fractionation of the crude enzyme would be required to elucidate this point with certainty. However, the low residual activity observed in the presence of pepstatin A (27%) could indicate the existence of one (or more) aspartic proteinase(s) in the crude enzyme mixture. However, it is interesting to observe that the activity of a purified serine alkaline protease from *B. mojavensis* was completely inhibited by more than one protease inhibitor agents; specifically PMSF (1mM) and iodoacetic acid (1mM) (Beg and Gupta, 2003). In contrast, the activities of milk-clotting protease from *B. licheniformis* USC13 (Ageitos *et al*., 2007) and novel protease from *B. licheniformis* K7A (Hadjidj *et al*., 2018) were completely inhibited only by PMSF. In other studies, the milk-clotting activities of purified milk-clotting aspartic protease from Withania coagulants fruit were exclusively suppressed by pepstatin A (0.08mM) (Salehia *et al*., 2017).

In the present study, the dialyzed enzyme from *B. subtilis* SMDFS-2B showed maximum activity at 55 ^{o}C. Similar to the present work, the purified milk-clotting enzyme from *B. sphaericus* (El-Bendary *et al*., 2007) and *B. subtilis* natto (Wu *et al*., 2013) showed optimal milk-clotting activity at 55 ^{o}C and 60 ^{o}C, respectively. However, milk-clotting activity from *B. licheniformis* 5A5 (Ahmed and Helmy, 2012) and *B. amyloliquefaciens* JNU002 (Ding *et al*., 2012) that showed maximum activity at 75 ^{o}C and 70 ^{o}C, respectively, are more thermo-tolerant than the proteinase from *B. subtilis* SMDFS-2B (this study). Different from the present study, the highest milk-clotting activity for milk-clotting protease from *B. licheniformis* USC13 (Ageitos *et al*., 2007) and purified enzyme from *B. subtilis* MTCC 10422 (Kumari *et al*., 2016) were obtained at 37 ^{o}C and 45 ^{o}C, respectively.

The thermal stability of the rennet enzyme was one of the most important criteria with respect to its potential applications. In this study, the result of the enzyme preparation from *B. subtilis* SMDFS-2B showed significant thermal stability. However, the MCA of the dialyzed enzyme was considerably reduced upon exposure at 60 ^{o}C. The enzyme retained 55% of its original activity after heating at 60 ^{o}C for 15 min; further incubation of the enzyme at 60 ^{o}C for 30 min caused a dramatic loss of its activity to 35%. The loss of milk-clotting activity upon increasing the reaction temperature may be interpreted to be due to the denaturation of the enzyme above the verified temperature (Wehaidya *et al*., 2016) and this maybe a technological advantage since no bitterness development can occur during cheese ripening due to the inactivated proteolytic action of the enzyme after the cooking process of the curd (Sousa *et al*., 2001).

The residual activity obtained for bacterial rennet from *B. licheniformis* 5A5 (73.4%) upon exposure for 1 h at 40 ^{o}C (Ahmed and Helmy, 2012), MCE from *B. subtilis* (81.3%) after incubation for 10 min at 55 ^{o}C (Ding *et al*., 2011), enzyme from *B. amyloliquefaciens* JNU002 (52.84%) after incubating for 20 min at 50 ^{o}C (Ding *et al*., 2012), enzyme from *B. sphaericus* (30%) after 20 min incubation at 60 ^{o}C (El-Bendary *et al*., 2007), enzyme from *B. subtilis* natto (47%) after incubation for 40 min at 55 ^{o}C (Shieh *et al*., 2009) and enzyme from *B. subtilis* natto (50%) after incubation at 60 ^{o}C for 20 min (Wu *et al*., 2013) were comparable with the present study.

The pH had a significant effect on the activity of the milk-clotting enzyme. The dialyzed enzyme obtained from *B. subtilis* SMDFS-2B showed the highest milk-clotting activity at pH 5.5. The result from the current study inferred that the enzyme works best at acidic pH than alkaline pH. Similar to the present study, the maximal activity for milk-clotting enzyme from *B. subtilis* (Wehaidya *et al*., 2016), *B. subtilis* B1(Ding *et al*., 2011), *B. amyloliquefaciens* D4 (He *et al*., 2011), *B. amyloliquefaciens* JNU002 (Ding *et al*., 2012), *B. subtilis* natto (Wu *et al*., 2013) and *B. subtilis* MTCC 10422 (Kumari *et al*., 2016) were shown at pH 5.0, 5.5, and 6.0, respectively. On the other hand, the highest activities for milk-clotting protease from *B. licheniformis* strain USC13 (Ageitos *et al*., 2007) and milk-clotting enzyme from *B. sphaericus* (El-Bendary *et al*., 2007) were obtained at pH 7.5 and 6.0-7.5, respectively.

The enzyme showed a narrow range of pH stability after incubation at different pH at room temperature. The dialyzed enzyme retained 50-80% of its milk-clotting activity upon incubation at pH 5.0-6.5 for 1.0 h. However, any change of pH at either end of these ranges caused a significant reduction in residual milk-clotting activity. These findings are

in accordance with values reported for purified milk-clotting enzymes from *B. amyloliquefaciens* JNU002 (Ding *et al*., 2012), *B. amyloliquefaciens* D4 (He *et al*., 2011), *B. subtilis* natto (Shieh *et al*., 2009) and *B. subtilis* natto (Wu *et al*., 2013). In another study, the milk-clotting enzyme from *B. subtilis* MTCC 10422 was shown stability in both acidic and basic pH (Kumari *et al*., 2016).

The enzyme showed an increment in its MCA with increasing skim-milk concentration. The highest milk-clotting activity was achieved at 20% skim-milk concentration. However, the maximal enzyme activity for milk-clotting enzyme (MCE) from *B. subtilis* (Wehaidya *et al*., 2016), *B. amyloliquefaciens* D4 (He *et al*., 2011), *B. licheniformis* 5A5 (Ahmed and Helmy, 2012) and *B. sphaericus* (El-Bendary *et al*., 2007) were attained at 0.4%, 5%, 6% and 9% substrate concentration, respectively. The differences between the present finding and others could be inferred to the impurity and handling of the crude enzyme used in this study.

The effect of several metal ions on the MCA of the dialyzed enzyme was determined. The MCE was activated by most of the metal ions tested except Ni^{2+}, Cu^{2+}, and Co^{2+}. The activity of MCE extremely stimulated by $MnSO_4$, $FeSO_4$, $MnCl_2$, $MgCl_2$, and $CaCl_2$. Similarly, MCE from *B. licheniformis* 5A5 (El-Bendary *et al*., 2007), *B. subtilis* (Ding *et al*., 2011), *B. amyloliquefaciens* D4 (He *et al*., 2011) and *B. amyloliquefaciens* JNU002 (Ding *et al*., 2012) were also strongly triggered by Mn^{2+}, Ca^{2+}, Ca^{2+}, and Ca^{2+}, respectively. In other studies, Mg^{2+} and Mn^{2+} also showed a stimulatory effect on the MCA of MCE from *B. subtilis* MTCC 10422 (Kumari *et al*., 2016). Whereas, Ni^{2+}, Cu^{2+}, and Co^{2+} inhibited the MCA of MCE from *B. sphaericus* (Ding *et al*., 2012; El-Bendary

et al., 2007). Likewise, Co^{2+} was found to have an inhibitory effect on the MCA of MCE from *B. subtilis* MTCC 10422 (Kumari *et al*., 2016).

Calcium had a positive effect on the activity of the MCE. It has been described as important in milk clot formation when its concentration is high enough (He *et al*., 2011). It is known that Ca^{2+} combines with ρ-casein to form a firm clot during the second phase of the clotting process. The addition of $CaCl_2$ to milk causes a reduction in milk-clotting time by rennet, this increases the rate of coagulation (Ahmed and Helmy, 2012). In the present study, the highest MCA of the dialyzed enzyme was obtained at 10 mM and 50 mM concentration of $MnSO_4$ and $CaCl_2$, respectively. Similar to this study, the maximum activity for MCE from *B. subtilis* B1 (Ding *et al*., 2011) and *B. amyloliquefaciens* JNU002 (Ding *et al*., 2012) were obtained at 50 mM and 60 mM $CaCl_2$ concentration respectively. On the other hand, the optimal milk-clotting activity for MCE from *B. licheniformis* 5A5 (Ahmed and Helmy, 2012) and *B. amyloliquefaciens* D4 (He *et al*., 2011) were detected at 10 mM and 25 mM $CaCl_2$ concentration respectively.

6.2.3. Conclusion

In the present study, the milk-clotting enzyme produced from *B. subtilis* SMDFS-2B by submerged fermentation revealed attractive properties. The partial characterization results have been shown that the enzyme exhibited optimum activity at 55 ^{o}C and pH 5.5. Further studies on the purification and full characterization of the enzyme(s) present in the crude preparation are in preparation.

Chapter 7

7. Cloning and expression of an active aspartic proteinase gene from *Aspergillus oryzae* DRDFS13 in *Pichia pastoris*

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Abstract

Pichia pastoris is a yeast widely used in expressing recombinant proteins from eukaryotic organisms. The aim of the present work is to study the expression of an aspartic protease gene from *A. oryzae* DRDFS13 into *P. pastoris*. In the present study, the total RNA was extracted from *Aspergillus oryzae* DRDFS13 and reverse transcribed into cDNA using first strand cDNA synthesis kit. The gene for aspartic protease was amplified, sequenced and then cloned into pGAPZαA for further expression in *P. pastoris*. The recombinant yeast (*P. patoris* X-33Ap) was cultivated in YPD media at pH 5 and 7 for 6 days and the production of recombinant proteins was checked by total protein determination, milk-clotting activity assay, and SDS-PAGE analysis. The gene sequence results showed 98% similarity with aspartic protease gene from *A. oryzae* RIB40. The aspartic protease gene cloned into pGAPZαA (later pMKAP) was successfully expressed in *P. pastoris* as an active extracellular protease with the highest MCA (190.47 U/mL) of secreted enzyme from the recombinant yeast was obtained at pH 5 and 6 days of incubation time. The major protein expressed by the recombinant *P. pastoris* X-33 AP has a molecular mass between 32 and 46 kDa. When analyzed for clotting activity, the protein was able to clot skim-milk in 2 min. The clotting activity was found to be 190.47 U/mL. Thus, the

obtained recombinant protein could be applied for cheese production. However, further study of the recombinant proteins need to be carried out and its application in cheese production by analyzing the organoleptic and chemical properties of the cheese produced.

Keywords: *Aspergillus oryzae*, Milk-clotting activity, Recombinant protein, Recombinant yeast

7.1. Results

7.1.1. cDNA synthesis and amplification of aspartic protease gene

The concentrations of RNAs and aspartic protease gene were 140.1 ng/µL and 75.4 ng/µL, respectively. The size of the aspartic protease gene showed a highly intensive clearly visible band between 1.2 kbp and 1.5 kbp (Fig. 7.1). According to the results from the amino acid sequencing (Fig. 7.2), the aspartic protease gene of interest showed 98% similarity with the aspartic protease gene from *Aspergillus oryzae* RIB40.

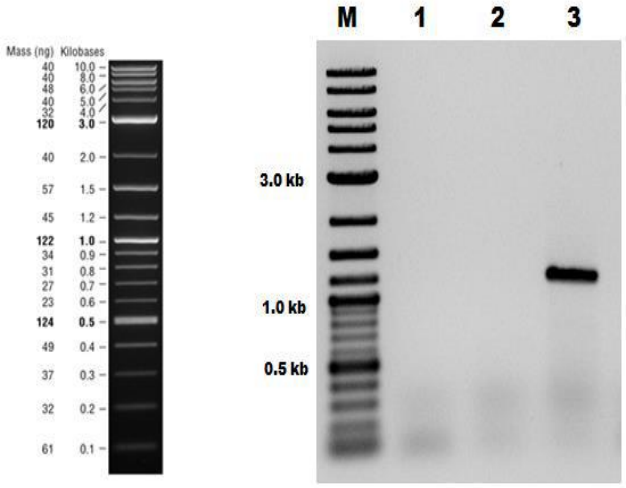

Figure 7.1: Agarose gel electrophoresis of marker protein and PCR product for aspartic protease gene. Bands on molecular marker indicated kilobases, Lane M is the marker protein, Lane 1 and 2: Negative control Lane 3: aspartic protease gene

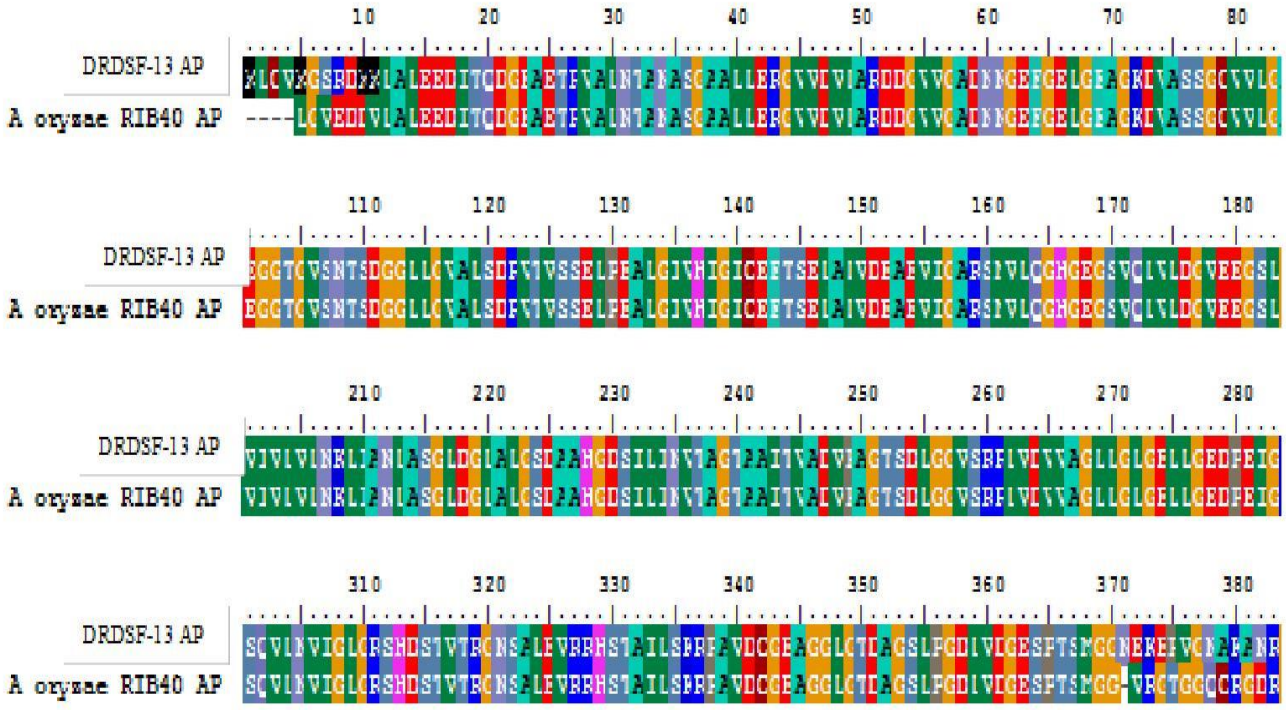

Figure 7.2: The amino acid sequence alignment of the aspartic protease gene, DRDFS-13 AP: the aminoacid sequence of aspartic protease gene from *Aspergillus oryzae* DRDFS13, *A. oryzae* RIB40 AP: the aminoacid sequence of aspartic protease gene from *Aspergillus oryzae* RIB40.

7.1.2. Determining the vectors concentration

The concentrations of pGAPZαA vector (Fig.7.3) expression vector pMK-AP (Fig.7.4)

were determined by NanoDrop 2000 and found 186.4 ng/ µL 55.8 ng/ µL.

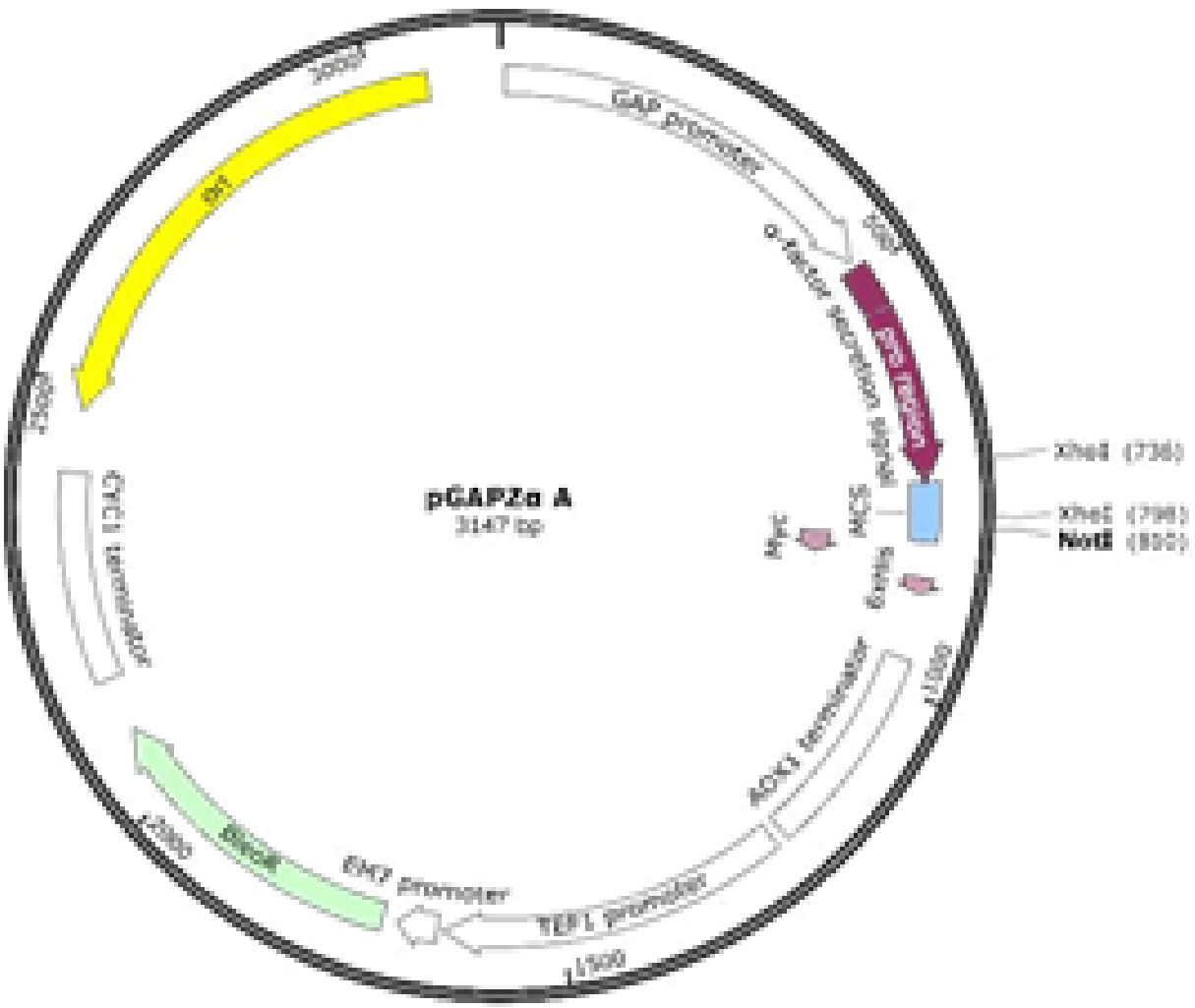

Figure 7.3: Representation of pGAPZαA vector including the site of the restriction enzymes XhoI and NotI used for cloning.

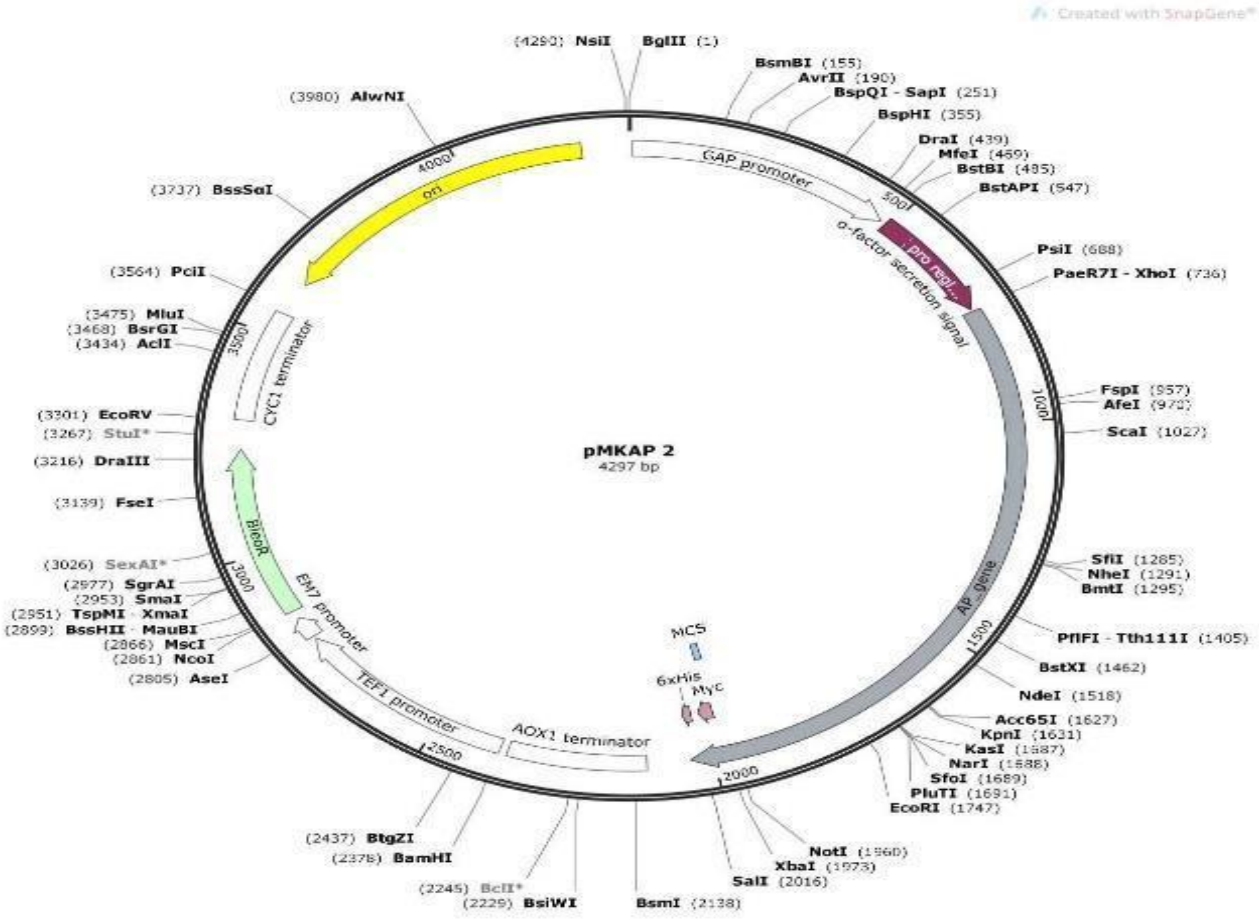

Figure 7.4: Schematic representation of the pMK-AP vector used for protein expression.

7.1.3. Milk-clotting activity

The milk-clotting activity of crude extract collected from *P. pastoris* X-33 AP (transformed) and *P. pastoris* X-33 (wild type, control) was determined at 2nd, 4th and 6th day. The crude enzyme extracted from *P. pastoris* X-33 AP (transformed) using YPD media at pH 7 and the *P. pastoris* X-33 (control) did not show milk-clotting activity up on 6 days of incubation. However, the crude enzyme extracted from *P. pastoris* X-33 AP (transformed) cultivated in YPD media at pH 5 showed amplified milk-clotting activities as the fermentation time increased. The specific activity of the crude enzyme was also improved as the fermentation time was increased (Table 7.1). On the other hand, the total protein contents of the crude enzyme did not show significant differences at the time of

incubation increased. But there is a slight increase in protein concentration for crude enzyme extracted from control on the 6th day (Table 7.1).

Table 7.1: The TP, MCA and specific activity of the crude enzyme from *P. pastoris* X-33 AP (transformed) at different pH and fermentation time in comparison with original fungi strain (*A.oryzae* DRDFS 13)

Microbes	Initial Media pH	Cultivation time (days)	MCA (U/mL) Mean±STD	Total protein (mg/mL)	Specific Activity (U/mg)	Cultivation time (days)	*A. oryzae* DRDFS13 (original Fungi) MCA (U/mL) Mean±STD
Recombinant *P. pastoris* X.33 AP	5.00	2	43.63±0.03^{e}	6.10	7.14	1	ND^{f}
	7.00	2	ND^{f}	5.84	_	2	ND^{f}
	5.00	4	92.31±0.30^{b}	6.24	14.79	3	ND^{f}
	7.00	4	ND^{f}	5.51	_	4	63.76±6.62^{d}
	5.00	6	190.47±0.20^{a}	6.19	30.75	5	79.60±3.16^{c}
	7.00	6	ND^{f}	5.44	_	6	70.20±1.20^{d}

*ND- milk clotting activity not determined within 40 min, STD: standard deviation, Mean: is average of two measurements, Different letters (a, b, c, d) designate significantly different means as determined by Duncan multiple mean comparison test (P<0.05).

7.1.4. SDS-PAGE analysis

The presence of an aspartic protease in the crude enzyme extract was checked by SDS-PAGE analysis. Two types of gels were run, one containing the un-concentrated samples and the other containing the concentrated samples (Fig. 7.5). As shown in figure 7.5A, there is a molecular marker in lane 1; and there are two slightly visible bands observed in lane 6 (pointed with the black arrow) with a molecular mass of between 32 and 46 kDa. Since the gel run with the un-concentrated samples, the two bands did not clearly visible. On the other hand, the other SDS-PAGE run with concentrated samples (Fig 7.5B) clearly showed visible bands between 32 and 46 kDa at lane 2, 4 and 6. However, some other protein bands with molecular mass above 80 kDa were also observed on the SDS-PAGE gel (Fig. 7.5B).

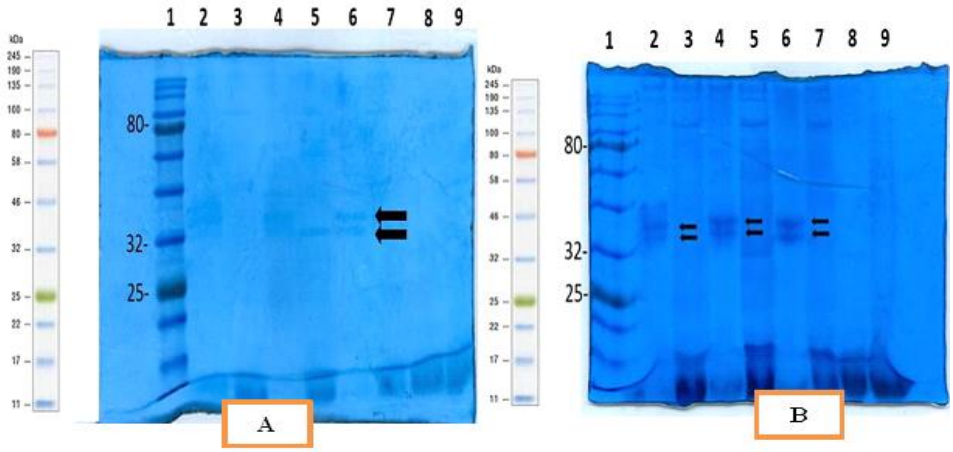

Figure 7.5: (A): Scanned picture of SDS-PAGE gel loaded with un-concentrated samples after staining with Coomassie Blue Brilliant solution. In lane 1, the molecular protein standard (kDa) was loaded. Lanes 2-9 were loaded with samples as follows: day 2 pH5, pH7, day 4 pH5, pH7, day 6 pH5, pH7 and day 6 controls pH5 and pH7. (B): depicts the SDS-PAGE gel after Coomassie Blue Brilliant staining. The molecular marker is shown in lane 1, and the lanes 2-9 are loaded with concentrated samples from day 2 pH5, pH7, day 4 pH5, pH7, day 6 pH5, pH7 and day 6 controls pH5 and pH. The black arrows in lanes 2, 4 and 6 indicate the bands between 32 and 46 kDa

7.2. Discussion

The expression of aspartic protease gene in *P. pastoris* was investigated by using plasmid harbouring AP gene sequence. Initially, an aspartic protease from *Aspergillus oryzae* DRDFS 13 was cloned into pGAPZαA, that expresses protein using a constitutive GAP promoter with a secretion signal sequence (the S. cerevisiae α-MF pre-pro signal sequence) for secreting needed protein in the culture media.

The concentration of RNA and aspartic protease gene was 140.1 ng/µL and 75.4 ng/µL, respectively. The amplified aspartic protease gene (fragment) had a size of about 1.2 kbp. Similarly, the gene encoding for milk-clotting aspartic protease (MCAP) from *M. circinelloides* strain DSM 2183 (Antonio *et al*., 2013), MCAP protein from *M. circinelloides* DSM 2183 (Kangwa *et al*., 2018), yak chymosin from Yak (Luo *et al*., 2016) and aspartic proteinase from *M. mucedo* DSM 809 (Yegin and Fernandez-Lahore, 2013) were 959 bp, 1229 bp, 1,008 bp and 1,200 bp long, respectively. This implies

that the gene amplified in the present study has a similar size with other aspartic protease gene indicating that the gene could be encode for aspartic protease enzyme.

The purified gene of interest was later sequenced. The results were analyzed in comparison to the gene sequence of aspartic protease from *Aspergillus oryzae* RIB40. Accordingly, the amino acid sequence alignment of the aspartic protease gene from *Aspergillus oryzae* DRDFS 13 showed 98% similarity with the aspartic protease gene from *A. oryzae* RIB40. This confirmed that the amplified gene from *A. oryzae* DRDFS13 may be a gene encoding aspartic protease enzyme (Antonio *et al*., 2013). Similarly, the deduced amino acid sequence for milk-clotting acid protease (MCAP) from *M. circinelloides* showed 88% similarity with *M. bacilliformis* pepA gene (Antonio *et al*., 2013). The deduced amino acid sequence of the pepA gene from *A. oryzae* has also shown 67% homology to the pep A gene of *A. awamori* (Gomi *et al*., 1993). In another study, the gene encoding aspartic proteinase of *M. mucedo* had 80 % similarity with the *Rhizopus niveus* gene for aspartic proteinase II and 73 % similarity with rhizopuspepsinogen precursor of *Rhizopus microsporus* var. *chinensis* (Yegin and Fernandez-Lahore, 2013).

The aspartic proteinase gene from *Aspergillus oryzae* DRDFS 13 was successfully expressed in *P. pastoris* under the control of the GAP promoter. The advantage of using a constitutive GAP promoter is that methanol is not required for induction, nor it is necessary to shift from one carbon source to another (Yegin and Fernandez-Lahore, 2013). This feature makes the cell growth and protein expression more convenient and straightforward (Yegin and Fernandez-Lahore, 2013). Antonio *et al*., (2013), reported the expression of the aspartic protease gene from *M. circinelloides* in *P. pastoris* under the

control of the constitutive GAP promoter. Similarly, the gene encoding an aspartic protease (MCAP) from *M. circinelloides* DSM 2183 cloned in *P. pastoris* was successfully expressed using both the native *M. circinelloides* signal peptide (mcSP) and α-factor secretion signal from *Saccharomyces cerevisiae* (α-MF) (Kangwa *et al.*, 2018). A novel aspartic protease gene (RmproA) from *Rhizomucor miehei* CAU432 was also cloned and successfully expressed in *P. pastoris* (Sun *et al*., 2018). In another study, an alkaline protease gene from *A. oryzae* and a serine protease gene from *Thermoascus aurantiacus* var. *levisporus* were cloned in *P. pastoris* GS115 were fruitfully expressed in *P. pastoris* (Guo and Ma, 2008; Li *et al*., 2011).

The milk-clotting activity of the crude extract collected from *P. pastoris* X-33 AP (transformed) and *P. pastoris* X-33 (wild type, control) was determined at 2^{nd}, 4^{th} and 6^{th} day. The highest MCA (190.47 U/mL) and specific activity (30.75 U/mg) of the secreted crude enzyme from the recombinant yeast were obtained at pH 5 after 6 days of incubation. The highest MCA of the crude enzyme from the recombinant yeast (190.47 U/mL) was better than the maximum MCA (79.60 U/mL) recorded for the crude enzyme extracted from the original organism (*A. oryzae* DRDFS13) used as a source of aspartic protease gene for cloning. Similarly, the maximum MCA recorded for recombinant enzyme (210 U/mL) from *M. mucedo* DSM 809 expressed in *P. pastoris* (Yegin and Fernandez-Lahore, 2013) and the recombinant MCAP (257 CU/mL) from *M. circinelloides* expressed in *P. pastoris* (Antonio *et al*., 2013) was comparable with this study. However, the milk-clotting activity recorded from recombinant *P. pastoris* with plant milk-clotting aspartic protease (23 CU) (Feijoo-siota *et al*., 2018) and recombinant

P. pastoris with bovine chymosin B (96 IMCU/mL) (Noseda *et al*., 2013) was lower than the present study.

The data also showed substantial milk-clotting activity (MCA) of the recombinant yeast was obtained only at pH 5; whereas, the crude enzyme from recombinant yeast cultivated on YPD media at pH 7 did not possess milk-clotting activity. This could be due to the fact that aspartic proteases may be produced under acidic conditions indicating that the initial pH of the media plays an important role in protein expression. Antonio *et al*., (2013) also reported the highest milk-clotting activity for aspartic protease from recombinant yeast (X-33/pGAPZα+MCAP-5) in YPD medium at pH 5.0. Interestingly, the enzyme was even induced in *P. pastoris* X-33 from *M. mucedo* DSM 809 at an initial medium pH of 3.5 (Yegin and Fernandez-Lahore, 2013). On the other hand, maximum enzyme activity was detected from recombinant *P. pastoris* at 72 h showing a difference in activity as a function of time (Antonio *et al*., 2013; Yegin and Fernandez-Lahore, 2013). The differences in the time of maximum MCA production could be due to the media and physicochemical parameters used for cultivation of the recombinant yeast. A component of media may have a positive or negative impact on the early induction of aspartic protease genes.

The MCA and specific activity of the transformed crude enzyme increased as the fermentation time increased. However, the total protein content of the crude enzyme did not show significant differences at the time of incubation increased. Though, there was a slight increase in protein concentration for the crude enzyme extracted from the control on the 6^{th} day. Comparatively, a slightly higher protein concentration from recombinant

yeast was noticed at 96 h of fermentation time. Likewise, the cultivation of recombinant *P. pastori*s under optimized conditions produced maximum protein concentration at 72 h (Yegin and Fernandez-Lahore, 2013).

The major protein expressed by recombinant *P. pastoris* X-33 AP has a molecular mass between 32 and 46 kDa. However, some other proteins with molecular mass above 80 kDa were also observed on the SDS-PAGE. This may infer to be either from the proteins contained in the media, or some other proteins expressed apart from the protein of interest. Similarly, the SDS-PAGE analysis for the recombinant Rmap (extracellular aspartic proteinase) secreted by *P. pastoris* transformants, MCAP secreted by recombinant yeast X-33/pGAPZα+- SyMCAP-6, MpAPr1 (an aspartic protease gene) secreted by recombinant *Komagataella pastoris* X-33, *Mucor pusillus* rennin expressed in *P. pastoris*, recombinant chymosin in *P. pastoris* and progaline B (novel plant milk-clotting aspartic protease) in *P. pastoris* showed a single band of approximately 33.5 kDa (Schoen *et al*., 2002), between 33 kDa and 37 kDa (Antonio *et al*., 2013), 43.3 kDa (Theron and Divol, 2017), between 30 to 45 kDa (Noseda *et al*., 2013) and 48 kDa (Feijoo-siota *et al*., 2018), respectively. However, the recombinant aspartic proteinase expressed in *P. pastoris* with MW of 52.4 kDa (Sun *et al*., 2018) and 46–58 kDa (Yegin and Fernandez-Lahore, 2013) by SDS-PAGE was different from the present study.

7.3. Conclusion

Based on the results it can be concluded that the aspartic protease gene from *Aspergillus oryzae* DRDFS 13 cloned and expressed in *P. pastoris* X-33 AP was a functionally active protein with significant milk-clotting activity. Therefore, the milk-clotting protease extracted from the recombinant yeast may be a suitable candidate for application in cheese and other food industries.

Chapter 8

8. Danbo cheese production using microbial aspartic protease enzyme from *Aspergillus oryzae* DRDFS13 and *Bacillus subtilis* SMDFS 2B

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Abstract

This study aimed to investigate the efficiency, biochemical composition and sensory quality of Danbo cheese produced using proteases derived from indigenous fungus and bacterium compared to the commercial product. A fungal enzyme and bacterial enzyme were produced by solid state and submerged fermentation from *A. oryzae* DRDFS13 and *B. subtilis* SMDFS 2B, respectively. The crude enzyme was partially purified by dialysis and used for Danbo cheese production using commercial rennet as a control. The Danbo cheese produced using dialyzed fungal enzyme (E1), dialyzed bacterial enzyme (E2) and commercial rennet (C) were analyzed for body property, organoleptic characteristics, proximate and mineral composition when fresh and after 2 months of ripening. There was no significant difference in the cheese yield among the three treatments. The overall organoleptic characteristics of Danbo cheese produced by the fungal enzyme (5.5) were similar with control cheese produced by commercial rennet (5.3). Both cheese types were significantly different in organoleptic properties from Danbo cheese produced by bacterial enzyme (4.9). There was no significant difference ($p>0.05$) in the proximate composition between the ripened Danbo cheese produced by fungal enzyme and the control cheese except for crude protein content. However, the ripened cheese products

showed significant difference in their mineral composition except for sodium. In conclusion, this study demonstrated that the fungal enzyme from *Aspergillus oryzae* DRDFS 13 is more appropriate for Danbo cheese production than the bacterial enzyme from *Bacillus subtilis* SMDFS 2B. However, it requires further application of the enzymes for production of other cheese varieties.

Keywords: Danbo cheese**,** Minerals, Proximate composition, Organoleptic characteristics, Ripened chees

8.1. Results

8.1.1. Characteristics of dialyzed enzyme

The physical and chemical properties of the enzymes derived from the newly isolated bacterial and fungal strains in Ethiopia are reported in Table 8.1. There was no significant difference in titrable acidity (TTA) and total protein content (TP%) between the enzymes extracted from the two sources ($p<0.05$). However, the bacterial enzyme had a 2.5- and 7.0-fold higher in milk-clotting and protease-activities than the fungal enzyme ($p<0.05$), respectively. In contrast, the MCA/PA ratio was significantly higher ($p<0.05$) in the fungal enzyme than the bacterial enzyme (Table 8.1).

Table 8.1: Characteristics of the dialyzed bacterial and fungal enzyme

Parameters	Dialyzed Bacterial Enzyme	Dialyzed Fungal Enzyme
pH	5.96 ± 0.00^{a}	4.81 ± 0.01^{b}
TTA (%)	0.04 ± 0.00^{b}	0.07 ± 0.00^{a}
TP (%)	0.30 ± 0.00^{a}	0.52 ± 0.02^{a}
MCA (U/mL)	521.99 ± 11.35^{a}	266.70 ± 2.96^{b}
PA (U/mL)	1623.62 ± 6.10^{a}	231.24 ± 4.80^{b}
Ratio (MCA/PA)	0.32 ± 0.01^{b}	1.15 ± 0.01^{a}

TTA: Titrabile Acidity, TP: Total Protein, MCA: Milk Clotting Activity, PA: Protease Activity, Data are expressed as mean ± SD. (n=2) on wet basis. Means in the same row with different superscripts are significantly different at $p<0.05$.

8.1.2. Aflatoxin determination

The aflatoxin types detected from the crude enzyme derived from *A. oryzae* DRDFS13 were B1, B2, G1, and G2 with <0.1 µg /Kg (Table 8.2).

Table 8.2: Aflatoxin concentration of crude enzyme extract from *A. oryzae* DRDFS13

Types of aflatoxins	Concentration (µg/Kg)
B_1	<0.1
B_2	<0.1
G_1	<0.1
G_2	<0.1
Total	<0.4

8.1.3. Cheese and whey yield

In this study, Danbo cheese was successfully produced using the enzymes from the two sources. The desirable characteristic of the different cheese products is reported in Table 8.3. There was no significant difference in the cheese yield upon the three treatments ($p<0.05$). The cheese made with commercial rennet was firm and acceptable. Similarly, the cheese made with the fungal enzyme was slightly firm and acceptable. However, the bacterial enzyme cheese was watery. In fact, the whey yield was significantly different ($p<0.05$) upon the three treatments, in order of CR>DFE>DBE.

Table 8.3: Cheese and whey yield from commercial rennet, dialyzed fungal & bacterial enzyme

Treatment	Whey yield (%)	Cheese yield (%)	Body properties
Commercial rennet	40.0	9.0	Firm and acceptable
Dialyzed fungal enzyme	37.0	8.6	Slightly firm and acceptable
Dialyzed bacterial enzyme	27.5	8.9	Watery body

8.1.4. Sensorial characteristics of fresh Danbo cheese

The result of the rating acceptance sensory test of the Danbo cheeses is reported in Table 8.4. The color score was in the acceptance order of CR>DFE>DBE. There was significant difference in flavor among the treatments. However, there was no significant difference in odor and texture scores between the three cheeses.

Table 8.4: Sensorial Characteristics of fresh Danbo Cheese

Parameters	Danbo cheese produced by CR	Danbo cheese produced by DFE	Danbo cheese produced by DBE
Color (7%)	5.63 ± 0.71^{a}	4.79 ± 1.13^{b}	4.28 ± 1.26^{c}
Odour (7%)	5.64 ± 1.09^{a}	5.64 ± 1.23^{a}	4.99 ± 1.32^{a}
Flavour (7%)	5.36 ± 0.88^{a}	4.98 ± 1.12^{ab}	4.82 ± 1.03^{b}
Texture (7%)	5.54 ± 0.94^{a}	5.34 ± 1.10^{a}	5.30 ± 1.10^{a}
Overall acceptability (7%)	5.54^{a}	5.29^{a}	4.84^{b}

Data are expressed as mean $\pm$ SD. Mean values in the same row with different superscripts are significantly different at $p<0.05$. CR: Commercial Rennet, DFE: Dialyzed Fungal Enzyme, DBE: Dialyzed Bacterial Enzyme

8.1.5. pH and TTA liquids samples produced during Danbo cheese production

The pH and titrable acidity (TTA) of the three liquid products are shown in Table 8.5. The inoculation of the raw milk with mesophilic *S. lactis* (LAB) reduced the pH of the milk by 0.7-1.0 unit. The renneting with the crude fungal and the commercial enzymes slightly decreased the pH. Also, there was significant difference in titrable acidity (TTA) among the treatments (Table 8.5).

Table 8.5: PH and TTA of raw milk and liquid samples During Danbo cheee processing

Parameters	Liquid samples produced during Danbo cheese processing						
	Raw Milk	CR after LAB addition	CR after Renneting	DFE after LAB addition	DFE after Renneting	DBE after LAB addition	DBE after Renneting
pH	6.65 ± 0.03^{a}	5.95 ± 0.01^{c}	5.82 ± 0.02^{d}	5.70 ± 0.02^{e}	5.60 ± 0.03^{f}	5.70 ± 0.00^{e}	5.87 ± 0.01^{d}
TTA	0.12 ± 0.00^{d}	0.14 ± 0.00^{c}	0.12 ± 0.00	0.15 ± 0.00^{b}	0.15 ± 0.00	0.16 ± 0.00^{a}	0.14 ± 0.00^{c}

Data are expressed as mean ± SD (n=2). Mean values with the different letters within a row are significantly different at $p<0.05$. LAB: Lactic acid bacteria, TTA: Titrabile Acidity, CR: Commercial Rennet, DFE: Dialyzed Fungal Enzyme, DBE: Dialyzed Bacterial Enzyme

8.1.6. Proximate composition of liquid samples produced during Danbo cheese production

The addition of the LAB drastically decreased the crude fat content by 12-30%, while the crude enzymes from the fungus and bacterium slightly decreased the crude fat contents after renneting. The renneting treatment also decreased the carbohydrate and energy contents of the samples; where the decrease in the latter was higher with the commercial rennet than the crude enzyme treatments (Table 8.6).

Table 8.6: Proximate composition of raw milk and liquid samples during Danbo cheese processing

Parameters	Liquid samples were taken during Danbo cheese processing						
	Raw Milk	CR after LAB addition	CR after Renneting	DFE after LAB addition	DFE after Renneting	DBE after LAB addition	DBE after Renneting
Moisture (%)	88.93±0.01^{de}	88.62±0.05^{de}	90.73±0.05^{b}	88.40±0.02^{e}	89.73±0.38^{c}	88.81±0.01^{de}	89.18±0.31^{dc}
Crude protein (%)	2.96±0.01^{cd}	3.15±0.01^{a}	3.06±0.01^{ab}	2.87±0.01^{de}	2.85±0.02^{e}	2.87±0.01^{de}	3.05±0.07^{bc}
Crude fat (%)	4.00±0.00^{a}	2.81±0.01^{e}	2.00±0.00^{g}	3.51±0.00^{b}	3.05±0.05^{d}	3.23±0.08^{c}	2.70±0.00^{f}
Ash (%)	0.48±0.01^{e}	0.62±0.01^{c}	0.79±0.01^{a}	0.69±0.00^{b}	0.70±0.00^{b}	0.48±0.01^{e}	0.64±0.02^{c}
Carbohydrates (%)	3.64±0.01^{f}	4.33±0.01^{d}	3.40±0.01^{g}	4.58±0.01^{bc}	4.07±0.05^{e}	4.53±0.02^{c}	4.32±0.11^{d}
Energy (Kcal/100 g)	62.38±0.02^{a}	55.22±0.08^{d}	43.84±0.08^{f}	60.85±0.38^{b}	55.13±0.17^{d}	58.61±0.65^{c}	53.76±0.74^{e}

LAB: lactic acid bacteria, TTA: Titrable acidity, CR: Commercial Rennet, DFE: Dialyzed Fungal Enzyme, DBE: Dialyzed Bacterial Enzyme Mean: is average of two measurements and expressed as mean ±SD, Means with same letters within row are not significantly different while means with different letters within row are significantly different at $P<0.05$.

8.1.7. pH and TTA of Danbo cheese

The fresh Danbo cheeses produced in all the three treatments were salted by dipping into 20% NaCl solution for 48 h and ripened in the curing room at 10 ^{0}C for 2 months. In all cases, pH and TTA significantly increased in the ripened cheese irrespective of the treatments (Table 8.7).

Table 8.7: pH and TTA of Danbo cheese (before press, salted and ripened)

Parameters	CR Fresh cheese before press	CR salted cheese	CR Ripened cheese	DFE fresh cheese before press	DFE salted cheese	DFE Ripened cheese	DBE fresh cheese before press	DBE salted cheese	DBE Ripened cheese
pH	5.39±0.00^{g}	5.77±0.01^{e}	7.31±0.00^{b}	5.22±0.02^{h}	5.61±0.01^{f}	6.56±0.00^{c}	5.63±0.02^{f}	6.06±0.02^{d}	7.34±0.00^{a}
TTA	0.05±0.00^{e}	0.03±0.00^{f}	0.11±0.00^{c}	0.06±0.00^{d}	0.02±0.00^{g}	0.27±0.00^{a}	0.05±0.00^{e}	0.03±0.00^{f}	0.16±0.00^{b}

Data are expressed as mean $\pm$SD(n=2). Mean values within a row with different superscripts are significantly different at $p<0.05$.

TTA: Titrable Acidity, CR: Commercial Rennet, DFE: Dialyzed Fungal Enzyme, DBE: Dialyzed Bacterial Enzyme.

8.1.8. Proximate composition of Danbo cheese

Moisture content decreased in salted and ripened cheese, in which in the former the rate was higher. Among the three fresh cheese types, CR had the highest protein content followed by DBE and DFE, respectively. The crude protein content in the ripened cheese was in order of CR>DFE>DBE. In contrast, there was no significant difference in crude protein content among the three salted cheeses. Among the three cheese types, CR ripened cheese had the highest crude protein content (Table 8.8). Salted CR and DFE cheese types had higher crude fat content, while the DBE salted cheese had the lowest amount. There was no significant difference in the crude fat content among all the ripened cheese types ($p<0.05$). The ash content in the fresh DBE was significantly lower than the amount in the other two cheese types. In all the cases the salted cheese had higher ash content than the fresh and ripened cheeses (i.e. CR>DBE>DFE) except for DFE. The ripened cheese types had shown the highest carbohdrates contents in all the three treatment. The salted cheese types had highest total energy compared with the fresh and ripened cheese.

Table 8.8: Proximate composition of Danbo cheeses (before press, salted and ripened)

Parameters	CR Fresh cheese before the press	CR salted cheese	CR Ripened cheese	DFE fresh cheese before the press	DFE salted cheese	DFE Ripened cheese	DBE fresh cheese before the press	DBE salted cheese	DBE Ripened cheese
Moisture (%)	44.95±0.01^{c}	24.60±0.05^{h}	36.13±1.67^{f}	49.92±0.05^{b}	27.40±0.05^{g}	37.28±0.45^{f}	52.90±0.20^{a}	39.53±0.57^{e}	42.06±0.86^{d}
Crude protein (%)	21.38±0.01^{d}	26.61±0.02^{c}	30.73±0.22^{a}	11.65±0.01^{f}	25.20±0.10^{c}	28.50±1.45^{b}	14.77±0.01^{e}	26.17±0.04^{c}	21.62±0.16^{d}
Crude fat (%)	21.40±0.10^{b}	35.90±0.10^{a}	12.75±1.25^{e}	21.15±0.15^{bc}	35.05±0.05^{a}	14.88±2.43^{de}	19.10±0.10^{bc}	18.00±0.00^{cd}	11.93±0.21^{e}
Ash (%)	1.86±0.01^{e}	5.05±0.02^{a}	3.83±0.01^{bc}	1.64±0.00^{e}	3.51±0.01^{d}	3.66±0.10^{cd}	0.63±0.01^{f}	4.96±0.01^{a}	3.95±0.21^{b}
Carbohydrates (%)	12.18±0.00^{bcd}	7.71±0.02^{d}	16.55±0.65^{ab}	15.74±0.02^{ab}	9.08±0.02^{cd}	15.68±0.00^{ab}	12.90±0.01^{bc}	12.04±0.08^{bcd}	20.44±0.28^{a}
Energy (Kcal/100	326.84±0.86^{b}	460.38±0.744^{a}	303.92±12.93^{c}	299.91±1.31^{cd}	452.57±0.93^{a}	310.67±9.97^{cb}	282.54±0.90^{de}	314.83±0.51^{bc}	275.61±3.63^{e}

Data are expressed as mean $\pm$ SD and all analysis are done in duplicate. Mean values in the same row with different superscripts are significantly different at $p<0.05$. CR: Commercial Rennet, DFE: Dialyzed Fungal Enzyme, DBE: Dialyzed Bacterial Enzyme

8.1.9. Mineral contents of ripened Danbo cheese

The ripened cheese products showed significant difference in their mineral composition except for sodium (Table 8.9). The DBE and DFE cheese had significantly the highest concentration of (potassium, zinc) and (calcium and manganese), respectively. Zinc and potassium concentrations were higher in the microbial enzyme made cheeses than in the control. Similarly, calcium content was higher in the DFE and DBE made cheese than in the control. Manganese was not detected in the control cheese but significantly higher concentration was found in the DFE and DBE, cheese respectively. Magnesium content in CR cheese was the highest. Iron was not detected in all the samples.

In the present study, the average value of Na, K, Ca and Mg recorded from ripened Danbo cheeses were between 357-366 mg/100 g, 2.1-3.8 mg/100 g, 3.7-10.9 mg/100 g and 7.0-49.8 mg/100 g, respectively. The average value of Zn, Mn, and Fe recorded from ripened Danbo cheeses were between 0.1-0.8 mg/100 g, 0.0-23.2 mg/100 g, and 0.00 mg/100 g, respectively (Table 8.9).

Table 8.9: Mineral concentrations of ripened cheese (after 2 months of ripening)

Types of cheese	Mineral concentration in mg/100g						
	Sodium	**Potassium**	**Calcium**	**Magnesium**	**Zinc**	**Manganese**	**Iron**
CR cheese	356.50 ± 0.02	2.10 ± 0.01	3.74 ± 0.01	49.80 ± 0.04	0.10 ± 0.07	ND	ND
DFE cheese	364.07 ± 0.00	2.88 ± 0.00	10.91 ± 0.00	24.17 ± 0.01	0.56 ± 0.02	23.21 ± 0.44	ND
DBE cheese	366.31 ± 0.02	3.78 ± 0.00	9.11 ± 0.01	6.99 ±0 .031	0.834 ±0.08	6.29 ± 0.20	ND

ND: Not Detected, CR: Commercial Rennet, DFE: Dialyzed Fungal Enzyme, DBE: Dialyzed Bacterial Enzyme, Data are expressed as mean $\pm$SD(n=3).

8.2. Discussion

The aflatoxin content of the crude enzyme from *A. oryzae* DRDFS 13 used for Danbo cheese production was below the standard limit set by European Union (Koe, 1999). This indicated the safety of the crude enzyme for application in food production. In fact, other studies also marked that enzymes extracted from *Aspergillus* species are safe and Generally Recognized as Safe (GRAS) (Castro *et al*., 2014; Ichishima, 2018; Kumar *et al*., 2014; Kumura *et al*., 2017). Thus, it can be used as rennin substitute in coagulation of milk and manufacture of cheese (Amer *et al*., 2015). Similarly, the milk-clotting enzyme obtained from *Rhizomucor miehei* NRRL 2034 used for white soft cheese was free from aflatoxins B1, B2, G1, G2 in the study by Amer *et al*., (2015). Fazouane-Naimi *et al*., (2010) also reported that the culture supernatants from *A. niger* FFB1 used for cheese production was free from Ochratoxin A (OTA).

The raw cow milk used for the Danbo cheese making had pH, TTA, water, crude protein, crude fat, total ash and total carbohydrate values of 6.65, 0.12%, 88.93%, 2.96%, 4%, 0.48% and 3.64%, respectively. Similar chemical composition of cow's milk used for production of white pickeled and prato cheeses was reported by Çepepoğlu & Güler-Akın (2013) and Alves *et al*. (2013), respectively.

Substantial volumes of whey were discarded during the Danbo cheese production. The highest whey yield was obtained from commercial rennet followed by fungal and bacterial enzymes, respectively. The physico-chemical characteristics of the whey in the present study was similar, with whey samples reported by Omole *et al*., (2012). Similarly, the highest amount of cheese was yielded from commercial rennet enzyme as compared to fungal and bacterial enzymes. In contrast, higher yield of UF-Domiati cheese was

obtained by *M. mucedo* KP736529 enzyme (E-cheese) than control cheese produced by commercial calf rennet *(*Ayana *et al*., 2015). The yield of semi-hard cheese produced using commercial chymosin was slightly higher than the value in present study (Darnay *et al*., 2003).

As per the rating acceptance sensory test on the Danbo cheeses, color and flavor score was in the acceptance order of CR>DFE>DBE. However, there was no significant difference in odor and texture between the three cheeses. The less rating acceptance score for the DBE could be due to the non-specific catalytic activity of bacterial enzyme on casein as compared to the fungal enzyme (Yegin *et al*., 2010).

In previous studies, white soft cheese produced using milk-clotting enzyme from *M. miehei* NRRL 3420 (Amer *et al*., 2015), UF-soft cheese produced by *Rhizomucor miehei* NRRL 2034 coagulant (Abbas *et al*., 2013), fresh cheese manufactured with *A. niger* FFB1 enzyme *(*Fazouane-Naimi *et al*., 2010) and fresh goat cheese produced by *M. miehei* microbial coagulant (Garc´ıa *et* al., 2012) had similar organoleptic characteristics with control cheese produced by calf rennet. Also Çepepoğlu & Güler-Akın (2013) reported a higher sensory acceptability of fresh Turkish white cheese made using *A. niger var. awamori* recombinant chymosin than commercially made cheese. Thus, the fungal enzymes might be considered as potential substituents for the commercial rennet.

The results also showed that, the titrable acidity of Danbo cheeses decreased after salting but increased after ripening in all the experimental and control treatments. The decrease in titrable acidity after salting could be associated with diffusion of lactic acid from the cheese into the brine (Çepepoğlu and Güler-Akın, 2013). Similarly, the titrable acidity of

all Turkish white cheeses produced using different coagulants decreased during storage time of 30 days and then increased (Çepepoğlu and Güler-Akın, 2013). Similarly, the total acidity of experimental Domiati cheese manufactured using the *M. mucedo* KP736529 enzyme and control cheese were gradually increased during ripening for 60 days (Ayana *et al*., 2015).

Among the three fresh cheese types, CR had the highest protein content followed by DBE and DFE respectively. The crude protein content in the ripened cheese was in order of CR>DFE>DBE. Similarly, a higher total nitrogen content was reported in a control cheese (Domiati cheese manufactured by calf rennet) than experimental cheese (Domiati cheese manufactured with *M. mucedo* KP736529 enzyme) (Ayana *et al*., 2015). On the other hand, the UF-white soft cheese manufactured by *B. stearothermophilus* coagulant (Ahmed *et al*., 2016), fresh white soft cheese made *Rhizomucor miehei* NRRL 2034 rennet (Amer et al., 2015b) and fresh goat cheese produced by *M. miehei* coagulant (Garc´ıa *et al*., 2012) were revealed comparable protein content with control cheese produced by calf rennet.

Salted CR and DFE cheese types had higher crude fat content, while the DBE salted cheese had the lowest amount. The ash content in the fresh DBE was significantly lower than the amount in the other two cheese types. In all the cases the salted cheese had higher ash content than the fresh and ripened cheeses (i.e. CR>DBE>DFE). The salted cheese types had highest total energy compared with the fresh and ripened cheese. This could be dueto the removal of moisture content during salting which leads to an increase in crude fat and crude protein contents of the cheese.

In the present study, the crude fat contents were increased after salting but decreased after ripening for Danbo cheeses produced from commercial rennet and dialyzed fungal enzyme. However, the crude fat content was continuously decreased after salting and ripening for Danbo cheese produced using dialyzed bacterial enzyme. The highest crude fat was noticed from ripened Danbo cheese produced by fungal enzyme. Whereas the lowest crude fat was recorded from ripened Danbo cheese produced by bacterial enzyme. The lipid contents (5.5-10%) noticed from cream cheeses produced using purified milk-clotting enzyme by *Bacillus sp.* P45 is lower than the present study (Lemes *et al*., 2016). The low lipid content noticed in cream cheese could be attributed to the high moisture contents the cream cheese as compared to Danbo cheese.

In contrast to the present study, the fat contents of Turkish white cheeses remained steady in all treatments throughout the ripening period (Çepepoğlu and Güler-Akın, 2013). In other study, a higher fat content was detected in control cheese than cheese produced by fungal enzyme (Ayana *et al*., 2015). However, the fat contents UF-white soft cheese produced using *B. stearothermophilus* coagulant (Ahmed *et al*., 2016), prato cheese produced by *Thermomucor indicae-seudaticae* N31 enzyme (Alves *et al*., 2013), white soft cheese produced by *Rhizomucor miehei* NRRL 2034 rennet enzyme (Amer *et a*l., 2015b and fresh goat cheese produced by *M. miehei* coagulant (Garc´ıa *et al*., 2012) were similar with control cheese produced using commercial enzyme.

The total carbohydrates contents of Danbo cheese were decreased after salting and increased after ripening whereas the energy produced (Kcal/100g) from Danbo cheeses were increased after salting but decreased after ripening for all the three treatments. The

increase in energy after salting could be due to the increase in crude fat contents due to the remioval of moisture content.

The highest carbohydrate content was detected from ripened Danbo cheese produced using bacterial enzyme in comparison with fungal enzyme and commercial rennet. However the highest energy in Kcal was obtained from ripened Danbo cheese made by fungal enzyme. The carbohydrate contents of (5.34 to 9.01 %) and calories of energy (113.14-139.16 Kcal/100g) obtained from cream cheeses produced using purified milk-clotting enzyme by *Bacillus sp.* P45 is lower than the present study (Lemes *et al*., 2016). The low nutrient noticed in cream cheese could be attributed to the high moisture contents the cheese.

In the present study, both the experimental and control Danbo cheeses showed variable ash contents. However, the highest ash content was recorded from fresh salted cheese produced using commercial rennet. In contrast to this study, higher ash content was noticed from Prato cheese produced by *Thermomucor indicae-seudaticae* N31 protease as compared to control cheese produced using commercial coagulant (Merheb-dini *et al*., 2012).

The crude fiber was not detected in both experimental and control Danbo cheeses. The absence of fiber in the Danbo cheese could be associated to the limited use of only milk and enzyme as ingredients for Danbo cheese production rather than different flours. In contrast, a fiber content of 3.00 to 4.96% was detected from cream cheeses produced using purified milk-clotting enzyme by *Bacillus sp.* P45 (Lemes *et al*., 2016).

In the present study, the average value of Na, K, Ca and Mg recorded from ripened Danbo cheeses were between 356-366 mg/100 g, 2.1-3.8 mg/100 g, 3.7-10.9 mg/100 g and 7.0-49.8 mg/100 g, respectively. The concentration of sodium was the haighest in all three types of cheeses. This may be due to diffusion of sodium in to cheeses during dipping of cheese into 20% NaCl.

Similarly, the Na content (462 mg/100 g) (Lee, 2017) noticed from full fat cheese and K and Ca contents (K:1.0 mg/100 g and Ca: 11.5 mg/100 g) (Sulieman *et al*., 2012) detected from mozzarella cheese was equivalent with this study. The Mg content (27 mg/100 g) (Gore *et al*., 2016), (406 ppm)(González-martín *et al*., 2011), (40 mg/100 g) (Jaoude *et al*., 2010), (33 mg/100 g) (Lee, 2017) and (41 mg/100 g) (Mattera *et al*, 2016) detected from hard cheese cantal, from ripened cow's, ewe's and goat's cheeses, double crème white brined cheese, full fat cheese and *Pecorino d'Abruzzo* cheeses was also comparable with the present study.

However, the major minerals composition recorded from ripened prato cheese (Na: 6.5, K: 1.2, Ca: 13 and Mg: 0.5 g/kg) (Cichoscki *et al*., 2002), hard cheese cantal (Na: 682, K: 93 and Ca: 668. mg/100g) (Gore *et al*., 2016), ripened cow's, ewe's and goat's cheeses (Na:7.9, K: 1.2 Ca:7.9 g/Kg) (González-martín *et al*., 2011), double crème white brined cheese (Na: 995, K:57, Ca: 378 mg/100 g) (Jaoude *et al*., 2010), full fat cheese produced by high pH method (K: 107 and Ca:729) (Lee, 2017), *Pecorino d'Abruzzo* cheeses (Na: 581, K: 84, Ca: 669 mg/100 g) (Mattera *et al*., 2016) and ripened cheddar cheese (Na:675, K:85 and Ca:739 mg/100g) (Murtaza *et al*., 2014) were higher than the present study.

The average value of Zn, Mn, and Fe recorded from ripened Danbo cheeses were between 0.104-0.834 mg/100 mg, 0.00-23.205 mg/100 g, and 0.00 mg/100 g, respectively. The Zn content noticed from double crème white brined cheese (2.0 mg/100 g) was comparable with this study (Jaoude *et al*., 2010). On the other hand, the Zn (31 mg/kg) and Fe (3.0 mg/kg) content recorded from ripened Prato cheese are higher than the present study, while the Mn content (0.26 mg/100 g) is lower than this study (Cichoscki *et al*., 2002). Different from the present study, 0.7 mg/100 g of Fe was noticed from full fat cheese produced by high pH method (Lee, 2017).

8.3. Conclusion

Therefore, the results obtained from the body property, organoleptic characterstics, proximate composition and partly from mineral composition revealed that the fungal enzyme from *Aspergillus oryzae* DRDFS 13 is more appropriate from Danbo cheese production than the bacterial enzyme from *Bacillus subtilis* SMDFS 2B.

Chapter 9

9. Summary, Conclusion, and Recommendations

The present study on " Characterization of Aspartic Protease Enzyme from Fungi and Bacteria and its Potential Application for Cheese Production" was conducted with the major objective for searching potential microbial strains producing aspartic protease enzyme with better milk-clotting activity and less protease activity. The crude enzyme extract was purified and characterized to improve its milk-clotting activity and hence used for commercial application in dairy industries. The results found from the current study have been summarized as follows:

9.1. Aspartic protease enzyme from *Aspergillus oryzae* DRDFS13

- A total of 188 fungal isolates were recovered from soil and dung samples collected from some hot areas of Ethiopia. Seventeen fungal isolates with better milk-clotting activity under solid-state fermentation were successfully identified to the strain level by sequencing ITS gene.
- Finally, *Aspergillus oryzae* DRDFS 13 was chosen for further study. The cultivation parameter for maximum production of aspartic protease enzyme from *Aspergillus oryzae* DRDFS 13 was optimized under solid-state fermentation. Optimization of cultural conditions for the maximum yield of aspartic protease enzyme (168 U/mL) was obtained when *Aspergillus oryzae* DRDFS 13 was cultivated at 30 ^{o}C, pH 6.0, 55% moisture content, 0.5 mL ($1*10^{6}$) inoculum level for 120 h under solid-state fermentation using wheat bran as a solid substrate.
- The aspartic protease enzyme from *Aspergillus oryzae* DRDFS 13 was purified by size exclusion (SEC) and ion-exchange chromatography (IEC) and the highest

milk-clotting activity (477.11 U/mL) was noticed from IEC fraction A_8. Using IEC techniques, the enzyme was purified by 6.2 fold with 9.20% recovery. The enzyme had a molecular weight of 40 kDa and a specific activity of 183.50 U/mg.

- The enzyme had temperature optima at 60 ºC and pH optima at pH 5.0. The enzyme was nearly completely inhibited by pepstatin A. The aspartic protease was shown stability at pH 4.5-6.5 and temperature 35-45 ^{o}C.
- Metals such as $MgCl_2$, $FeCl_3$, $MnCl_2$, $MnSO_4$, and $FeSO_4$ showed a pronounced effect on the milk-clotting activity of the enzyme. The milk-clotting activity of the aspartic protease enzyme was increased uniformly as the concentration of skim-milk increased from 25 to 200 g/L.
- The dialized enzyme showed a Km and Vmax values of 17.50 mM and 1369 U, respectively.
- The gene encoding for aspartic protease of *Aspergillus oryzae* DRDFS13 was amplified, sequenced and cloned into *Pichia pastori.* The aspartic protease gene cloned into pGAPZαA (later pMKAP) was successfully expressed in *P. pastori* as an active extracellular protease.
- The highest MCA (190.47 MCU/mL) of the secreted enzyme from the recombinant yeast was obtained at pH 5 and 6 days of incubation time.
- The major protein expressed by the recombinant *P. pastori* X-33 AP has a molecular mass between 32 and 46 kDa.

9.2. The milk-clotting enzyme from *Bacillus subtilis* SMDFS 2B

- A total of 49 bacterial isolates were recovered from soil and dung samples collected from some hot areas of Ethiopia, and 14 bacterial isolates that exhibited

better milk-clotting enzyme production under SmF were successfully identified to the strain level by sequencing 16s rRNA gene. Finally, *B. subtilis* SMDFS 2B was selected for further study.

- The milk-clotting enzyme from *B. subtilis* SMDFS 2B was partially purified and characterized. The enzyme was purified by 2.07 fold using a dialysis tube and showed a specific activity of 36.56 U/mg.
- The MCE had temperature optima at 55 ^{o}C and pH optima at pH 5.5. The dialyzed enzyme showed stability at 35–40 ^{o}C for 15 min and at pH 5.5.
- The addition of Mg^{2+} and Mn^{2+} significantly increased the milk-clotting activity of the enzyme.
- The enzyme also showed an elevation in its milk-clotting activity with increasing the concentration of skim milk.

9.3.Application of partially purified enzyme

The application of milk-clotting enzyme from *A. oryzae* DRDFS 13 and *B. subtilis* SMDFS 2B was investigated by the production of Danbo cheese and analyzed for biochemical and nutritional changes during ripening up to 2 months. Comparatively, the Danbo cheese produced using aspartic protease enzyme from *A. oryzae* DRDFS 13 exhibited body property, organoleptic characteristics, proximate composition and mineral composition similar to commercial rennet.

9.4. Conclusion

The milk-clotting activity, specific activity, purification fold, biochemical characteristics, inhibition study, deglycosylation assay and the results obtained from organoleptic and proximate analysis of Danbo cheese confirmed that the aspartic protease enzyme from *Aspergillus oryzae* DRDFS 13 is more appropriate for commercial application in the dairy industry than the milk-clotting enzyme from *B. subtilis* SMDFS2B.

9.5. Recommendations

- Whole-genome sequencing and microbial safety analysis should be conducted for *Aspergillus oryzae* DRDFS 13 and *Bacillus subtilis* SMDFS 2B.
- The aspartic protease enzyme from *A. oryzae* DRDFS13 should be produced on large scale with the developed strain.
- The production of mycotoxins other than aflatoxins by *A. oryzae* DRDFS13 should be determined by standard methods. The isoelectric point of the purified aspartic protease enzyme from *A. oryzae* DRDFS13 should be determined.
- The MCE from *B. subtilis* SMDFS 2B should be purified by IEC and SEC chromatography and its molecular weight will be determined by SDS-PAGE.
- The MCE from *A.oryzae* DRDFS 13 and *B. subtilis* SMDFS 2B should be immobilized using calcium alginate beads and checked for the biochemical characteristics of immobilized enzyme.
- The recombinant protein produced from transformant *P. pastori* X-33 AP should be used in Danbo cheese production in comparison with commercial rennet.
- Further application of the aspartic protease enzyme from *A.oryzae* DRDFS 13 and *P. pastoris* X-33 AP for food industries other than Dairy (Brewery, Winery, and Bakery), and Pharmaceutical industries.

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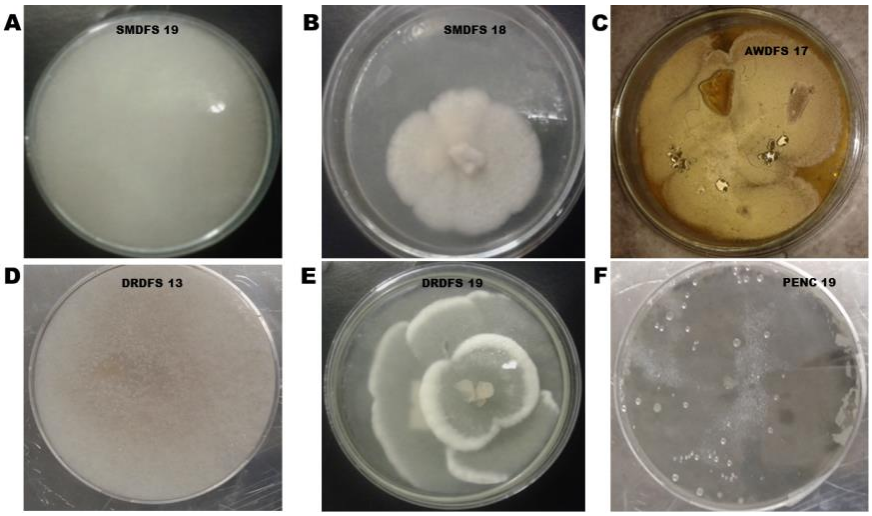
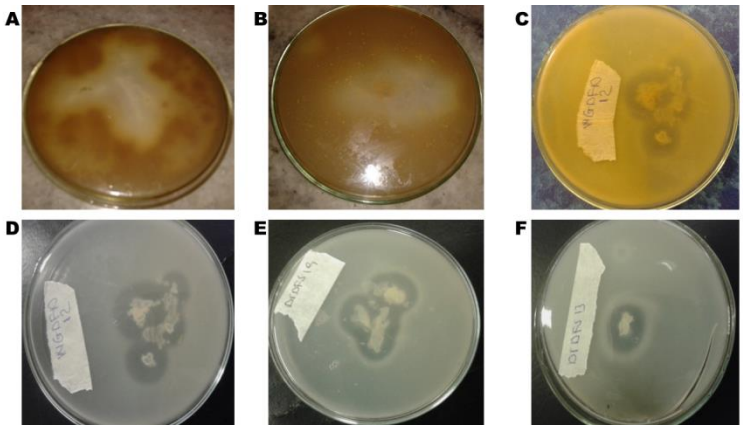
Appendices

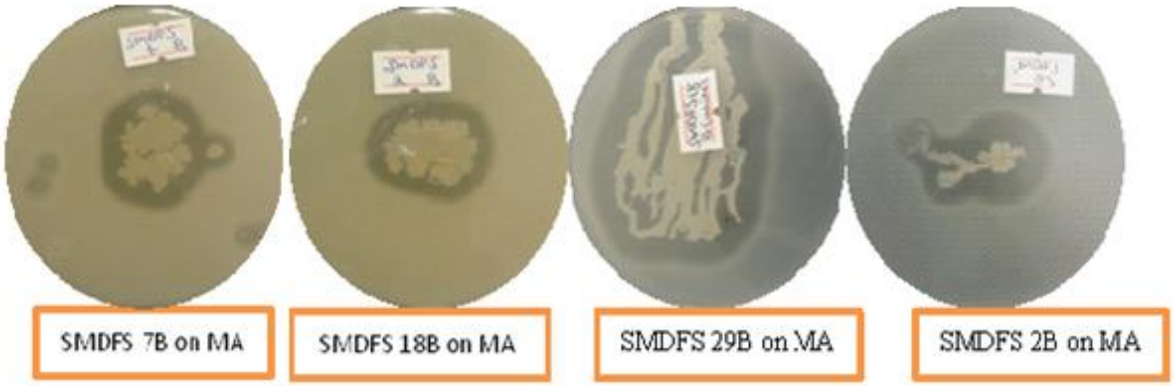
Appendix 1: Soil and cow dung sample collection



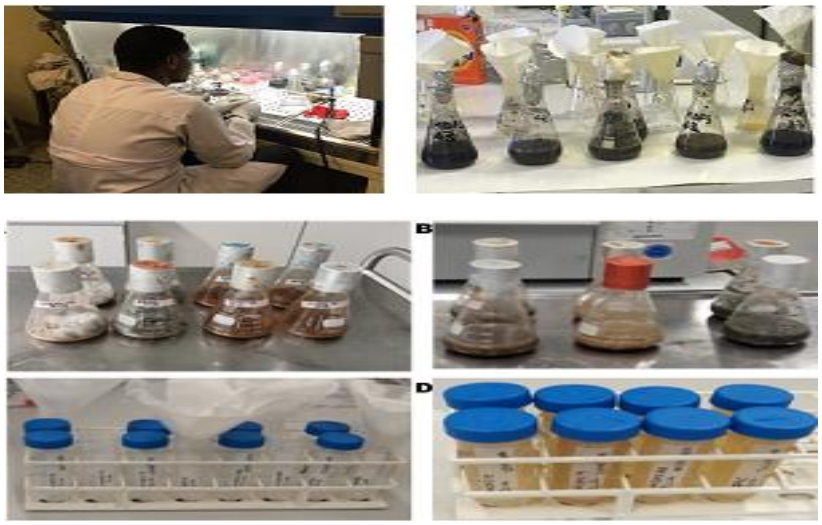
Appendix 2: Agar plate culture of some of the purified potential fungal isolates



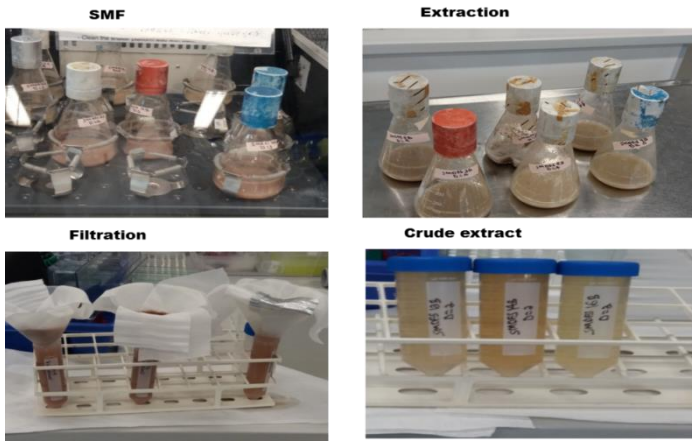
Appendix 3: Primary screening of protease production fungi on Skim- milk Agar using tannic acid (A, B, C) and without tannic acid (D, E, F)



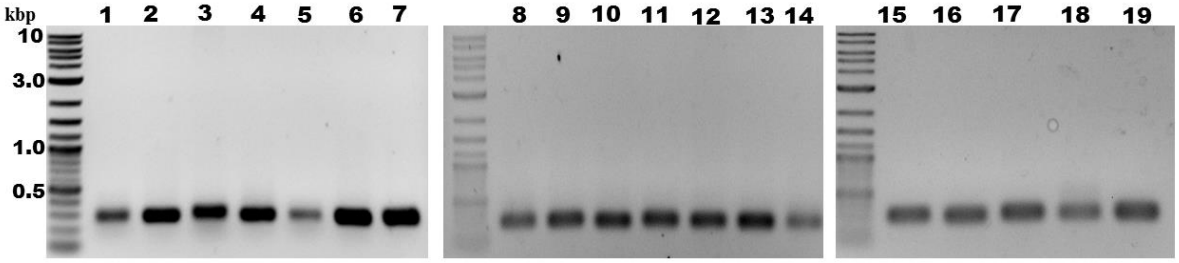
Appendix 4: primary Screening protease enzyme from bacteria using Skim-milk Agar media



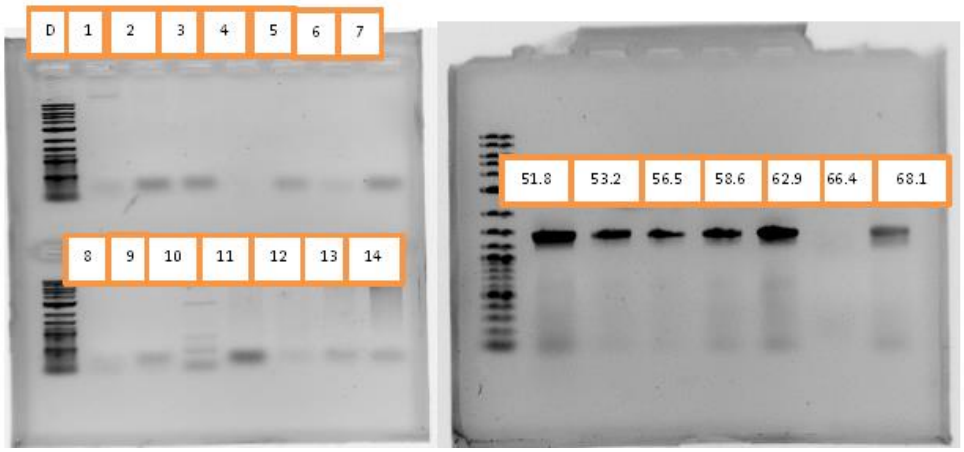
Appendix 5: Production and extraction of crude enzyme from filamentous fungi in SSF



Appendix 6: Production and extraction of crude enzyme from Bacteria in SmF



Appendix 7: An Agarose gel electrophoresis of PCR product of 19 fungal Isolates [1)] DRDFS13, 2) SMDFS19, 3) PENC, 4) SMDFS18, 5) SMDFS42, 6) DRDFS19, 7) AWDFS12A, 8) AWDFS17, 9) AWDFS18A, 10) SMDFS49, 11) SMDFS50, 12) SMDFS52, 13) SMDFS55, 14) SMDFS51, 15) AWDFS5, 16) WGDFD6, 17) SMDFS 57, 18) SMDFS58, 19) SMDFS19)



Appendix 8: (A): Agarose gel electrophoresis of PCR product of 14 Bacterial Isolates (1=SMDFS2B, 2=SMDFS3B, 3=SMDFS5B, 4=SMDFS6B, 5=SMDFS7B, 6=SMDFS8B, 7=SMDFS11B, 8=SMDFS12B, 9=SMDFSFS14B, 10=SMDFS15B, 11=SMDFS16B, 12=SMDFS18B, 13=SMDFS29B) (B): gradient PCR temperature for SMDFS16B

Appendix 9: Concentration of gDNA and purified PCR products of 17 fungal isolates

S.No	Isolates	Conc of gDNA (ng/µl)	Conc. of purified PCR products (ng/µl)
1.	DRDFS13	429.00	33.50
2.	SMDFS19	375.60	66.50
3.	PENC	3037.60	45.60
4.	SMDFS 18	8419.60	51.70
5.	SMDFS42	394.00	24.80
6.	DRDFS19	241.40	92.70
7.	AWDFS12A	3896.00	59.00
8.	AWDFS 17	3257.60	47.40
9.	AWDFS18A	2382.10	79.40
10.	SMDFS49	111.70	67.60
11.	SMDFS50	5473.70	62.80
12.	SMDFS52	730.60	63.30
13.	SMDFS55	10048.70	80.10
14.	AWDFS5	662.40	66.40
15.	WGDFD6	1216.20	82.70
16.	SMDFS57	504.70	55.00
17.	SMDFS59	239.40	67.80

Appendix 10: Concentration of gDNA and purified PCR products of 14 Bacterial isolates in ng/µl

S.No	Isolates	Conc. of gDNA (ng/µl)	Conc. of purified PCR products (ng/µl)
1.	SMDFS2B	957.90	78.60
2.	SMDFS3B	366.00	57.90
3.	SMDFS5B	3471.300	54.60
4.	SMDFS6B	6181.30	54.70
5.	SMDFS7B	653.60	114.30
6.	SMDFS8B	13859.00	52.60
7.	SMDFS11B	10161.00	48.50
8.	SMDFS12B	8866.60	65.10
9.	SMDFS14B	18055.20	69.50
10	SMDFS15B	15668.60	70.50
11.	SMDFS16B	17946.10	71.70
12.	SMDFS18B	1218.30	49.70
13.	SMDFS29B	1956.90	56.80
14.	DRDFS13B	181.70	69.30

Appendix 11: Biochemical and morphological characteristics of potential bacterial isolates

S.No	Isolates	Gram rxn	Cell shape	Spore staining	Catalase test
1.	SMDFS 2B	+ve	Rod	+ve	+ve
2.	SMDFS 3B	+ve	Rod	+ve	+ve
3.	SMDFS 5B	+ve	Rod	+ve	+ve
4.	SMDFS 6B	+ve	Rod	+ve	+ve
5.	SMDFS 7B	+ve	Rod	+ve	+ve
6.	SMDFS 8B	+ve	Rod	+ve	+ve
7.	SMDFS 11B	+ve	Rod	+ve	+ve
8.	SMDFS 12B	+ve	Rod	+ve	+ve
9.	SMDFS 14B	+ve	Rod	+ve	+ve
10	SMDFS 15B	+ve	Rod	+ve	+ve
11.	SMDFS 16B	+ve	Rod	+ve	+ve
12.	SMDFS 18B	+ve	Rod	+ve	+ve
13.	SMDFS 29B	+ve	Rod	+ve	+ve
14.	DRDFS 13B	+ve	Rod	+ve	+ve

Appendix 12: Biomass determination by glucose amine method

Appendix 13: Determination spore concentration using Hemocytometer

Appendix 14: Slant of some potential fungi

Appendix 15: Macroscopic Characteristics of Potential Fungal Isolates

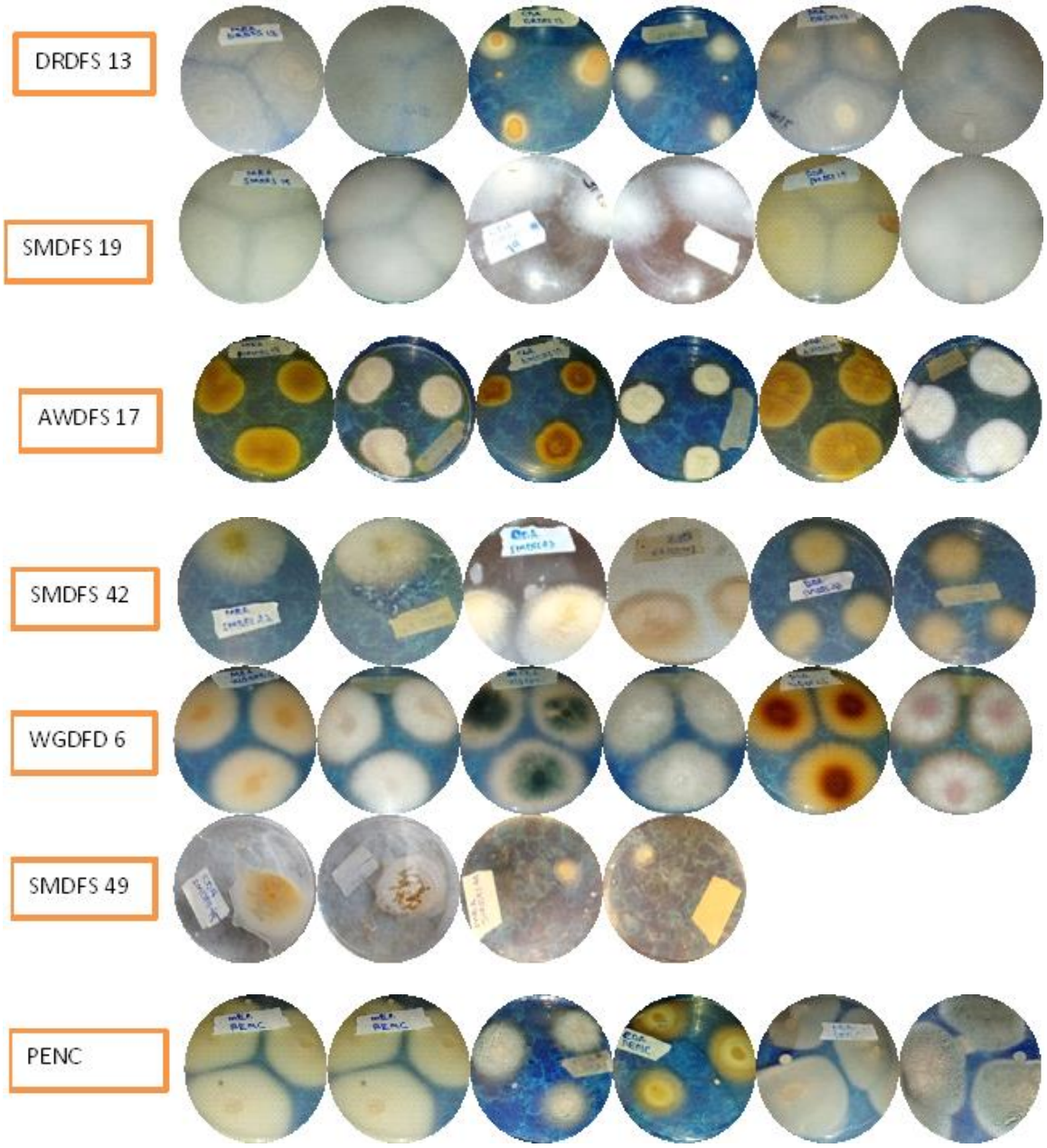
Isolates	Media	Colony growth	Colony texture	Ave.Colony Diameter	Obverse Colony color	Reverse colony color	Degree of sporulation
DRDFS 13	MEA	Very high	Floccose (cottony)	48.33±2.36	Dark white to gray	White	Low
	CDA	Very low	Floccose (cottony)	11.00±2.94	White	Pink	Low
	PDA	Very high	Floccose (cottony)	50.83±1.18	Dark white to gray	Dark white	Low
SMDFS 19	MEA	Very high	Floccose (cottony)	50.67±0.94	White	White	Low
	CDA	Medium	Floccose (cottony)	29.67±8.81	White with gray at	White with gray at the center	_
	PDA	Very high	Floccose (cottony)	50.00±4.08	white	White to slightly yellow	Low
AWDFS 17	MEA	Medium	Rough	31±0.82	white to brown	Brown	Low
	CDA	Low	Rough	24.50±3.19	white to brown	Dark brown	Low
	PDA	High	Rough and velvety	38.17±2.09	Dark white	Brown	Low
PENC	MEA	High	Rough and powdery	44.00±0.82	Blue	white to slightly yellow at the	High
	CDA	Low	Rough and powdery	24.83±5.63	Blue covered with	white to brown	Low
	PDA	Very high	Rough and powdery	45.33±4.94	Blue	blue with white at center	High
DRDFS 19	MEA	High	Rough and powdery	40.50±2.68	green with white at	white and brown at the center	High
	CDA	Medium	Rough and powdery	33.50±1.78	yellow and powdery	yellow at center and white at the	High
	PDA	High	Rough and velvelty	41.67±2.36	Mixed (green and	Light yellow	High
AWDFS 12A	MEA	High	Rough, velvelty&	41.67±3.40	Mixed (green and	Light brown	High
	CDA	Medium	Rough and powdery	30.00±0.00	yellow with slightly	Brown	High
	PDA	High	Rough and powdery	44.00±2.83	Dark green	Pale white	High
AWDFS 18A	MEA	High	Rough and powdery	39.83±1.84	Green with white at	Pale white with brown at the center	High
	CDA	Medium	Rough and powdery	34.83±2.25	Yellow with slightly	Brown	High
	PDA	High	Rough and powdery	38.50±1.08	Green with white at	Pale white	High
SMDFS 18	MEA	Very low	Floccose (cottony)	9.67±2.05	White with scattered	White to yellow	High
	CDA	Very low	Floccose (cottony)	10.00±1.63	White	White	High
	PDA	High	Floccose (cottony)	39.67±1.25	Gray with fully	Pale with slightly yellow at the	High
SMDFS 52	MEA	High	Floccose (cottony)	38.00±2.45	white with fully	Pale white	Very high
	CDA	Low	Floccose (cottony)	23.67±1.70	White with scattered	Pale white	Medium
	PDA	High	Floccose (cottony)	39.67±2.05	white with fully	Pale white	Very high

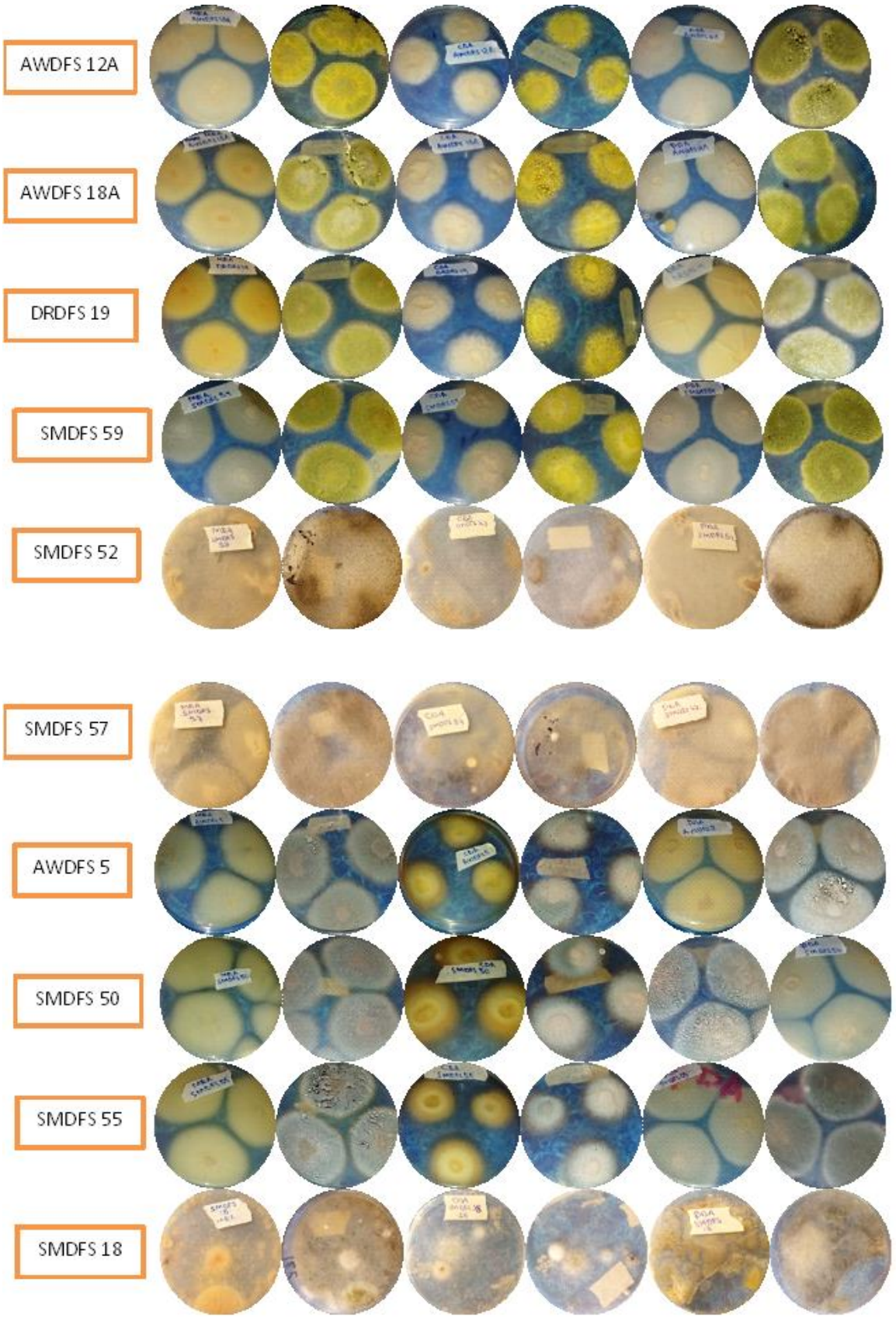
Appendix 15 continued

SMDFS 59	MEA	High	Rough and powdery	41.00±2.94	Green with a brown	Pale white	High
	CDA	High	Rough and powdery	35.33±0.47	Yellow with green at	Brown with green at the periphery	High
	PDA	High	Rough and powdery	40.67±1.70	Dark green	Pale white	High
SMDFS 50	MEA	High	Rough and powdery	41.67±2.36	Blue with fine	Pale white	High
	CDA	Medium	Rough and powdery	34.67±2.05	Light brown with	Brown	High
	PDA	High	Rough and powdery	42.83±1.65	Blue with fine gray	Pale white	High
SMDFS 55	MEA	High	Rough and powdery	44.67±2.05	Blue with white at	Pale white	High
	CDA	Medium	Rough and powdery	31.33±1.25	Mixed (Blue and	Light brown	High
	PDA	Very high	Rough and powdery	47.33±4.50	Dark Blue	Pale white with blue at the	High
AWDFS 5	MEA	Very high	Rough, velvelty&	45.00±2.45	Blue with white at	Pale white	High
	CDA	Medium	Rough, velvelty&	31.67±1.25	Mixed (Blue and	White	High
	PDA	High	Rough, velvelty&	42.83±1.65	Dark gray with blue	Write to Gary	High
SMDFS 57	MEA	Very high	Floccose (cottony)	57.33±2.05	White to gray	Pale white	Low
	CDA	Low	Floccose (cottony)	24.33±2.05	White with Black	White	Low
	PDA	Very high	Floccose (cottony)	46.67±4.99	White to gray	White to gray	Low
SMDFS 42	MEA	Very high	Rough and cottony	50.00±1.63	White with brown at	White with brown at the center	Low
	CDA	Low	Rough and cottony	24.00±2.16	White	White	Low
	PDA	Medium	Rough and cottony	32.00±2.45	White with Pink at	White with brown at the center	Low
SMDFS 49	MEA	Very low	Rough	9.67±1.25	White with slight	White with slight yellow at the	Low
	CDA	Medium	Rough	27.33±1.70	Very white	White	Low
	PDA	No growth	_	_	_	_	_
WGDFD 6	MEA	High	Rough & cottony	40.17±3.47	White with brown at	White with brown at the center	Low
	CDA	High	Rough & cottony	36.67±1.25	Mixed (white & blue)	Blue with white at the periphery	Low
	PDA	High	Rough & cottony	43.67±1.70	White and pink at the	White with dark brown at the	Low

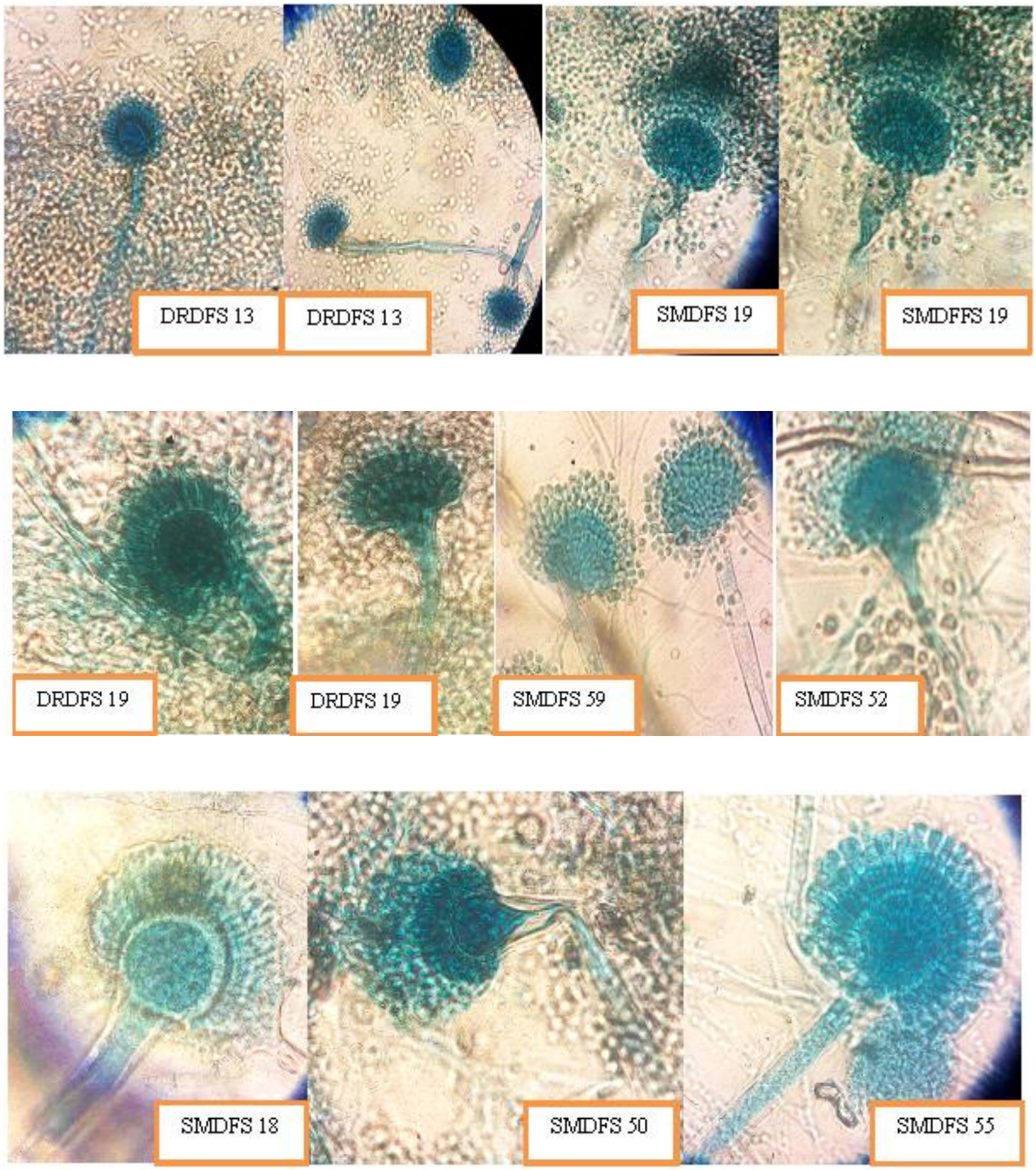
A: Malt Extract Agar; CDA: Czapek Dox Agar; PDA: Potato Dextrose Agar; Very low :< 15mm; Low: 15-25mm; Medium: 25-35mm, High: 35-45mm; Very high :> 45mm

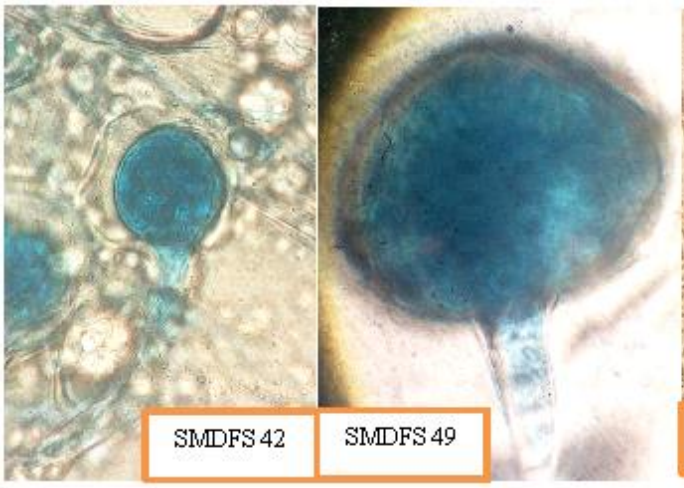
Appendix 16: Morphological Characterization of Potential Fungi

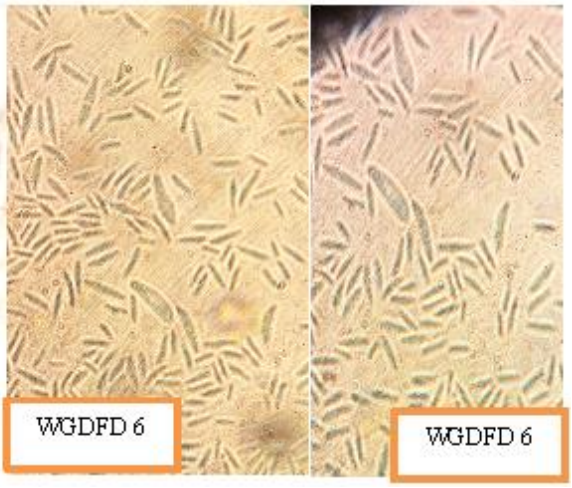


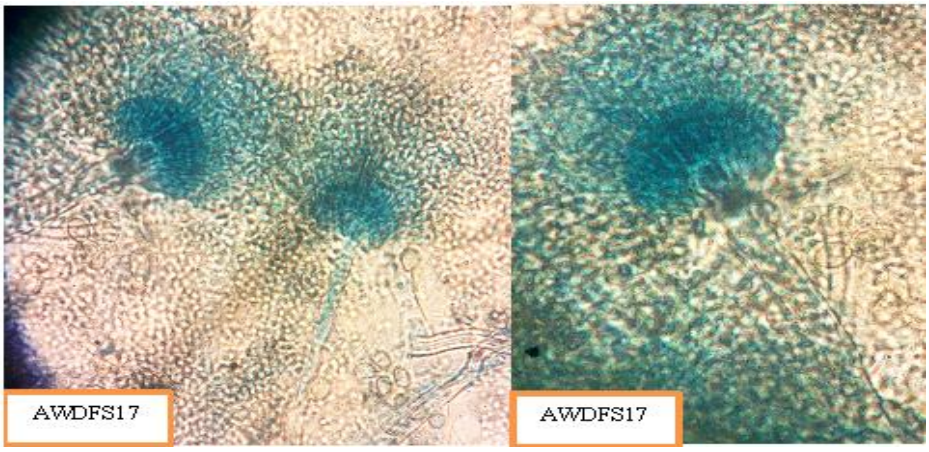

AWDFS 12A
AWDFS 18A
DRDFS 19
SMDFS 59
SMDFS 52
SMDFS 57
AWDFS 5
SMDFS 50
SMDFS 55
SMDFS 18

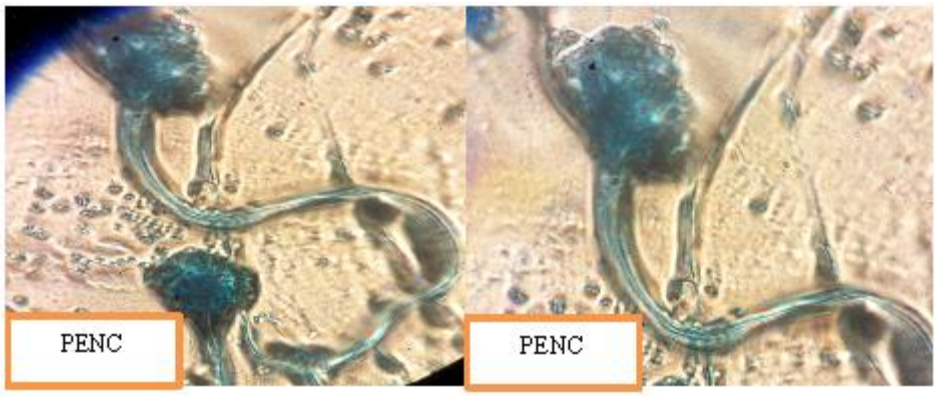
Appendix 17: Microscopic structures some potential fungi strains




SMDFS 42
SMDFS 49


WGDFD 6
WGDFD 6


AWDFS17
AWDFS17


PENC
PENC

Appendix 18: API 50 CHB/E results

SMD FS 2B	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
24 hrs	-	-	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-	-	-	+	-	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-
48 hrs	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	+	-	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	-	-	+	+	+	-	-	-	-	-	-	-	-	-	+	-	-
	0	Gly	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LANT	GNT	2KG	5KG

SMD FS 6B	0	1	2	3	4	5	6	7	8	9	10	11	12	13	`4	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
24 hrs	-	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
48 hrs	-	+	-	-	+	+	+	-	-	-	-	-	+	+	-	-	-	+	-	-	-	-	+	-	+	+	-	+	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
	0	Gly	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LANT	GNT	2KG	5KG

SMD FS 7B	0	1	2	3	4	5	6	7	8	9	10	11	12	13	`4	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
24 hrs	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	-	+	-	+	+	-	+	-	+	+	+	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	-	-
48 hrs	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	-	-	+	-	+	-	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-
	0	Gly	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LANT	GNT	2KG	5KG

SMD FS 11B	0	1	2	3	4	5	6	7	8	9	10	11	12	13	`4	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
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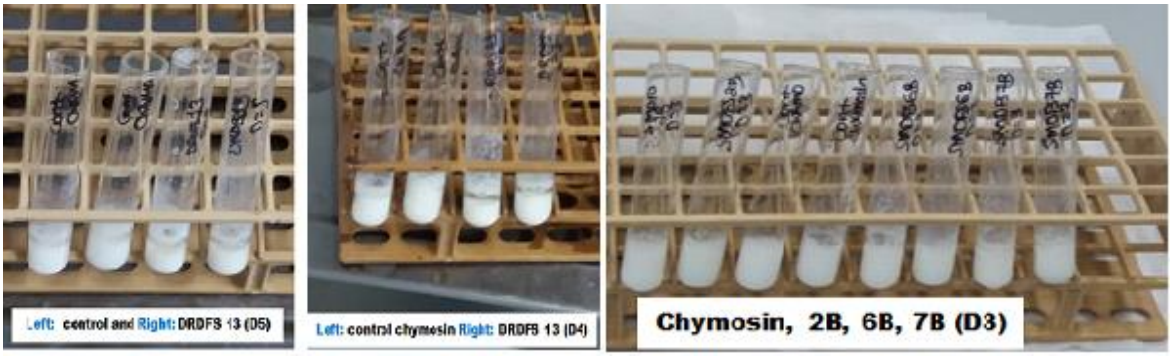
24 hrs	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
48 hrs	-	+	-	-	+	+	+	-	-	-	-	+	+	+	+	-	-	+	+	+	-	+	-	+	-	+	-	+	+	-	+	-	+	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-
	0	Gly	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG

SMD FS 12B	0	1	2	3	4	5	6	7	8	9	10	11	12	13	1`4	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
24 hrs	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	-
48 hrs	-	-	-	-	+	+	+	-	-	-	-	+	+	-	-	-	-	+	+	+	-	+	-	+	-	+	-	-	+	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	0	Gly	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG

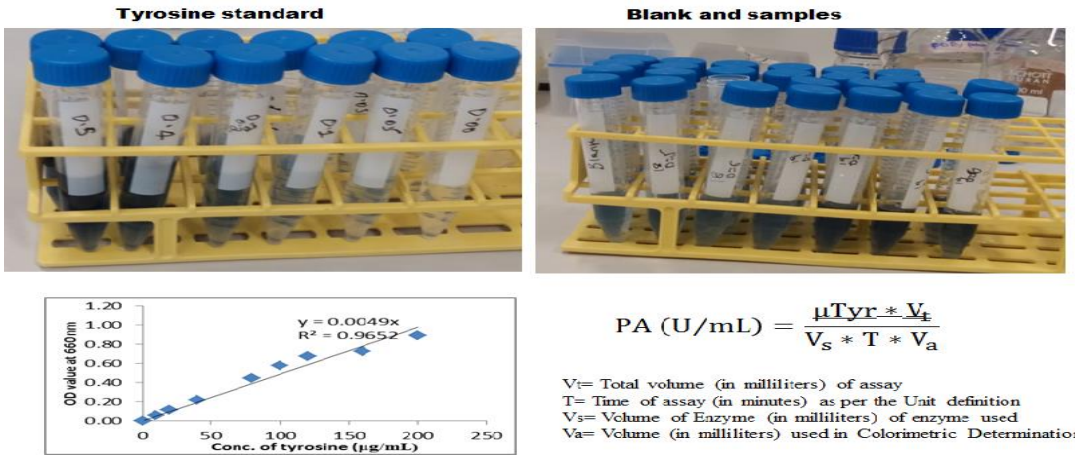
SMD FS 14B	0	1	2	3	4	5	6	7	8	9	10	11	12	13	1`4	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
24 hrs	-	+	-	-	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	+	-	+	+	+	+	+	+	-	+	+	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
48 hrs	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
	0	Gly	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG

SMD FS 16B	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
24 hrs	-	+	-	-	+	+	+	-	-	-	-	+	+	+	-	-	-	+	+	+	-	+	-	+	-	+	-	+	+	-	+	-	-	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-
48 hrs	-	+	-	-	+	+	+	-	-	-	+	+	+	+	-	+	-	+	+	+	-	+	-	+	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	?	?
	0	Gly	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUC	LFUC	DARL	LARL	GNT	2KG	5KG

Appendix 19: Color changes observed at 24h and 48 h during API CHB/E test

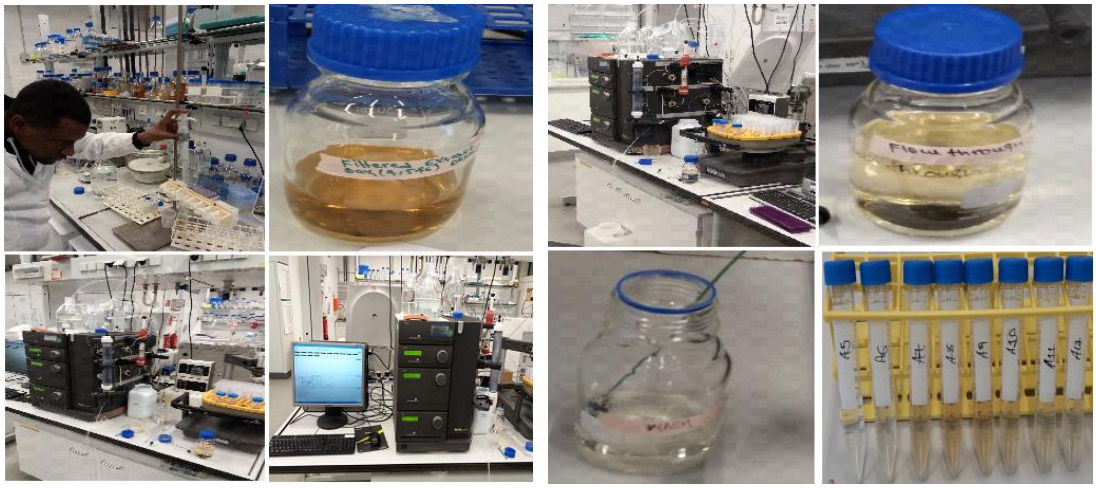


Appendix 20: MCA test for some potential bacterial and fungi strains

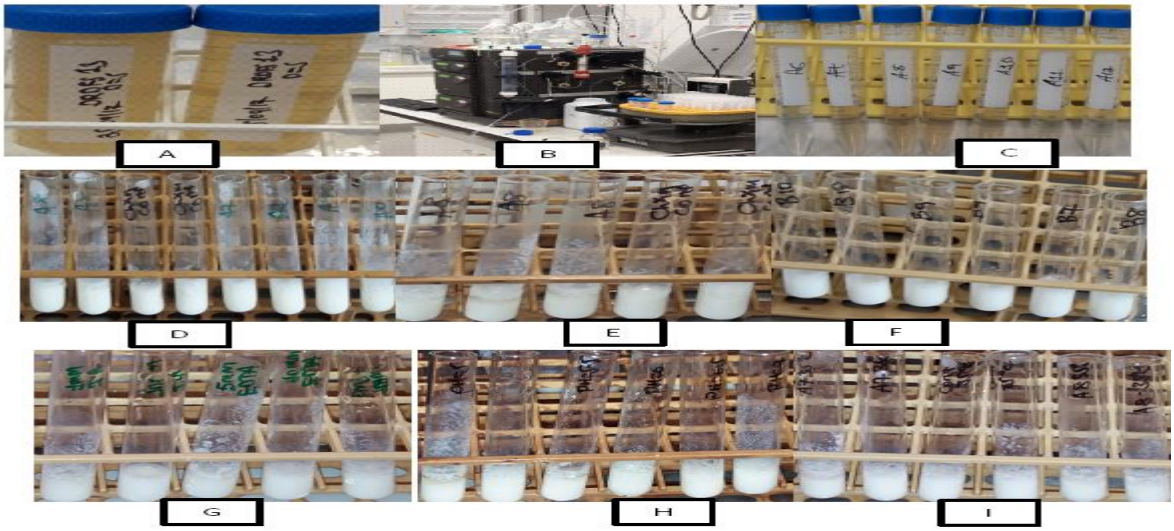


Appendix 21: Protease assay

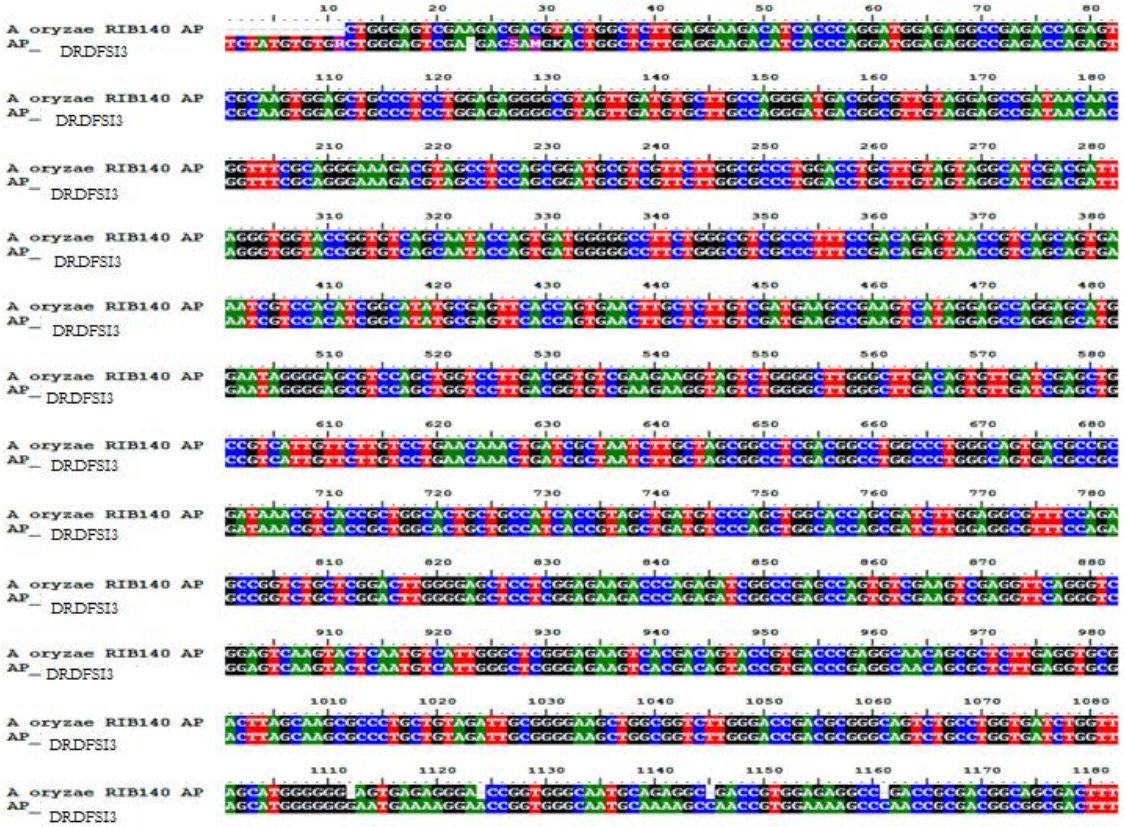
Appendix 22: Determination of total protein and Soluble carbohydrate



Appendix 23: Enzyme purification steps using AKTA purifier



Appendix 24: (A): crude extract (B): Purification and fractionation of enzyme by IEC (C): IEC fractions (D) and (E): MCA of active fractions (F): MCA of Inactive fractions (G): Inhibition study for active fractions (H): pH optimization (I): Temp optimization



Appendix 25: Complete nucleic acid sequence alignment of aspartic protease gene from *Aspergillus oryzae* RIB140 and *A. oryzae* DRDFS 13.

Appendix 26: Sensory evaluation score sheet

Demography data

Occupation	Health status	Age	Sex

Product					
Date					
Panellist No					

Instructions: Taste the given samples, and then place "√" mark on the scale point which best describes your feeling.Thank you in advance.

Characteristics	Sample code				
	Colour	Taste	Texture	Odour	Overall acceptability
Extremely liked					
Moderately liked					
Slihtly liked					
Neither liked Nor disliked					
Slightly disliked					
Moderately disliked					
Extremely disliked					

Appendix 27: Danbo cheese production process

Appendix 28: : (A) Different Danbo cheeses (BR: Bacterial Enzyme FR: Fungal Enzyme and R: Commercial Rennet (B) The respectivewhey produced using R, F, and B

Appendix 29: Proximate analysis of Danbo cheeses