CHARACTERIZATION AND BIOACTIVITIES OF CARBOXYMETHYL CELLULASE PRODUCED BY *ALCALIGENES FAECALIS* USING DISPOSED X-RAY FILM AS THE SUBSTRATE

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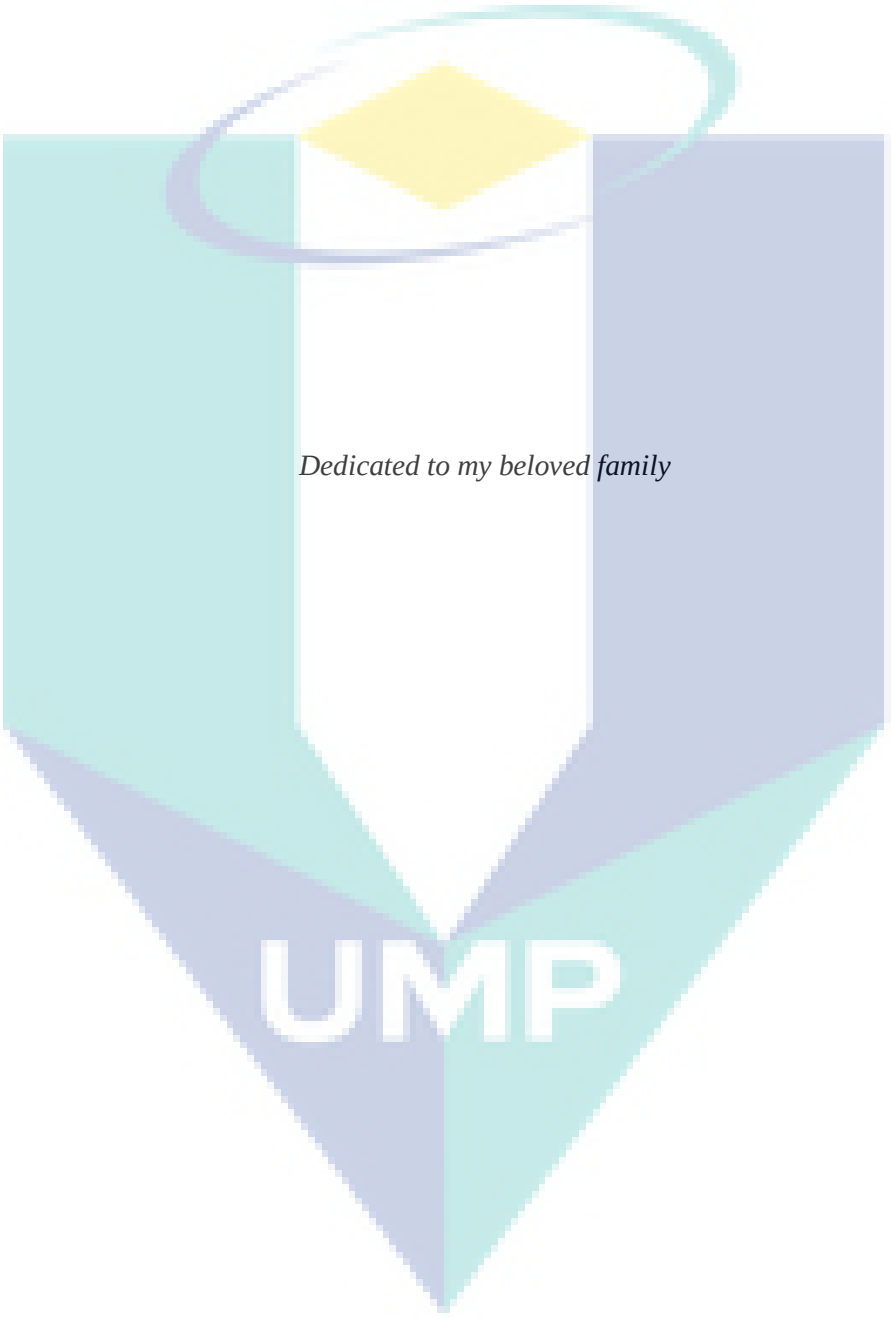

Dedicated to my beloved family

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ABSTRAK

Selulase adalah enzim yang bermanfaat yang telah lama digunakan dalam pengeluaran makanan haiwan, dalam perumusan bahan pencuci, penjernihan jus, pembuatan kertas dan pengeluaran wain. Pada masa kini, pengeluaran enzim selulase dari biopertukaran sisa selulosa telah diamalkan secara meluas. Enzim biasanya boleh diperolehi daripada mikroorganisma terutamanya bakteria dan kulat. Keupayaan potensi mikroorganisma yang tidak diketahui untuk mencerna bahan selulosa dapat dieksploitasi menggunakan substrat baru, iaitu sisa filem sinar-x. Filem sinar-x adalah sejenis sisa berbahaya. Ia mengandungi sisa perak di samping selulase yang boleh dihasilkan oleh mana-mana mikrob yang memakan selulosa yang terdapat dalam sisa filem sinar-x. Objektif projek ini adalah (i) untuk memencil, menyaring, dan mengenalpasti mikroorganisma dari sumber air, tanah dan makanan, dengan itu memilih pencilan yang paling kuat (ii) untuk mengoptimumkan kesan pencilan bakteria menggunakan filem sinar-x yang dilupuskan sebagai substrat pada pengeluaran selulase (iii) untuk menulenkan dan mencirikan bioaktiviti selulase. Sampel Tanah Panching (PS1) dan sampel Hati Ayam (CL8A) dipilih sebagai pencilan yang paling kuat selepas pemeriksaan daripada 27 isolat awal. Selepas pengoptimuman penghasilan CMCase dan avicelase, hanya CMCase dipilih untuk proses selanjutnya kerana ia lebih gemar filem x-ray sebagai substrat. Keadaan yang optimum yang meningkatkan penghasilan CMCase disiasat dengan menggunakan kaedah "Satu Faktor Pada Satu Masa" (OFAT), yang melibatkan 7 faktor yang berbeza; keadaan inkubasi, sumber karbon, sumber nitrogen, pH awal, jumlah substrat, saiz inokulum dan vitamin. PS1 mencapai aktiviti CMCase tertinggi sebanyak 0.934 U / ml dalam keadaan bergetar, dengan 0.4 % w/v kanji, 0.1 % w/v ekstrak malt, 2.5 g jumlah substrat, 2 ml saiz inokulum dan 2 % w/v tiamin dalam pH 8 media penghasilan. Sedangkan CL8A mencapai aktiviti tertinggi CMCase sebanyak 4.559 U/ml dalam keadaan bergetar, dengan 0.4 % w/v laktosa, 1.5 g jumlah substrat, pH 9 media penghasilan dan faktor-faktor lain menunjukkan keputusan serupa dengan PS1. Walau bagaimanapun hanya CL8A dipilih untuk penulenan separa enzim kerana ia menunjukkan produktiviti enzim yang lebih tinggi. CL8A dihasilkan secara besar-besaran untuk melaksanakan pemendakan ammonium sulphate selepas proses pengoptimuman. Hasil CMCase sebanyak 6.49% telah diperolehi dan berat molekul enzim telah dianalisis menggunakan kaedah elektroforesis dengan hasilnya 60 kDa. Mikroorganisma PS1 dikenalpasti sebagai bakteria Gram negatif *Providencia rettgeri*, sementara CL8A dikenalpasti sebagai bakteria Gram negatif *Alcaligenes faecalis.* Pecahan enzim CMCase telah dimangkinkan menggunakan 2 faktor yang berbeza dan didapati stabil pada 25 ° C dan pH 5. Kesimpulannya, pencilan yang berasal dari sisa makanan (*A. faecalis*) dan tanah (*P. rettgeri*)) memperlihatkan potensi yang baik sebagai mikroorganisma selulosa dan dapat degradasikan filem sinar-x dan menghasilkan CMCase.

ABSTRACT

Cellulase is a beneficial enzyme that has been long used in production of animal feed, in the formulation of detergents, juice clarification, paper manufacturing and wine production. Nowadays, production of cellulases enzyme from the bioconversion of cellulosic waste has been extensively practiced. The enzymes typically can be acquired from microorganisms especially bacteria and fungi. Furthermore, potential capability of unknown microorganisms to digest cellulosic material can be exploited using a new substrate, which is x-ray film waste. X-ray film is a form of hazardous waste which contains silver residue, alongside cellulase which can be produced by any microorganisms that feed on cellulose found in x-ray film waste. The objectives of this project are (i) to isolate, screen and identify microorganisms from water, soil and food sources, thus select on most potent isolates, (ii) to optimize the effect of bacterial isolates using disposed x-ray film as the substrate on cellulase production, and (iii) to purify and characterize the bioactivity of cellulose. The samples of Panching Soil (PS1) and Chicken Liver (CL8A) were selected as the most potent isolates after screening from 27 initial isolates. After optimization of CMCase and avicelase productions, only CMCase was selected for further process as it preferred the x-ray film as substrate. The optimum condition that enhanced the CMCase production was investigated using "One Factor at a Time" (OFAT) method, involving 7 different factors; incubation condition, carbon source, nitrogen source, initial pH, amount of substrate, inoculum size and vitamin. PS1 achieved highest CMCase activity of 0.934 U/ml in shaking condition, with 0.4 % w/v starch, 0.1 % w/v malt extract, 2.5 g amount of substrate, 2 ml inoculum size and 2 % w/v thiamine with pH 8 of production media. Whereas, CL8A achieved highest CMCase activity of 4.559 U/ml in shaking condition, with 0.4 % w/v lactose, 1.5 g amount of substrate, pH 9 of production media and the rest of the factors were similar to PS1. However only CL8A was selected for partial purification of enzyme as it displayed higher enzyme productivity. CL8A was produced on a large scale to carry out ammonium sulphate precipitation after the optimization process. CMCase yield of 6.49 % was obtained and the molecular weight of the enzyme has been analysed using electrophoresis method resulting in 60 kDa. Microorganism of PS1 was identified as Gram negative bacteria *Providencia rettgeri*, while CL8A was identified as Gram negative bacteria *Alcaligenes faecalis*. The CMCase enzyme fraction was catalysed using 2 different factors and was found to be stable at 25 °C and pH 5. In conclusion, the isolates derived from food waste (*A. faecalis*) and soil (*P. rettgeri*) displayed a good potential as cellulolytic microorganisms and were able to degrade x-ray film and produce CMCase.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

%	Percent
°C	Degree Celsius
#	Highest value
/	Per
:	Ration
=	Double-bonded
β	Beta
×	Multiplication

LIST OF ABBREVIATIONS

APS	Ammonium persulfate
AZCL	Azurine-Crosslinked
A. faecalis	*Alcaligenes faecalis*
A. niger	*Aspergillus niger*
A. sulphate	Ammonium sulphate
BGL	Beta-glucosidases
BSA	Bovine serum albumin
B. subtilis	*Bacillus subtilis*
CBH	Cellobiohydrolase
CC	Corn cob
CFF	Cell free filtrate
CMC	Carboxymethyl cellulose
CMCase	Carboxymethyl cellulase
DNA	Deoxyribonucleic acid
DNS	Dinitrosalicylic
DEAE	Diethylaminoethyl cellulose
EDTA	Ethylenediaminetraacetic acid
EG	Endoglucanases
EH	Epoxide hydrolase
E.coli	*Escheria coli*
E. faecalis	*Enterococcus faecalis*
FDA	Food and drug administration
FPase	Filter paperase
g	Gram
GRAS	Generally recognised as safe
h	Hour
hrs	Hours
JDC	*Jatropha curcas* deoiled cake
km	kilometre
K. pneumonia	*Klebsiella pneumonia*
L	Litre

M	Molar
mg	Milligram
min	Minute
ml	Millilitre
MUC	Methyl-umbelliferyl-cellobioside
MWCO	Molecular weight cut-off
NA	Nutrient agar
NB	Nutrient broth
OBR-HEC	Ostazin brilliant red-hydroxyethyl cellulose
OD	Optical density
PASC	Phosphoric acid swollen cellulose
PET	Polyethylene terephthalate
pH	Potential hydrogen
ppm	Parts per million
P. aerogenosa	*Pseudomonas aerogenosa*
P. variotii	*Paecilomyces variotii*
rpm	Revolutions per minute
RS	Rice straw
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SD	Standard deviation
SEM	Scanning electron microscope
SmF	Submerged fermentation
sp.	Species
SSF	Solid state fermentation
S. typhii	*Salmonella typhii*
S. aureus	*Staphylococcus aureus*
TAE	Tris-acetate-EDTA
TEMED	Tetramethylethylenediamine
T. reesei	*Trichoderma reesei*
T. viride	*Trichoderma viride*
UMP	Universiti Malaysia Pahang
U/ml	Units per millilitre
w/v	Weight per volume

LIST OF CHEMICAL FORMULAS

$CaCl_2$	Calcium chloride
$CuSO_4.5(H_2O)$	Copper (II) sulphate pentahydrate
KH_2PO_4	Potassium dihydrogen phosphate
$KNaC_4H_4O_6 \cdot 4H_2O$	Potassium sodium tartrate
KNO_3	Potassium nitrate
$MgSO_4$	Magnesium sulphate
NaOH	Sodium hydroxide
$Na_2HPO_4.7H_2O$	Disodium phosphate heptahydrate
Na_2HPO_4	Disodium phosphate
$NaNO_3$	Sodium nitrate
NH_4Cl	Ammonium chloride
$(NH_4)_2SO_4$	Ammonium sulphate
NaCl	Sodium chloride
Na_2CO_3	Sodium sulphite
Na_2(tartrate)$2H_2O$	Sodium tartrate dehydrate

CHAPTER 1

INTRODUCTION

1.1 Background of Study

An enzyme is a type of protein that serves the purpose as accelerator of any related chemical reactions provided with substrate that allow the enzyme to be a fully-functioned component. Among the natural existing substrate that has been used, lignocellulosic substrate is the common substrate as which can be easily acquired from inedible municipal waste or plant residue. Lignocellulosic is a complex material consists of mostly cellulose, hemicelluloses and lignin. Cellulose is a fibrous, insoluble, crystalline polysaccharide that made up the structure of x-ray film sheet, that can be used as feeder to microorganism which acts as cellulase producer (Li et al., 2009). The bioconversion of cellulosic materials has been receiving a great attention in recent years and the development of large-scale bioconversion process would alleviate shortages of food and animal feeds, solve modern waste disposal problems and diminish the dependency on fossil fuels by providing an energy source in the form of glucose.

Maximum utilization of these cellulosic materials is crucial as it can directly incline with pollution if the materials are not properly managed. In this situation, an assistant is desirable as it can bring down the probability to a certain degree. With this intention, cellulose-degrading microorganisms or any microorganisms that are able to hydrolyze cellulose are used in producing various economically important products in food technology (Gautam et al., 2011). A good example is microbial enzymes which have advantage in large scale production as it can be closely controlled in microorganisms. Cellulase that can be produced by any potential microorganisms is the enzyme responsible in cellulose breakdown (Sadhu et al. 2013). Commonly, the enzyme also involved in conversion of agricultural waste materials to useful products, such as

single cell protein, fuels and chemical feed stocks (Juturu & Wu, 2014). More significant uses of enzyme has been investigated despite being a very expensive material for it can only be produced in a small amount for every batch, thus, making enzyme in high demand.

Also, in term of using alternative way, this study deals with possibility of using hazardous waste as a recovery material which is x-ray film that consists of major part of cellulosic material (Houston, 2007). Studies using this material are not extensive yet till this very date. As a cellulosic-based waste, x-ray has been used as the substrate as it provides carbon source. This way, it is not only contribute to minimum environmental pollution, but enzymatic hydrolysis can also help in producing useful products (Hamza, 2017). The x-ray film that was used as substrate is common waste that may come from dental offices, photographic processors and metal plating industries. More importantly, a setback that were not given a serious attention that, in 1994 a total of 420 000 tons of hazardous waste were created annually by variety of productions in Peninsular Malaysia and has been increasing since then given by a study conducted for the Department of Environment of Malaysia (Jain, 2017). Over a regional group, Asia has been releasing the most silver directly to land and water (Eckelman & Graedel, 2007).

Thus, a biologically-assisted way is a preferable strategy to handle this problem on account of microorganism which plays a key role in biotechnology field. Varieties of cellulose-degrading microorganisms were isolated from different natural sources and screened for cellulase production ability. The microorganism synthesizes the cellulose to produce cellulases (CMCase and avicelase) with addition x-ray film as the substrate. Also, different parameters (incubation condition, carbon source, nitrogen source, pH value, amount of substrate, inoculum size and vitamin) was optimized for optimum production of cellulase.

1.2 Problem Statement

Disposed x-ray film is a cellulose-comprised waste that has been investigated in producing different enzymes which served on different purpose. The structure of cellulose is complex and triggered different diversity of degradative enzyme. Enzymatic hydrolysis of cellulosic waste can produce different cellulase enzyme depending on the mode of enzymatic actions and selection of substrate. Furthermore, due to cellulose

complexity, the mechanism of cellulose-degrading differentiates between the sources of cellulase. The source may come from bacteria or fungi to help in breakdown of this polysaccharide. Study of metagenomic was applied which includes the isolation of microorganisms from different origin and microorganism was optimized in term of enzyme production ability. So, in this study different factors have been emphasized to maximise cellulase production from microorganisms derived from different sources.

Cellulase market has been growing and surpasses other enzymes comparing current market size for industrial enzymes making it the future largest industrial enzyme (Zhang & Zhang, 2013). Workload minimization, environment pollution prevention, land usage preservation and subsequently producing valuable products out of low-cost substrate making it recommendable to grab an opportunity with the project. This involved fermentation process by optimizing production medium and further improvise using an x-ray film as sole carbon source (Gautam et al., 2011). Ultimately, experimental investigation was conducted using biological approach to explore biodegradation of low-cost cellulosic waste and maximise cellulase production.

1.3 Objectives

i. To isolate, screen and identify microorganisms from water, soil and food sources, thus select on most potent isolates
ii. To optimize the effect of bacterial isolates using disposed x-ray film as the substrate on cellulase production
iii. To purify and characterize the bioactivity of cellulase

1.4 Overview of the Report

This thesis consists of 5 chapters. Chapter 1 reviews the content of the project including problem statement and objectives. Chapter 2 compiles the idea behind past research of other scientist, the discoveries and future hurdles. Chapter 3 starts with sample and substrate collection, screening, media culture, enzyme optimization, bacterial identification, purification and characterization. Chapter 4 discusses all the results which related to previous chapter. Finally, Chapter 5 summarizes the findings which include conclusion and recommendations.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

In this chapter, results of previous research, relevant methodological courses, key ideas and concepts that built up the project were reviewed. The areas included the cellulosic waste and the structure of the cellulose break down. Enzyme potentials in the new era of technology have also been discussed. The method of using microorganism and its essential value which formed the frame of this study has also been highlighted.

2.2 Enzyme

Enzyme is a protein with an ability to catalyse a particular biological compound (substrate) into a product at intensively high rate. The reaction can be thousands time faster than a normal reaction depending on the substrate and specific condition of the enzyme. To review, Goyal & Phutela (2018) expressed three main types of enzyme:

i. Metabolic enzymes (enzymes which our body produces that work in blood, tissues and organs)
ii. Digestive enzymes (enzymes that break down food into usable material)
iii. Food enzymes (enzymes that are contained in raw food)

Equally important, there are some other industrially essential enzymes including cellulolytic, lignolytic and proteolytic (Goyal & Phutela, 2018).

2.3 Mechanism of Enzyme Activity

Cellulose, the most common natural renewable biopolymer, is commonly degraded by the hydrolytic action of a multicomponent enzyme system- the cellulase and represents the key step for biomass conversion (Sadhu et al., 2013). Cellulose provides to 8 % of the worldwide industrial enzyme needs and it is anticipated to grow larger by 100 % within 2014 (Gaur & Tiwari, 2015). Manavalan et al. (2014) and Malik et al. (2016) explained that among worldwide biomass, cellulosic material is well known available throughout biosphere which comprises of 4 sub-polymer; lignin, pectin, cellulose and hemicelluloses.

Cellulase is any of several enzymes produced commonly by fungi, bacteria, and protozoans that catalyse cellulolysis, the decomposition of cellulose and of some related polysaccharides. Generally, cellulase can be an association of free inducible enzymes which are synthesized by microorganism during their growth on cellulosic materials (Ghosh & Roy, 2016). This was described by Saini et al., (2015) that open up an idea of cellulose degrading enzymes. A group of enzymes in cellulose hydrolysis is usually called cellulose complex. It was split up by few components of enzymes: endoglucanases (EG) [EC 3.2.1.4] , exoglucanases (cellobiohydrolases, CBH) [EC 3.2.1.91] and beta-glucosidases (BGL) [EC 3.2.1.21].

Youssef (2011) and Ghosh and Roy (2016) also agreed that a single enzyme cannot accomplish the task of extensive cellulose degradation, hence, multiple enzymes are required. There are three essential reactions catalysed by cellulases which are the endogucanase breaking the noncovalent interaction, secondly chain ends breakage by cellobiohydrolase and finally disaccharide dispersion into glucose by β-glucosidase. They hydrolyzed the β-1,4 linkages in cellulose and converted into soluble sugars or glucose (Caf & Arikan, 2017). Figure 2.1 works as an illustration to describe how three different enzymes synergistically conduct complete alteration by operating different characters. The process begins where endoglucanase strikes in to dispose chain randomly on amorphous cellulose fibre and produces small fibers with free reducing and non-reducing end. Later on, cellobiohydrolase takes charge by releasing cellobiose on free ends which then hydrolyzed by β-glucosidase and produce glucose as end product.

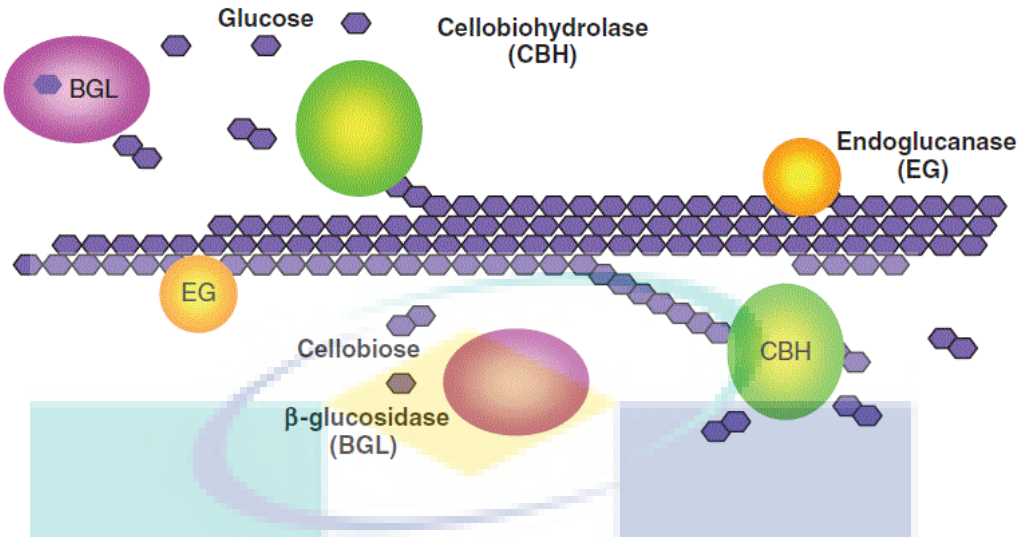


Figure 2.1 Action of different component of cellulase

Source: Singhania (2009)

There are various parts of the complex cellulosic structure and the main structures are hemicelluloses, cellulose and lignin. The target for a cellulose degrading process is to hydrolyse the hardest part of the cellulose which is the lignin. However, during the process, other parts of the cellulosic material might come together to form barrier but will be least hindrance as the target part has been the first priority. Due to unproductive binding in lignin structure, it can be a major reason in slowing down the enzymatic degradation (Alvarez et al., 2016) .

Moreover, the effieciency of the cellulose being breakdown to glucose molecules to be converted to ethanol can be hindered by the crystallinity and association of cellulose with hemicellulose. In the same way, cellulose and hemicellulose hydrolysis generated a variety of simple sugar monomers which can add to difficulty (Rose & Bennett, 1999). Therefore, enzymatic hydrolysis can be restrained by the following substrate-related factors: cellulose contains highly resistant crystalline structure, lignin and hemicellulose surrounding cellulose form a physical barrier and sites available for enzymatic attacks are limited (Aliyu et al., 2017).

2.4 Biotechnological Potential of Cellulase Enzyme

One of the potential in biotechnology is biofuels production which used enzyme technology in different bioconversion and upgraded fermentation processes. Based on

the recent research by Manavalan et al. (2014), present day is the generation of new biofuel research using lignocellulosic biomasses being used as a major source of biofuel production. Similarly, Saini et al. (2015) informs that new generation bioethanol is produced from lignocellulosic feedstock by its saccharification, followed by microbial fermentation and product recovery products (Ballardo et al., 2017). Bioethanol production can be hugely exploited from the residues of agricultural industries which resulted with renewable lignocellulosic wastes. Lignocellulosic waste is renowed due to their abundance, recycle ability and also high carbohydrates content particularly in the production of biofuels (Monlau et al., 2014).

In the recent year, renewable energy source has been seriously explored as to conserve earth's fossil energy as well as maintain the existing energy. Using technology, biodegradable material will be a big help in handling mass production of energy flow as it require less worker, less waste material and cost effective. Equally important, some biobased discoveries are in fast demand comparing to old ways such as geomics and preteomics, metabolic engineering, bioinformatics and recombinat DNA technology, metabolic engineering, functional genomics and proteomics and bioinformatics, which are rapidly outpacing the more traditional and catalytic-based chemical processes (Rogers et al., 2005).

Until the present, enzyme industry has been producing highly used cellulase worldwide including the microbial source as highlighted in Table 2.1. Liquid and powder form celulases were produced commercially with naturally found bacterial and fungal strain. While in this case, cellulases were found to produce genetically enhanced enzymes to convert cellulose to sugar. One of the benefits for enzymes to be available two states which are cellulase and hemicellulase, it will cut the cost in pretreated lignocellulosic material to be converted to fermentable sugars. This factor contributes to an increase in enzyme activity as claimed by these enzyme producers companies; Genencor International and Novozymes A/S.

Table 2.1 Commercially available cellulases

Product name	Company	Source	pH	Temp (°C)	Form
Biocellulase TRI	Quest Intl. (USA)	*T. reesei*	4.0-5.0	50	Liquid
Biocellulase A	Quest Intl. (USA)	*A. niger*	5.0	55	Powder
Celluclast 1.5 L	Novo Nordisk, (Danbury, CT)	*T. reesei*	5.0	50	Liquid
Cellulase TAP10[6]	Amano Enzyme (Troy, VA)	*T. viride*	5.0	50	Powder
Cellulase AP30 K	Amano Enzyme (Troy, VA)	*A. niger*	4.5	60	Powder
Cellulase TRL	Solvay Enzymes (Elkhart, IN)	*T. reesei*	4.5	50	Liquid
Econase CE	Alko-EDC (USA)	*T. reesei*	5.0	50	Liquid
Multifect CL	Genencor Intl. (USA)	*T. reesei*	4.5	50	Liquid
Multifect GC	Genencor Intl. (USA)	*T. reesei*	4.0	50	Liquid
Spezyme	Genencor Intl. (USA)	*T. reesei*	4.0	50	Liquid
Ultra-Low Microbial (ULM)	Iogen, (Ottawa,Canada)	*T. reesei*	NA	NA	Liquid
Cellic Ctec 2	Novozymes (Bagsvaerd, Denmark)	Enzyme cocktail	NA	NA	Liquid
Cellic Ctec 3	Novozymes (Bagsvaerd, Denmark)	Enzyme cocktail	NA	NA	Liquid

NA: Not available

Source: Saini et al. (2015)

Cellulases are commonly used in many industrial applications and the demands for more stable, highly active and specific enzymes are growing rapidly (Vimal et al., 2016). The cellulase prevalence can found in animal feedstock industry, starch processing, paper industry, extraction of fruit, malting and brewing, textile industry and grain alcohol fermentation (Abo-State et al., 2010).

Source of cellulase enzymes may come from *Trichoderma* and *Aspergillus* that have been frequently used by enzyme companies worldwide. Due to different fermentation method, different yield of enzyme can be harvested. However, when manufacturing costs are taken into account, submerged fermentation is chosen as the best way. After all, the outcome for this method which is dry protein weight exceeds total yield for normal enzyme production. Purchasing rate of the enzyme was estimated at the range of US $1.00 to $1.50 per gallon of cellulosic ethanol. Nowadays, a wide species of bacteria and fungi have been exploited for their capability in producing cellulase enzyme. More new species has been revealed for the potential and high value enzyme produced despite being underestimated. Generosity of fungi in producing large amount of cellulase and hemicellulase have been widely known and emphasised. Moreover, the enzymes from fungi are more stable and less-complex than bacterial-produced enzyme. With the help of rapidly growing bacterial host, the enzyme can be regenerated using recombination process (Kunamneni, 2016).

One of the main contributions of cellulase is in food industries which is extraction of juices from fruits or vegetables. To extract and clarify the juices from fruit and vegetables, a combination of enzymes is used to ensure the juices are macerated. The enzymes involved are hemicellulases, pectinases and cellulase. The enzymes also can improve the yield and save the production cost. In fact, these enzymes can also amplify antioxidant ad vitamin E content in olive oil production (Kunamneni, 2016). Furthermore, celluases are used in the production of colouring agents which are extracted from different type of plants. One of the colours is carotenoid which can be generated from carrot, sweet potato and orange peel. Carotenoid helps in releasing red to yellow colour pigments and has null toxicity, high versatility and desirable properties due to natural origin. Cellulase and pectinase involve in the disruption of cell wall of the carotenoid sources and the pigment has been a high demand in the market (Behera et al., 2017). Apart from the significances of cellulases, a lot can be improved such as in

production cost. In addition, numerous studies are conducted in term of increasing the cellulases' effectiveness henceforth only a small amount of enzyme required in every production. In fact, progressions in microbial genetics and protein engineering have come in long way and expected to be more developed in the future (Sukumaran et al., 2005).

In pharmaceutical industry, cellulase which associated with digestive enzyme is used to digest cellulose fibres. This is important in order to maintain healthy cells (Behera et al., 2017). Equally important, Kunamneni (2016) clarifies that cellulase can also treat phytobezoars, a type of indigestible food fibres such as cellulose which come from fruits or vegetables. Phytobezoars are the most common type of bezoars and can found in human stomach.

Hence, cellulase helps in curing the blockage in digestive tract. Moreover, cellulase can be found in textile making industry to digest the small fibre ends. This process is named as biostoning where enzymatic process is introduced instead of using harsh chemical agents. The fabrics resulted in a uniform aged look for denim and various visual effects can be experimented. Besides, utilizing cellulase in pulp and paper industry can save around 20 to 40 % energy through improving strength of the handsheets. For paper mills, recycled fibres undergo deinking and at the same time, the drainage and runnability can also be increased using cellulase. Also, cellulase assists in the removing of ink, toners and coating of the papers (Sukumaran et al., 2005).

However, in many bioconversion strategies, the cellulases required for biomass conversion may still account for as much as 40 % of the total process cost. Thus, large-scale and low-cost production of cellulase is a significant key for the overall process economics of lignocellulosics bioconversion (Trinh et al., 2013). Based on ideas of Shuangqi et al. (2011), there are three main problems in the determination process of cellulase activity:

i. Different components of a multi-component cellulase hinder the substrate characteristics from being expressed therefore standardised method of determining the enzyme activity become hard.

ii. Different source of cellulose makes the determination inconsistent. This is due to the contradict composition and proportion of components.

iii. The complex structure of cellulose biochemical characteristic, its composition and enzymatic effects makes the purity of components is inconsistent. Thus, the classification of cellulase system not really interflow.

Complementary to the idea mentioned earlier, the difficulty in comparison between cellulases activities depends on several factors including the assay determination, different microorganism strains used in production and conditions of production which are submerged fermentation (SmF) or solid state fermentation (SSF).

2.4.1 Carboxymethyl Cellulase (CMCase)

Carboxymethyl cellulase contributes as one of the main classes of enzyme systems (endoglucanases). Endoglucanases are called as CMCase and only degrade carboxymethyl cellulose. This is because endoglucanase cleave the beta-1,4-glycosidic linkage present in CMC. Therefore as it cleaves the bond in the inner part of the CMC it is called as endoglucanase. There are many applications of this enzyme such as production of animal feed, formulation of detergents, juice clarification, paper industry, beauty treatment (Botox) and wine production (Kiio et al., 2016).

Sharma et al. (2015) reported that cellulose degrading bacteria *Bacillus tequilensis* S28 was successfully isolated from soil and optimization of the cultural conditions was done to enhance CMCase production. Using these Plackett Burman and Response Surface Methodology statistical designs, maximum CMCase activity was observed to be higher than CMCase activity in unoptimized media. List of other microorganisms that have been studied to produce carboxymethyl cellulase was illustrated in Table 2.2.

Table 2.2 Bacterial and fungal strains used in the production of carboxymethyl cellulase

	Bacterial and fungal strains	Reference
1	*Peniophora* sp.	(Trinh et al., 2013)
2	*Bacillus tequilensis* S28	(Sharma et al., 2015)
3	*Penicillium digitatum*	(Sadiqa & Irshad, 2018)

Table 2.2 Continued

	Bacterial and fungal strains	Reference
4	*Fusarium oxysporum*	(Dar et al. 2013)
5	*Bacillus sonorensis* CY-3	(Caf & Arikan, 2017)
6	*Bacillus* sp	(Padilha et al., 2015)
7	*Pycnoporus sanguineus*	(Jalil et al. 2017)
8	*Bacillus pumilus* MGB05	(Bhuyan et al., 2018)
9	*Trichoderma viride*	(Ahmed et al., 2012)
10	*Aspergillus spp*	(Abrão et al., 2017)

2.4.2 Avicelase

Avicelase degrades crystalline cellulose to cellobiose. It cleaves disaccharide (cellobiose) units either from non-reducing or reducing ends. Avicelase is therefore commonly regarded as synonymous with exoglucanase or cellobiohydrolase (CBH) (Ray, 2015).

Walter and Schrempf, (1996) showed that avicelase is bound to the culture pellet consisting of the insoluble crystalline substrate (Avicel) and mycelia. However, an addition of Tween to the culture medium resulted in the release of a major portion of the enzyme to the culture filtrate. In contrast, soluble hydroxyethylcellulose (which can also be hydrolyzed by avicelase) did not give a measurable induction of the enzyme.

The only carbon source that causes avicelase synthesis is crystalline cellulose. Although pure microcrystalline cellulose supplemented culture medium induces the production of avicelase, a number of bacterial and fungal strains are reported to synthesize extracellular avicelase utilizing indigenous cellulose or cellulosic wastes such as wheat straw or rice bran.

A number of exoglucanase or avicelase producing bacterial strains were reported from a strain of *Geobacillus stearo thermophilus* (Ray, 2015). Avicelases are found to have potential applications in the bioconversion of agricultural waste materials to useful products, such as single cell protein, fuels and chemical feed stocks (Mukherjee et al., 2011). List of other strains that have been studied for avicelase production was depicted in Table 2.3.

Table 2.3 Bacterial and fungal strains used in the production of avicelase

	Bacterial and fungal strains	References
1	*Streptomyces reticuli*	(Walter & Schrempf, 1996)
2	*Geobacillus stearothermophilus*	(Makky, 2009)
3	*Paenibacillus chitinolyticus* CKS1	(Mihajlovski et al., 2015)
4	*Serratia marcescens* L4	(Shashidhar et al., 2018)
5	*Pycnoporus sanguineus*	(Jalil et al., 2017)
6	*Escherichia coli*	(Comlekcioglu et al., 2017)
7	*Streptomyces sp*	(Pinheiro et al., 2017)
8	*Penicillium chrysogenum P33*	(Yang et al., 2018)
9	*Trichoderma* and *Penicillium*	(Zhang et al., 2014)
10	*Aspergillus niger N402*	(Pensupa et al., 2013)

2.5 Substrate for Enzyme Production

Substrate is a chemical reactant which regulates its life dependency of presence of any suitable enzyme. On a situation substrates may react as a single component or in other condition substrates may flock by combining with each other to form a larger component. Many enzymes process tens or hundreds of reactions every second instead of thousands. Initially, a higher substrate concentration will increase enzyme activity, but when the enzymes become saturated, there is no further increase in processing activity no matter how much substrate is present. There are important factors for substrates to optimize the enzyme production including substrates concentration and substrates size. It has been shown experimentally that if the amount of the enzyme is kept constant and the substrate concentration is then gradually increased, the reaction velocity will increase until it reaches a maximum peak (Sharma, 2012).

Substrate acts as a pillar in enzyme production other than active site by giving an easy access for enzyme to hydrolyse specific reaction and produce a specific product. The enzyme will always return to its original state at the completion of the reaction. One of the important properties of enzymes is that they remain ultimately unchanged by the reactions they catalyse. After an enzyme is done catalyzing a reaction, it releases its products.

There were a variety of substrates that is accessible, low cost and sustained. Examples of substrate used in enzyme production which derived from agricultural are rice straw (Akhtar et al., 2017) and carrot grass (Bharti et al., 2018). Many cellulolytic waste products which otherwise are inedible, are converted into useful products by the aid of microorganisms. The biological degradation of cellulose has a great importance in the activity of living system (Gautam et al., 2011). Whereas, substrates consumption in enzymatic hydrolyzing of cellulose plays a quite essential points such as to avoid conflict whether a harsh pretreatment is needed to achieve a satisfactory result for enzyme production. Three basic factors of substrates for a reliable enzymatic reaction are as follow. Firstly, substrate accessibility that can involves the roles of certain component removal and size reduction by pretreatment. Secondly, Substrate reactivity limited by component inhibition such as binding site annihilation. Thirdly, reaction conditions such optimization of substrate-specific components (Bech & Herbst, 2015).

2.5.1 Substrate Derived from Industrial Waste

Global scenario of enzyme market emphasizes technical improvements and quest of better enzyme in accordance to the development of small and medium enzyme market. To elaborate, small steps has been taken to relate the development using recycling method which is the practice of reusing items that would otherwise be discarded as waste. Variations of recycling include upcycling, which involves adding value to an item for reuse, and downcycling, which involves breaking down an item or substance into its component elements to reuse anything that can be retrieved (Shrestha et al., 2017).

Back in the days, burning of any undesired wastes was way too common among the citizen. This also includes industrial wastes by unlicensed companies or small emerging companies. Burning practices are problematic due to the environmental concerns and negative health effects. Thus, there is an increasing trend towards more environmental friendly management practices in terms of recycling of agricultural residues and waste-to-energy concepts (Hamza, 2017). Devi & Kumar (2013) explained that sawmill industrial wastes (sawdust and paper cellulose) have been selectively chosen for the carbon source content and microorganism's isolation in producing cellulases. The study also emphasised on using readily available and cheap substrate to

produce cellulases using fungi that was isolated from industrial waste. Simultaneously, recycling practice can also be conducted using industrial cotton wastes (polymer, chemical, food, paper and textile industries). Usage of the waste gives good social values since the research can make profitable outcome from the numerous cotton industries (Thambiraj & Ravi, 2017).

2.5.2 Substrate from X-ray Film Sheets

Amidst all the potential substrates, cellulose-composed substrate (disposed x-ray film) which has vast possibility in enzyme production has been gradually disclosed. X-ray film sheets was used in radiography to capture and reveal internal organs condition and being basic necessities such as in dispensaries, hospitals and laboratories also some can be discovered in municipal waste (Verma et al., 2017).

X-ray film sheets comprises of film base (60 %), emulsion (20 %), protective coating (15 %) and adhesive coating (5 %). Based on Figure 2.2, film base which made up the majority of the x-ray sheet was used for sub-coating element. The base of this element consists of cotton and acetic acid. The cellulosic ingredient in this part intrigues some of the researcher to invigilate further whether it can be used in enzyme producing industry. As a part of recycle technology, this cellulose can be converted into usable products such as cellulase enzymes (Houston, 2007).

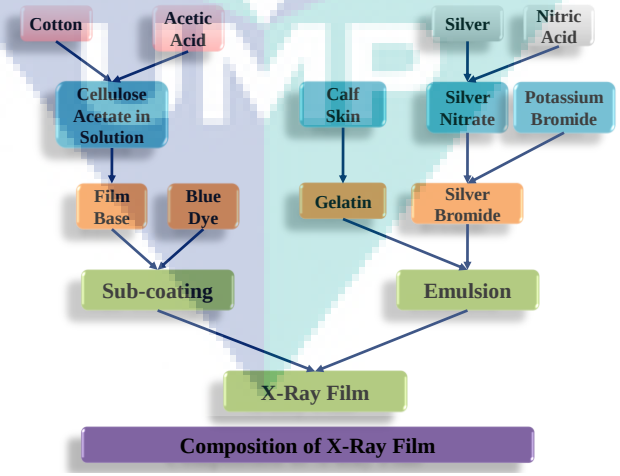


Figure 2.2 Composition of x-ray film

Source: Houston (2007)

Most prevalence microbial enzyme that used x-ray as substrate is protease which is regularly produced using bacteria derived from different natural sources. Protease has a lot of benefits for future industrial application as a cleaning bio additive in detergent formulations. Also, *Bacillus* species have been widely explored for its ability to tolerate alkaline condition (Mhamdi et al., 2017). Table 2.4 exhibited type of enzyme and different species of microorganisms involved in biodegradation where x-ray film was used as substrate.

Table 2.4 X-ray film used as substrate in enzyme production

	Type of enzyme	Microorganisms	Reference
1	Alkaline protease	*Bacillus* sp.	(Masui et al., 1999)
2	Protease	*Bacillus* sp	(Hamza, 2017)
3	Serine proteases	*Purpureocillium lilacinum* LPS # 876	(Cavello & Hours, 2013)
4	Alkaline protease	*Conidiobolus coronatus*	(Shankar et al., 2010)
5	Alkaline protease	*Bacillus subtilis* (NCIM 2724)	(Parpalliwar et al., 2015)

Back in the days, awareness of the treatment of wastes is very low but due to lack of space and pollution prevalence, more disposal ideas are generated. Furthermore, Malaysia is not included in the list of countries that have mastered proper and integrated plan of waste management. This is due to lack of budget that contribute to facilities restrain, lack of authorities cooperation and also inadequate treating plant (Che Jamin & Mahmood, 2015). Emergence of various technologies including biotechnology in treating the waste slowly copes up with the abundance of waste. In Figure 2.3 different methods of treating one of the waste which is x-ray film has raised many issues primarily due to cost effectiveness. Also, the effluents that come from the incineration method can cause harmful effect to human and environment.

Although most of the usage of the x-ray sheet waste was to recover silver, the cellulosic content in the x-ray film sheet can be introduced as a new key ingredient. For example in enzyme making industry, cellulosic waste either semi or full dialysed were able to amplify the interest of research for a cheaper and more convenient enzyme production. To further cut the whole complicated process, cellulose-degrading microorganism can be one of the options. It has been long discussed that microorganisms used as a vital source of discovery for example in pharmaceutical, genetically-modified product and bioremediation.

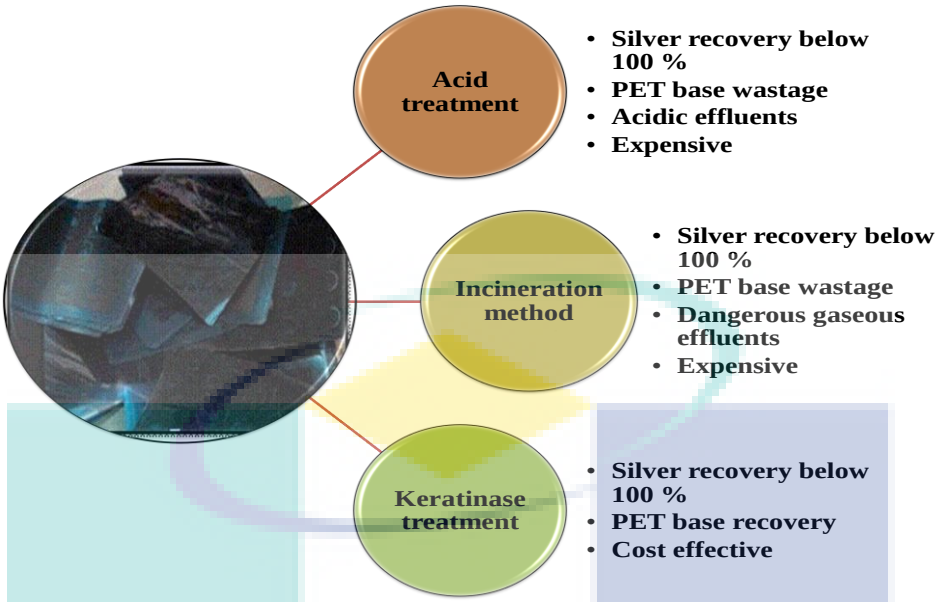


Figure 2.3 Common ways of disposing x-ray film sheet from dispensaries, hospitals and laboratories

Source: Verma et al. (2017)

2.6 Cellulosic Pretreatment

Prior to enzymatic reaction it is preferable to pretreat the cellulose waste to remove hindrance for metabolic process to take place. The goal of pretreatment is to make the cellulose accessible to hydrolysis for conversion to simple sugars. This can be achieved by physical, chemical, physicochemical and biological processes. During pretreatment, the matrix of cellulose and lignin bound by hemicellulose should be broken to reduce the crystallinity of cellulose and increase the fraction of amorphous cellulose, the most suitable form for enzymatic attack (Saini et al., 2015). The existence of pseudo lignin has been recognized because the amount of Klason lignin in pretreated biomass has often been found to be higher than in untreated biomass and has furthermore been reported to increase with pretreatment severity (Rasmussen et al., 2014)

There were multiple studies reporting the profile of degradation products formed from a particular pretreatment regime (Rasmussen et al., 2014). Unfortunately, even within the same type of pretreatment, several different process conditions such as time

and temperature are applied, which makes it difficult to unravel correlations between pretreatment conditions and degradation product formation. Identification of the factors such as production, timings, activities of lignocellulolytic enzymes, and their functions are important to maximise the delignification and recovery of simple sugars after pretreatment (Meehnian & Jana, 2017).

Steam explosion, dilute acid, ammonia fibre explosion, and organosolv pretreatments are currently being investigated for their ability to improve the hydrolyzability of lignocellulosic biomass (Maobing et al., 2009). With this intention, pretreatment of biomass before the enzymatic hydrolysis causes biomass to be easily and effectively converted into sugars (Srivastava et al., 2018).

2.6.1 Purpose of Cellulosic Pretreatment

Pretreatment of cellulosic material can be an alternative to disguise the biomass as a material that is ready to be used. The readiness of biomass will be depending on the accessibility of the material to different techniques till a certain degree. There are also materials that are not accessible to produce high yield even after complicated technique. Significantly, pretreatment is used to convert poor quality material to a profitable material that can generate worthy products (Lee et al., 2014).

Ghosh et al. (2016) has revived the techniques to facilitate the conversion of cellulosic biomass which is cereal straw by turning them to biofuels through fermentation process. Through that, several types of biofuels can be produced and most important thing is that the unmanageable status of cellulosic biomass can be solved by cutting back the amount of process using pretreatment as a brilliant approach. It can be seen during the conversion phase, enzymes have been slowed down by the cellulose biomass partly from the following aspects which cause a demand of pretreatment process before it can be enzymatically deconstructed into simple sugars. First aspect is lack of accessibility causes by the complicated structure of lignocellulosics. Second aspect is the condensed quantity of cellulose, hemicelluloses and lignin (Bali et al., 2015).

2.6.2 Types of Cellulosic Pretreatment Process

Due to complex structures and ultrastructure, Bali et al. (2015) summarizes 3 methods that can be used to treat cellulosic biomass:

i. Chemical pretreatment.
ii. Physical pretreatment.
iii. Combination of physical and chemical pretreatment.

Pretreatment has several aims, such as disrupting the physical structure of the biomass by breaking the lignin barriers, disrupting cellulose crystallinity, and removing noncellulosic components in order to increase the cellulose accessibility.

The pretreatments of the lignocellulosic biomass are done either through physical, chemical or combination of physical and chemical methods Ghosh et al. (2016). The pretreatment of cereal straws are conventionally done either by physical (steaming, grinding, milling, irradiation, exposure to temperature and pressure) or chemical (acid, alkali, ammonia, solvent and oxidizing agent) processes or through the combination of physical and chemical processes. The physical processes are highly energy intensive, whereas the chemical processes are often associated with equipment corrosion and generation of several inhibitors. Alkali was also used for the pretreatment of lignocellulosic biomasses and its action depends upon the lignin content present in the biomass (Irfan., 2014)

2.7 Cellulose Degrading Microorganism

Cellulolytic enzymes may come from a various of bacteria and fungi species, aerobes and anaerobes, mesophiles and thermophiles, and even by virus (Sharma & Yazdani, 2016). Extensive study has been done on cellulolytic enzymes produced by fungi and bacteria. Productions of enzyme from different microorganisms (algae, bacteria, fungi) will help in saccharification/hydrolysis and released reducing sugars thus making the final products (Baig et al., 2004).

It has been found that most researches are focusing on cellulase-producing fungus (Sandhu et al., 2018) However, few detailed information on cellulase-producing

bacteria is available up to date, such as *Cytophaga sp*. LX-7, Bacteroidaceae and thermostable *Escherichia coli* (Li & Gao, 1997). For example, Hong-li et al. (2015) isolated two strains (FY2 and MY6 strains) derived from fresh cow dung and fermentation biogas slurry.

Based on this, microorganisms were acquired from sources which were believed to have potential cellulase producers. Thorough investigation was carried out to intensify the probability of finding most potent cellulase producers. To study the morphological characteristics of the cells, two different cellulase-producing bacteria were identified as rod-shaped after Gram staining method after being observed with a microscope (1000 ×) (see Figure 2.4).

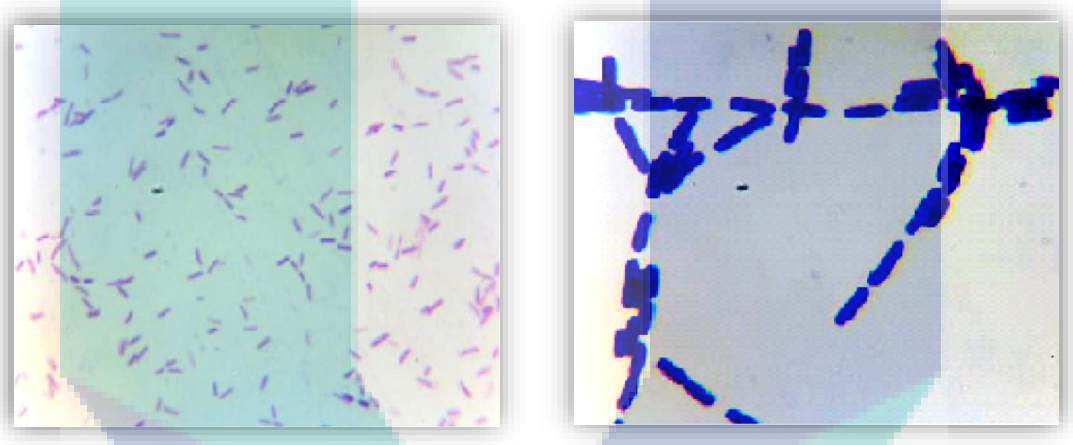


Figure 2.4 FY2 and MY6 strains under microscope observation
Source: Hong-li et al. (2015)

Cellulase can be found from vast species of microorganism from environment but more undeclared rare species are waiting to be harnessed. Metagenomics is a widespread study that focus on recovering genetic material from environmental samples, has an immense ability to uncover novel uncultured microbes from different region of the world. Hence, new strains which have been gradually discovered were classified according to distinctive traits. Table 2.5 sum up cellulase type with the source and environment, substrate and identified cellulase (Sharma & Yazdani, 2016).

Table 2.5 The cellulase enzyme from different metagenomic libraries obtained by function driven screening cellulase

Cellulase Type	Source and Environment	Substrate	Identified Cellulase	References
Endoglucanase	Anaerobic digestor	CMC, MUC	GH5	Healy et al. (1995)
Endoglucanase	Enrichment of lake sediment	CMC	GH9	Rees et al. (2003)
Endoglucanase	Enrichment culture of alkaline lake sediments and soil	CMC	GH9, GH79	Grant et al. (2004)
Endoglucanase	Contents of cow rumen	OBR-HEC	GH5, GH6	Ferrer et al. (2005)
β-Glucosidase	Soils from wetland	MUC	GH1	Kim et al. (2007)
Endoglucanase	Cow-rumen fluid	Dye-linked AZO-xylan	GH5, GH26	Palackal at al. (2007)
Endoglucanase/β-glucosidase	Contents of rabbit cecum	CMC, MUC, EH-FAC	GH3, GH3	Feng at al. (2007)
Endoglucanase	Contents of hindgut of higher termite	PASC	GH5, GH9, GH45	Warnecke et al. (2007)
Endoglucanase	Soil	CMC	GH44	Kim et al. (2008b)
Endoglucanase/β-glucosidase	Forest soil, elephant dung, cow rumen, rotted tree	CMC, MUC, EH-FAC	GH5,GH9, GH3	Wang et al. (2009)
Endoglucanase	Contents of cow rumen	CMC	GH5	Shedova et al. (2009)
Endoglucanase	Aquatic community and soil	CMC	GH5, GH9	Pottkämper et al. (2009)
β-Glucosidase	Sludge from biogas reactor	EH-FAC	-	Jiang et al. (2010)
β -Glucosidase	Marine environment	-	GH1	Fang et al. (2010)
Endoglucanase	Soil	-	GH5	Liu et al. (2011)
β -Glucosidase	Alkaline-polluted soil	EH-FAC	-	Jiang et al. (2009)
Endoglucanase	Enrichment culture	CMC	GH5	Ilmberger et al. (2012)
Cellulase	Termite gut	-	GH5	Nimchua et al. (2012)
Cellulase	Cattle rumen	AZCL-HE-cellulose	GH5	Nguyen at al. (2012)
Endoglucanase	Rice-straw compost	CMC	GH12	Yeh et al. (2013)
β -Glucosidase	Gut of *Globitermes brachycerastes*	Esculin-FAC	GH1	Wang et al. (2012)
Endoglucanase/ β -glucosidase	Cow rumen	Filterpaper, Avicel,xylan	GH5, GH8, GH9	Gong et al. (2012)
Endoglucanase	Bovine rumen	CMC	GH5	Rashamuse et al. (2013)

Source: Sharma and Yazdani (2016)

2.7.1 Cellulose Degrading Bacteria

In reality, the factors such as can withstand high acidic condition, high alkali condition and high temperature making bacteria as successful candidate in industrial needs. The bacterial isolates from different environment allows screening for efficient cellulases to help overcome challenges in application of cellulases (Vijayaraghavan et al., 2016). These features can be found in cellulases that were produced by bacteria that have wider organismal level (Sadhu et al., 2013). Species of the genus *Bacilli* are industrially important as they have a high growth rate, able to secrete proteins extracellularly and considered relatively safe to use with regard to health and environmental aspects (Beukes & Pletschke, 2006).

Vimal et al. (2016) and Gaur & Tiwari (2015) found that one of the good properties of bacteria is higher growth potential than fungi that results in higher rate of enzyme production which can be applied in cellulases production. Moreover, bacteria have the ability to degrade cellulose to obtain better cellulases from different sources such as organic matters, decayed flora, springs, soil, composts and faeces of ruminants. Previous studies have reported about cellulase production of bacterial isolates that were collected from various natural origins. They were from isolated from gut of termites (Shinde et al., 2017), soil samples (Picart et al., 2007), decayed wood of the papaya tree (Ponnambalam et al., 2011) and fresh cow dung (Hong-li et al., 2015).

2.7.2 Cellulose Degrading Fungi

Cellulases are feasible enzymes that fungi, bacteria or actinomycetes can produce during their consumption on cellulosic materials but the most prevalence generator is fungi (Samira et al., 2011). Novotný et al. (2004) and Aliyu et al. (2017) describe that filamentous fungi are preferred for commercially important enzymes production, because the level of the enzymes produced and also high biodegradative capabilities than those obtained from yeast and bacteria. Cellulases from fungi are commercially important as these enzymes are secreted outside the cells and robust. Generally, it is considered that thermophilic cellulases have commercial applications as they are robust and able to resist high temperatures and extreme pH values (Juturu & Wu, 2014).

These days, the pretreatment and hydrolysis steps of biorefinery systems had employed white-rot fungi-derived lignocellulolytic enzymes in various industrial needs. The lignin in wood was broke down by white-rot fungi, resulting in lighter stain (white) cellulose behind; some of these fungi produced powerful extracellular oxidative and hydrolytic enzymes by breaking down both lignin and cellulose. Different species of *Trichoderma spp.* are excellent cellulolytic model organisms to study for cellulose degradation since they release huge amounts of cellulases (Saini et al., 2015).

Among fungi, Trichoderma viride (Ahmed et al., 2012) and Aspergillus hortai (El-Hadi et al., 2014) have been studied for the CMCase production. The remarkable interest was found in Aspergillus niger, a species of great commercial interest with a highly promising future and already widely applied in modern biotechnology. Moreover, Soares et al. (2012) and Mukherjee et al. (2011) also point out that A. niger not only produces various enzymes but it is one of the few species of the fungus kingdom classified as GRAS (Generally Recognized as Safe) by the Food and Drug Administration (FDA). The species can produce cellulase freely, its cell mass is used as a component in animal feed and its fermentation produces organic acids and other compounds of high economic value

2.8 Parameters Involved in Enzyme Production

Generally, production of cellulase in laboratory is closely related with predetermined conditions. This is important as the the activity of the enzyme works well in an enriched and suitable medium. Various important environmental factors have been employed including incubation condition, carbon source, nitrogen source, pH value, substrate concentration, inoculum size and vitamin

In the study, One Factor at a Time (OFAT) approach was used to optimized the cellulase production by reducing overall fermentation process and cost. Furthermore, by optimizing fermentation conditions, the yield of the enzymes can be maximised (Adhyaru, et al., 2017). The approach was conducted by changing one component at a time while maintaining the others at the constant level. The study focuses on the process development in optimizing different conditions (physical and nutritional) of cellulase production media (Devi & Kumar, 2013).

2.8.1 Incubation Condition

One of physcial factors in enzymatic reaction is the incubation condition in which the media is either involved in static or shaking condition during fermentation process. The mixing of all elements in the media influence the yield by ensuring sufficient mixing of the cell and biomass which indirectly enhance the enzyme production (Jung et al., 2017).

Cellulase production from marine *Streptomyces* depicted a high activity at 100 rpm with 0.013 U/ml and low enyzme production under static condition with 0.004 U/ml. The enzyme was fermented under submerged condition using carboxymethyl cellulose (CMC) as carbon source (Fatokun et al., 2016). In a reserach of CMCase production using plant waste as the carbon source, the fermentation media were placed under static and shaking conditions. The enzyme produced by *Aspergillus terreus* DSM 826 showed more than 100 % of activities in shaking condition (150 rpm) compared to static condition (Abdel-Fatah et al., 2012).

However in some cases at the National Renewable Energy Laboratory (NREL), the implementation of shaking condition towards high insoluble loadings (≥ 15%) becomes ineffective. This presumably causes limitations in mass transfer such as mixing up of saccharification evaluation results, low enzyme dispersion and building up in localized hydrolysis output (Roche et al., 2009).

2.8.2 Carbon Source

Production of cellulase is also directly linked with the consumption of carbon in the culture medium. The carbon source of the medium is the vital source of energy which helps in product formation and cell growth. However the degree of production depends on the solubility, chemical structures of the carbon source and the availability of precursors for protein synthesis (Goswami & Pathak, 2013).

Trinh et al. (2013) justifies that the additional complex carbon slightly increased the CMCase production from 32 to 40 %. But sugars as other additional carbon sources including glucose, mannose, lactose, and sucrose, decreased the CMCase production up to two thirds compared with the control medium. This might be explained that certain strain preferred to consume simple sugar before utilizing other complex polysaccharides

as cellulose. Deswal et al. (2011) expressed that CMCase production *by Fomitopsis sp*. RCK2010 produce (71.526 IU/g) maximum activity when wheat bran was used as the carbon source. The physical properties such as particle size, geometry and compactness of the carbon source may influenced the enzyme production.

This was contradicted with Hagaggi (2018) which demonstrated media supplemented with 1% (w/v) lactose or CMC produced highest yield of enzyme production. While production of enzyme using glucose as carbon source was not stable. Meanwhile, cellulase production by *Aspergillus niger* using various carbon sources did not give positive effect. This was due to the substrate itself (banana peel) provides ample carbon saource needed for the growth which suggested media may not need additional carbon source to produce higher cellulase activity (Mandal & Ghosh, 2017).

2.8.3 Nitrogen Source

Nitrogen source is one of the important components in fermentation media. In enzyme production, nitrogen source encourages speedy growth and high yield of product. There are two types of nitrogen source which is inorganic such as ammonium salts and another one is organic such as amino acids, proteins or urea (Costa et al., 2002). In one of the reports, organic nitrogen source which is urea promotes better CMCase production by *Fomitopsis sp.* RCK2010. Whereas, no positive result acquired when inorganic nitrogen source associated in the media for CMCase production (Chakraborty et al., 2016).

Other than that, study of CMCase production by *E. aurantiacum* AN2 used different types of nitrogen sources which were NaNO3, peptone, yeast extract, NH4Cl, beef extract, tryptone, urea, meat casein, and casein. The CMCase production for media containing organic nitrogen source showed better improvement than inorganic nitrogen source. The best organic nitrogen source from the study was yeast extract followed by peptone which produce CMCase in presence of *E. aurantiacum* AN2 (Hagaggi, 2018).

Trichoderma sp. RCK65 produced a high yield of cellulose hydrolysing enzymes using different types of nitrogen source under solid state fermentation. Casein, corn steep liquor, urea, yeast extract, and soy bean meal have been tested using OFAT method. The results indicated the presence of soy bean meal; an organic nitrogen source gave out a maximum yield of cellulases. The condition may be contributed by the pH

medium regulated by the nitrogen source which enhanced the microbial metabolism (Chakraborty et al., 2016).

2.8.4 pH Value

One of the physiological factors in fermentation for enzyme production is pH value. Optimal pH value will ensure metabolism process and transports of nutrients run at desired rate. In fermentation process, maintaining pH value can promote functional proteins and support microorganism growth. Most organism grow over broad range of pH value 5.5-9.0 (Padan et al., 2005).

Besides, variation in yield of glucose from cellulose bioconversion at different pH demonstrates that pH is a critical parameter that affects enzymatic degradation process (Olajuyigbe, 2017). *E. aurantiacum* AN2 produced maximum yield of CMCase within pH values ranged from 7 to 10. However, in an acidic condition, the enzyme production was not impactful and CMCase production dropped starting from pH 10 (Hagaggi, 2018). The active sites of enzymes usually rely on the existence of ionic species that support conformations and facilitate effective binding to the substrate. This strong effect of pH makes it as an important factor to enzyme production by any microorganisms (Kheiralla et al., 2018).

Bharti et al. (2018b) observed the endoglucanase production was at maximum point in pH 7 media with (34.20 IU/gds). In solid state fermentation media, mixing mechanism was needed to maintain the pH. Eventually, enzyme activity declined as the pH moved to higher number. Whereas cellulase production from *Sporothrix carnis* grown on corn cob released an optimum activity at pH 5 (Olajuyigbe & Ogunyewo, 2016)

2.8.5 Amount of Substrate

Enzymatic hydrolysis happens in fermentation process of cellulase require several components. The fundamental components of enzymatic process either by hydrolysis or oxidation is the substrate itself. Biodegradation of cellulose requires the substrate to be available for enzymatic binding which takes place after the substrate is ready to cooperate. In optimization process of cellulase enzyme, amount of substrate has to be quantified according to the target enzyme to produce a maximum yield. In

addition, substrate can be regarded as nutritious feed that includes soluble sugars which accommodate the growth and propagation of the microorganisms (Sarkar & Aikat, 2014).

Chakraborty et al. (2016) presented *Trichoderma* sp. RCK65 was grown under solid state fermentation condition with a range of 2.5 to 20 g of wheat bran. Different concentration of wheat bran which was used as the substrate in cellulases production resulted in 5 g as the optimum concentration for all the tested enzymes. Maximum enzyme activities for FPase (41.09 U/g), CMCase (146.55 U/g) and β-glucosidase (104.64 U/g) were achieved with 5 g of substrate concentration which translated as a considerably low. The unfitting physical properties of subtrates such as geometry, particle size and density contributed to heat-mass transfer and aeration problems that were important for the fungus growth. Additionally, the amount of the substrate adds the complexity of the enzyme reaction which required quantification method to produce optimum production.

Hagaggi (2018) demonstrated the extracellular CMCase production produced by *Exiguobacterium aurantiacum* using CMC as the substrate. Eight substrate concentrations were studied 0.1, 0.25, 0.5, 1, 1.5, 2, 3, and 4% (w/v) where 0.5 % CMC was the optimum concentration for highest enzyme activity. Whereas, 1.5 % substrate concentration was expressed in optimum production of by *Bacillus subtilis* isolated from soil (Verma et al., 2012).

2.8.6 Inoculum Size

Commonly, most microorganisms can take up a wide range of substances as nutrients to produce various enzymes in proper time (Guo et al., 2017). This factor also related with the dose of the inoculum used in enzymatic production. A study on CMCase production from *P. variotii* produced a maximum activity (383.95 IU/g) when JDC (*Jatropha curcas* deoiled cake) was used as nutrient substrate and 10% inoculum dose was associated in solid state fermentation.

Other dose of inoculum which ranged from 5 to 25 % did not showed high yield with the possibility of increased competition for the substrate when higher doses were mixed with the fermentation media. The implications caused an exhaustion of macro

and micro nutrients that was supposed to facilitate the cell growth and enzyme production (Pathak et al., 2016).

This was in accordance Hagaggi (2018) which expressed that CMCase production by *E. aurantiacum* was found to be optimum using 2 % (v/v) of inoculum. Larger size of inoculums may cause the depletion in oxygen and nutrient in the production media which in return inhibit the cell growth and reduce enzyme product ion. The distinguished effect of the inoculum size on cellulase production by *S. fungicidicus* RPBS-A4 in solid state fermentation can be seen from the actual result. A range of 0.2 to 0.8 ml inoculum size was used and resulted in 12.59 U/ml maximum enzyme activity from 0.6 ml inoculum (Akurathi & Thoti, 2018)

2.8.7 Vitamin

Vitamin also one of important factors as it acts as co-factor for many enzymes. The absence of vitamins in culture may lead to decrease in cell growth, cell death or loss of productivity. Furtermore, the presence of vitamins usually affects the rate of biosynthesis of many metabolites (Büntemeyer & Lehmann, 2001).

The usage of vitamin was mentioned by Deswal et al. (2011) as a good accelerator as it gave utmost enzyme activities. Different vitamins were tested including retinol, pyridoxine, folic acid, thiamine, cyanocobalamine, ascorbic acid, riboflavin and biotin. Among those vitamins, folic acid pointed out maximal CMCase activity with 68.978 IU/g followed by riboflavin (65.716 IU/g), ascorbic acid (64.779 IU/g), biotin (52.561 IU/g) and retinol (51.050 IU/g). From the study also, all the vitamin has impacted in CMCase production either low or high effect which gave an advantage to CMCase exploration in the future.

That aside, from the data achieved by Kheiralla et al. (2018), the hemicellulase production from *Fusarium moniliforme* did not showed any significant results for the enzyme activities and protein content. Tween80, wheat bran and glucose that were used as the additives at different concentrations in submerged fermentation method, did not enhance enzyme production.

CHAPTER 3

METHODOLOGY

3.1 Introduction

This chapter explained the experimental design that has been used in this project. This chapter begins with screening of most potent isolates, pre-treatment of x-ray film, optimization for CMCase productivity, identification of selected bacterial isolates, purification and characterization of dialysed enzyme.

3.2 Methodology Flow Chart

The chronology of this research was illustrated in Figure 3.1 (page 25). The experiment started by pretreatment of disposed x-ray film, followed by screening of microorganisms. Following that, optimization of cellulase activity was carried out using OFAT method for 7 different factors which were incubation condition, carbon source, nitrogen source, initial pH, amount of substrate, inoculum size and vitamin. Subsequently, the most potent isolates resulted from previous test were identified using partial and full identifications. After that, purification process of supernatant from the optimized crude enzyme took place. Different percentages of ammonium sulphate were tested in the precipitation process and were brought to dialysis to obtain the dialysed sample with the highest enzyme activity. Then, the dialysed sample was eluted using diethylaminoehtyl DEAE-cellulose column which was the 1st chromatography. Immediately following that was Sephadex G-50 column chromatography. Later on, the molecular weight of partially purified enzyme was determined using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lastly, the partially purified enzyme was characterized using two factors which were temperature and pH.

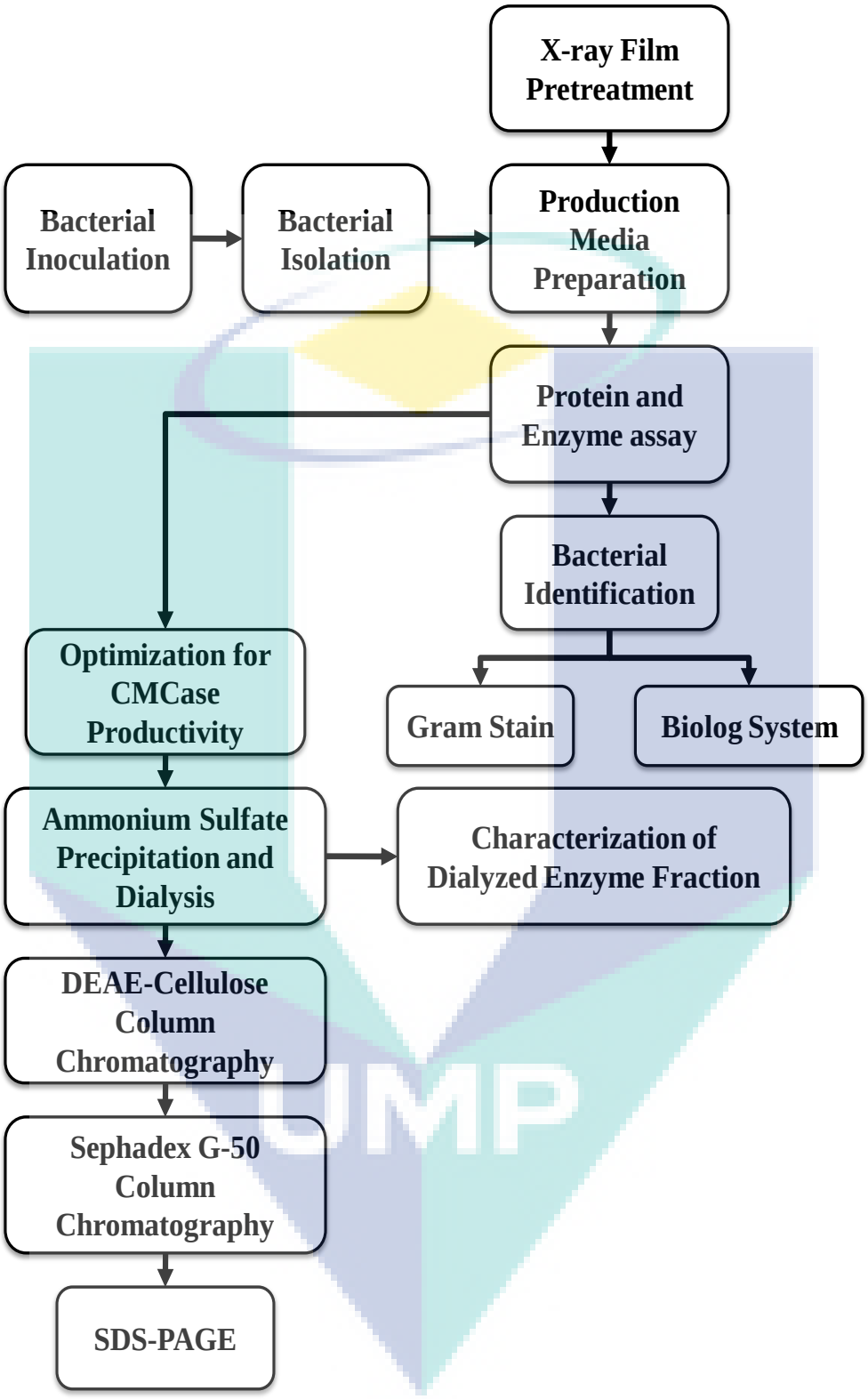


Figure 3.1 Methodology flow chart of the research

3.3 X-ray Film Pretreatment

Samples of used x-ray film were collected from local clinics around Kuantan area. For x-ray pretreatment purpose, 2 N sodium hydroxide (NaOH) was prepared by weighing 32 g NaOH and mixed with 400 ml distilled water (dH_2O) in a conical flask. X-ray film that was cut into 0.5 cm per 0.5 cm small pieces was added into a smaller conical flask and x-ray film was allowed to submerge in 2 N NaOH aliquot. The suspension was left to shake overnight at room temperature at 140 rpm using Lab Companion S1-600R Benchtop Shaker. The suspension was then washed with water to wash away the intense colour. The pretreated x-ray film was rinsed until the colour of the water become clear (refer Figure 3.2). The pH of the suspension was adjusted to 7 using pH meter and x-ray film was dried in the 50 °C oven for 48 hrs.

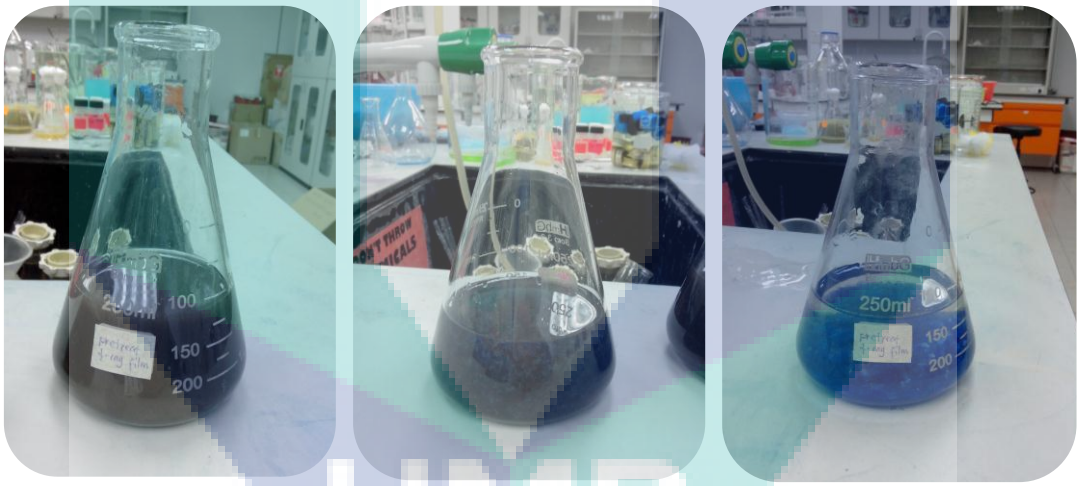


Figure 3.2 An illustration of x-ray film pretreatment before washing, after first rinse and after multiple rinsing.

3.4 Sample Collection from Different Sources

Bacterial isolates from different sources were collected in local area of Univerisiti Malaysia Pahang, Gambang in 23 km proximity. Table 3.1 described the source of the bacterial isolates, code used and GPS of the origin.

Table 3.1 Sources of the bacterial isolates

Source	Code used	GPS of origin
River Near Sekolah Sukan Malaysia Pahang	RSS	3.720552, 103.118033
Gambang Lake	GL	3.693899, 102.987476
Panching Waterfall	PS	3.793274, 103.143368
Kolej Kediaman 2 UMP	KK2S	3.729544, 103.124695
Chicken Liver	CL	NA
Spoiled Apple	A	NA

NA: Not available

3.4.1 Water Samples

River near the Sekolah Sukan Malaysia Pahang, and Gambang Lake were selected as water sources to get the samples. Both places were chosen from places near Universiti Malaysia Pahang, Gambang. Water samples were attained from deep region using plastic bottle. As the water gathered, the bottle was closed in the water after it was filled to the brim. The water samples were placed in the refrigerator prior to the next procedure.

3.4.2 Soil samples

As for soil samples, Panching Waterfall and Kolej Kediaman 2 UMP Gambang were chosen to attain fresh soil samples. Both places were chosen from places near Universiti Malaysia Pahang, Gambang. Clean plastic bags were prepared to gather the soil by digging to the bottom region and 300-500 g soil was collected. Rubber bands were used to tightly secure the soil samples in the plastic bags. The soil samples were safely placed in the room temperature prior to the next procedure.

3.4.3 Food Samples

Two food samples namely Apple fruit and Chicken Liver were left spoilt in room temperature for a week. Rotten apple and spoiled chicken liver was used as they represented degradable kitchen wastes. After that, both food samples were directly isolated on nutrient agar using streaking method.

3.5 Isolation of Microorganisms

One litre of nutrient agar (NA) was prepared on 60 Petri dishes for bacterial isolation. Twenty g of NA was prepared and topped up to 1 L with dH_2O mixed with magnetic stirrer and then autoclaved for 20 min at 121 °C. The media were poured into Petri dishes and were left to solidify.

For isolation method from water sample; Gambang Lake (GL) and River near Sekolah Sukan Malaysia Pahang (RSS), 1 ml of water sample was transferred to a sterile 15 ml conical centrifuge tube which contained 9 ml of dH_2O and mixed. The serial dilution method was used to get a desired concentration of samples. This conical centrifuge tube represents 10^{-1} dilution. It was labelled with: 10^{-1} dilution, date and sample name. Next, 1 ml of water sample from 10^{-1} dilution was transferred into another tube also contained 9 ml dH_2O, mixed and labelled with: 10^{-2} dilution, date and name. These steps were repeated until 10^{-5} dilution was made.

An L-shape glass rod was dipped into alcohol and flamed over a Bunsen burner From each dilution (10^{-1} to 10^{-5}), 0.1 ml of sample was transferred onto the surface of different NA plates. After that, the sample was spread over NA surface evenly (refer Figure 3.3). Each Petri dishes was labelled with: dilution factor (10^{-1} to 10^{-5}), date, and source of sample. The samples were then incubated at 37 °C for 24 hrs.

Figure 3.3 Isolation using spreading-plate techniques
Source: Sanders (2012)

For isolation of soil sample, Panching Soil (PS) Kolej Kediaman 2 UMP Soil (KK2S), 1 g of soil sample was mixed with 30 ml distilled water (dH_2O). The mixture was placed under constant agitation in a shaker for 5 min. By using the aseptic technique, 1 ml of the mixture was transferred into 15ml conical centrifuge tube contained 9 ml dH_2O and mixed by shaking for 1 min. The serial dilution method was done for all samples as previously mentioned. The same spreading-plate technique was used.

For isolation method from food samples: Apple (A) and Chicken Liver (CL), streaking method were used. Using sterilized inoculation loop, a loopful of sample was removed from the sources. The loopful of sample was streaked on a small area (refer Figure 3.4) at one edge of the plate (1). Then, the streaking continued to second quadrant leaving some space for another 2 quadrants (2). Only the tip of the streak from first quadrant was touched by the inoculation loop to form second quadrant. The space in third and forth quadrants (3, 4) were filled using same pattern making sure only pure culture formed in the last quadrant to be studied, tested and identified afterwards. The plate was then labelled with date, name and temperature. The plate was incubated at 37 °C for 24 h.

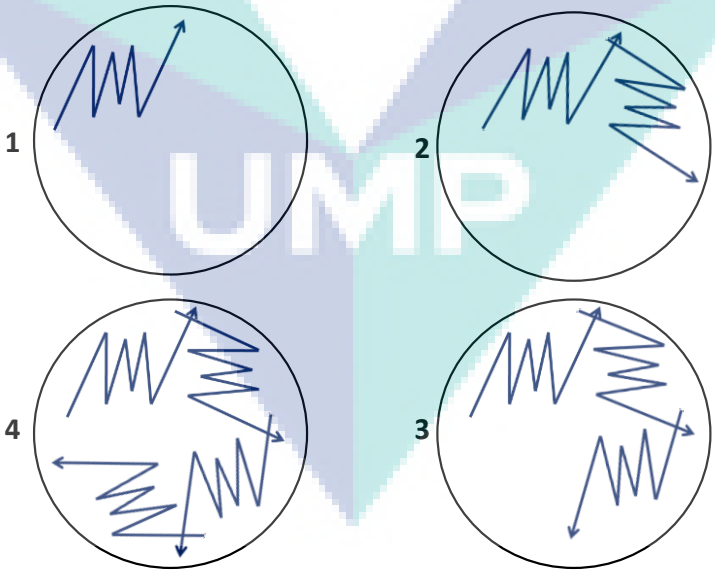


Figure 3.4 Isolation using streaking method

3.6 Purification of Bacterial Isolates Using Streaking Method

All the bacterial colonies obtained were purified on different plate. Using an inoculation loop and aseptic technique, the loop touched a colony of microorganisms. Then, the microorganisms were streaked on NA on a plate to get pure culture. The purified microorganisms incubated at 37 °C for 24 hrs.

3.7 Microorganisms Used in the Study

In this study, 20 different isolates derived from 3 different sources; water, food and soil were used. The selected isolates were updated in the form of group as in Table 4.1 (refer Section 4.2). Other than that, this study also used identified bacterial strains that have been used by former researchers and were obtained from Faculty of Industrial Sciences & Technology Laboratory, Universiti Malaysia Pahang in Gambang Campus. There were 7 identified bacterial strain namely *Enterococcus faecalis* ATCC 14506, *Pseudomonas aerogenosa* ATCC 15442, *Klebsiella pneumonia* ATCC BAA 1444, *Staphylococcus aureus* ATCC BAA-1026, *Bacillus subtilis* ATCC 11774, *Salmonella typhii* and *Escheria coli* ATCC 10536.

3.8 Inoculum Preparation

Nutrient broth (NB) was prepared prior to incubation of microbial isolates by mixing 13 g NB powder in 1 L dH_2O. The NB was measured with 50 ml measuring cylinder and poured into 100 ml conical flask before autoclaved for 20 min at 121 °C by using Hirayama autoclave machine. Aseptically, microbial isolates were transferred using inoculation loop from slant agar to the nutrient broth. Finally, the microbial isolate was incubated at 37 °C for 48 hrs.

3.9 Preparation of Modified M9 Minimal Salts (2×) Solution

Without glucose, modified M9 minimal salt media (2×) was prepared (refer manual sheet in Appendix A). First, 25.6 g disodium phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), 2.0 g of ammonium chloride (NH_4Cl), 6.0 g of potassium dihydrogen phosphate (KH_2PO_4), and 1.0 g of sodium chloride (NaCl) were weighed and placed into a 1 L beaker containing 500 ml dH_2O. Then, ingredients in the dH_2O were dissolved using magnetic stirrer. The solution was topped up to 1 L with dH_2O. The

solution was autoclaved at 121 °C for 20 min. The prepared solution was covered with aluminium foil and sealed with parafilm. It was then kept at room temperature**.**

3.10 Preparation of M9 Minimal Complete Medium

Prior to usage, 500 ml of M9 minimal salt media (2×) solution were transferred to a new 1 L beaker. Then, 2 ml 1.0 M magnesium sulphate ($MgSO_4$) solution and 0.1 ml 1.0 M calcium chloride ($CaCl_2$) solution were added into the 500 ml of M9 minimal salt media (2×) solution and topped up to 1 L with dH_2O and mixed until homogenous. The pH of M9 minimal complete medium was adjusted to 7 (unless otherwise stated) using pH meter and used immediately after prepared.

3.11 Production Media Preparation

Fifty ml of M9 minimal complete medium was measured using 50 ml measuring cylinder and poured into 100 ml conical flask. X-ray film which served as substrate was weighed to 0.5 g (unless otherwise stated) and added to the media and then autoclaved at 121 °C for 20 min. After that, media was left to cool down at room temperature.

3.12 Cell-Free Filtrate Preparation

At the end of incubation period, the media was transferred into 50 ml of conical centrifuge tube and centrifuged at 5000 rpm for 6 minutes at 4 °C using freeze centrifuge machine (Heraeus Biofuge Primor) to obtain cell-free filtrate (CFF) which had been used as crude enzyme.

3.13 Standard Curves

3.13.1 Protein Standard Curve

The albumin fraction V (from bovine serum) also known as bovine serum albumin (BSA) was used as standard. The protein standard curve experiment was carried out in dark environment because albumin fraction V is a light sensitive chemical. Different concentrations (refer Table 1 in Appendix B) of BSA were prepared as 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg/ml and the protein determined using Lowry

Method. Absorbance reading for each concentration was recorded at 700 nm and a standard curve (refer Figure 2 in Appendix C) was plotted (Lowry et al. 1951).

3.13.2 Enzyme Standard Curve

Glucose standard solution was prepared by mixing glucose and distilled water for 5 mg/ml concentration. From the standard solution, different concentrations (refer Table 2 in Appendix D) of glucose 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg/ml were prepared. Dinitrosalicylic (DNS) procedure was carried for these standard solutions and absorbance reading for each concentration was recorded at 575 nm. Using the absorbance reading, a standard curve (refer Figure 4 in Appendix E) was plotted (Miller, 1959).

3.14 Protein Determination

Protein determination procedure was carried out using the "Lowry Assay: Protein by Folin Reaction" (Lowry et al. 1951). This method was used to estimate the amount of proteins in biological sample and the protein content after assay method was expressed as mg/ml. Solution A (alkaline solution) was prepared by mixing 2.8598 g NaOH and 14.3084 g sodium sulphite (Na_2CO_3) in 500 ml distilled water. Solution B was prepared by dissolving 1.4232 g copper (II) sulphate pentahydrate ($CuSO_4.5(H_2O)$) in 100 ml distilled water. Solution C was prepared by dissolving 2.85299 g sodium tartrate dehydrate ($Na_2(tartrate)2H_2O$) in 100 ml distilled water. Folin reagent was prepared by mixing 2 N Folin and Ciocalteu's Phenol Reagent with distilled water with a ratio of 1:1. Lowry solution is a mixture of solution A, solution B and solution C with a ratio of 100:1:1 that were prepared earlier and mixed freshly before used.

Five ml of Lowry solution was added into a test tube before adding 0.5 ml of sample which was the supernatant resulted from production media. For the blank solution, 5 ml of Lowry solution was mixed with distilled water. The mixtures were left at room temperature for 15 min. After that, 0.5 ml Folin reagent was added to the mixtures and left for 30 min at room temperature. The colour changes can be clearly seen from colourless to blue indicated the presence of protein and the intensity of the colour marked as the higher content of protein (refer Figure 3 in Appendix C). Consecutively, the resulted sample reaction was transferred and measured using

microplate reader (Infinite M200 Pro Tecan). The absorbance value at 700 nm of the blank solution and sample solution was recorded. Triplicates of each sample were prepared to give more accurate results.

3.15 Enzyme Assay

Dinitrosalicylic (DNS) colorimetric method (Miller, 1959) with slight modification is a method used to detect the presence of free carbonyl group (C=O) or reducing sugars. Using this method, the equivalent enzyme used in the reaction can also be detected and was expressed as U/ml. One unit of enzymatic activity was defined as the amount of enzyme required to produce one micromole of reducing sugar per minute of reaction. The DNS principle is used widely these days to determine cellulose activity consider that the reducing sugar may reduce the nitro of 3,5-dinitrosalicylic acid (DNS) to amino after enzymatic hydrolysis was built. So, the yellowish colour of suspension turned to dark brown indicating amino compound (Shuangqi et al. 2011).

DNS reagent solution 1 % was prepared by mixing 10 g of dinitrosalicylic acid, 2 g phenol, 0.5 g Na_2CO_3 and 10 g NaOH in 1 L of dH_2O. Potassium sodium tartrate solution, 40 % was prepared by dissolving 40 g of potassium sodium tartrate ($KNaC_4H_4O_6 \cdot 4H_2O$) in 100 ml distilled water. A procedure for 0.1 M phosphate citrate buffer pH 6.6 was carried out by mixing 36.4 ml 0.2 M disodium phosphate (Na_2HPO_4) and 13.6 ml 0.1 M citrate in a 100 ml beaker. This buffer was labelled and stored at room temperature before further use. Meanwhile to produce 0.1 M sodium acetate buffer pH 5.0, 357 ml of acetic buffer was mixed with 643 ml sodium acetate in a 1 L beaker. This buffer was labelled and stored at room temperature.

Different testing buffers for CMCase and avicelase were used using method from (Abo-State et al. 2010). For avicelase assay, 1 ml of cell-free filtrate was incubated with 1 ml of 2 % Avicel in 0.1 M phosphate citrate buffer pH 6.6 at 40 °C for 2 hours. For CMCase assay, 1 ml of cell-free filtrate was incubated with 1 ml of 1 % CMC in 0.1 M sodium acetate buffer solution pH 5.0 (unless otherwise stated) for 30 min at 63 °C (unless otherwise stated). For the blank solution, distilled water is used instead of cell-free filtrate. The resulted reducing sugars were determined using DNS method. To avoid the liquid from evaporating, 1.5 ml of DNS reagent was added to 1.5 ml sample in a capped tube. The mixture was heated at 90 °C for 5-15 min to develop the dark brown

colour (refer Figure 5 in Appendix E). One ml of 40 % potassium sodium tartrate solution was added to stabilize the colour. After cooling at room temperature, the absorbance was measured using microplate reader at 575 nm and recorded. Triplicates of each sample were prepared to give more accurate results.

3.16 Identification of Bacterial Isolates

3.16.1 Identification of Bacterial Isolates by Gram Staining Method

A thin smear was made on a clean glass slide. It was dried in air and fixed by passing through flame of a burner. The smear was covered with crystal violet, kept for one minute then washed with water. Some Lugol Iodine was dropped on the slide, left for one minute and washed with water. The slide was decolorized with acetone, rocking the slide gently for 10-15 seconds till the violet colour comes off the slide. It was then washed with water immediately. The slide was counterstained with saffranin. The counterstained slide was left to stand for 30 seconds. It was then washed with water. The slide was blot dried and examined under the oil immersion lens of a microscope.

3.16.2 Identification of Bacterial Isolates Using Biolog System

Two plates of NA were prepared prior to sample inspection using Biolog System by Central Laboratory, Universiti Malaysia Pahang in Gambang Campus. Using inoculum loop, selected isolates were inoculated onto NA plates prepared earlier and incubated at 37 °C for 72 h. Then, the freshly prepared bacterial isolates were sent to Central Laboratory for full bacterial identification.

3.17 Parameters Controlling CMCase Productivity

The optimum condition that enhanced the CMCase production was investigated using one-factor-at-a-time (OFAT) method. The constant variables throughout this process were temperature (37 °C) and incubation period (72 hours) of the production media. It involved 6 different factors namely incubation condition, carbon source, nitrogen source, initial pH, amount of substrate, inoculum size and vitamin.

3.17.1 Effect of Incubation Condition on CMCase Production

The procedure began by allocating 50 ml M9 media (refer section 3.11) and 1 g of pretreated x-ray film in flasks. The control for each isolates (PS1 and CL8A) was 50 ml M9 media without pretreated x-ray film. The flasks were separated for 2 different incubation conditions which were shaking (140 rpm) and static. The cell-free filtrate was obtained from each flask (refer Section 3.12) and went through to the next phase, CMCase assay (refer Section 3.15).

3.17.2 Effect of Carbon Source on CMCase Production

Seven flasks containing 50 ml M9 media (refer Section 3.11) were filled with 1 g pretreated x-ray film and 0.4 % w/v carbon sources namely CMC, sucrose, starch, yeast extract, maltose, sodium acetate and lactose. A control flask was prepared by absence of any carbon source. The cell-free filtrate (supernatant) was obtained from each flask (refer Section 3.12) and went through to the next phase, CMCase assay (refer Section 3.15).

3.17.3 Effect of Nitrogen Source on CMCase Production

Seven flasks containing 50 ml M9 media (refer Section 3.11) were filled with 1 g pretreated x-ray film and 0.1 % w/v nitrogen sources namely yeast extract, beef extract, malt extract, ammonium sulphate $(NH_4)_2SO_4$, sodium nitrate ($NaNO_3$), potassium nitrate (KNO_3) and ammonium chloride (NH4Cl). The control for nitrogen source parameter was 50 ml M9 media with absence of any nitrogen sources. The cell-free filtrate was obtained from each flask (refer Section 3.12) and went through to the next phase, CMCase assay (refer Section 3.15).

3.17.4 Effect of Initial pH Value on CMCase Production

The optimum initial pH parameter was investigated for CMCase production using 0.1 N NaOH and 0.1 N HCl solutions to alter the pH. Seven 50 ml M9 media containing 1 g pretreated x-ray film (refer Section 3.11) with pH 3, 4, 5, 6, 7, 8 and 9 were prepared. The control for initial pH parameter was pH 7 M9 media. The cell-free filtrate was obtained from each flask (refer Section 3.12) and went through to the next phase, CMCase assay (refer Section 3.15).

3.17.5 Effect of Amount of Substrate on CMCase Production

Seven flasks containing 50 ml M9 media (refer Section 3.11) were filled by different amount of pretreated x-ray film weighed as 0.2, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 g. The control for amount of substrate parameter was 50 ml M9 media with the absence of substrate. The cell-free filtrate was obtained from each flask (refer Section 3.12) and went through to the next phase, CMCase assay (refer Section 3.15).

3.17.6 Effect of Inoculum Size on CMCase Production

The procedure began by allocating 1 g pretreated x-ray film and M9 media (refer Section 3.11) into 4 flasks with different amount of M9 media of 48, 45, 43 and 40 ml respectively. To complete a total of 50 ml media culture, another 2, 5, 7 and 10 ml of microorganism was transferred from inoculum preparation (refer Section 3.8) to the M9 media prepared earlier. The control amount for inoculum size parameter was 50 ml media containing 5 ml inoculum. The cell-free filtrate was obtained from each flask (refer Section 3.12) and went through to the next phase, CMCase assay (refer Section 3.15).

3.17.7 Effect of Vitamin on CMCase Production

Three flasks containing 50 ml M9 media (refer Section 3.11) were filled with 1 g pretreated x-ray film and 2 % w/v vitamins namely, thiamine, β-carotene and pyridoxine. The control for vitamin parameter was 50 ml M9 media with the absence of any vitamin. The cell-free filtrate was obtained from each flask (refer Section 3.12) and went through to the next phase, CMCase assay (refer Section 3.15).

3.18 CMCase Production at Optimum Fermentation Conditions

From each parameter, selected results were taken to optimum fermentation procedures. All steps for optimum fermentation that has been described in details (refer Section 3.17.1 until 3.17.6) were studied and carried out. Also, the constant variables that were kept constant throughout this study were temperature (37 °C) and incubation period (72 hours) of the production media.

3.19 Partial Purification of CMCase

3.19.1 Precipitation Using Ammonium Sulphate and Dialysis

Modified method of Kumar and Borah, (2012) was used for the purification of crude enzyme. After 72 hrs at 37 °C incubation, the bacterial suspension was filtered and the remaining solid was discarded. The remaining suspension was centrifuged for 5000 rpm, 8 min at 4 °C. Ammonium sulphate was weighed according to preferred percentages which were 50, 60, 70 and 80 % and added to flasks containing 100 ml supernatant for each percentage. The suspensions were then mixed well and covered with aluminium foil before brought to refrigerator and were kept inside overnight. After a day, the flasks were taken out and mixed again before transferred to Beckman tube then centrifuged for 12000 rpm, 20 min and 4 °C. The supernatant was discarded and the pellet was dissolved with 0.5 M acetate buffer pH 5.4. The mixture was mixed using vortex mixer till it appeared as semi-viscous. The resulted crude enzyme was transferred to falcon tube and stored in the refrigerator until further usage.

A set of dialysis bag (16 000 MWCO), a thin rope and 1 L beaker was needed to carry out dialysis or salting out process of crude enzyme sample. Distilled water was used to wet the dialysis bag and open the bag. First, one end of the bag was fasten using the thin rope. Slowly, pipette crude enzyme into the dialysis bag to prevent leaking out. Then, the other side of the bag was tied. A layer of sucrose was placed inside the beaker before the filled dialysis bag was buried in the sucrose. The bag was ensured to be covered well by pouring another layer of sucrose on top. It was left for 3 hours. The sucrose was removed and the end of dialysis bag was removed.

3.19.2 DEAE-Cellulose Column Chromatography

The dialysed sample was loaded on diethylaminoehtyl (DEAE)-cellulose column (1.5 × 20 cm; 50 ml). The size of the column is dependable on the target protein or enzyme. This can be explained through the fact the smaller column size causes a narrower peak. However, the smaller column size entails greater pressure in the column. Before loading the sample, the column was packed with 20 % DEAE cellulose that was prepared using 0.5 M acetate buffers pH 5.4. Before packing the beads, the column was initially rinsed 3 times with deionised water and subsequently with two column volume of 0.5 M acetate buffers pH 5.4. In the study, isocratic elution was used

throughout chromatography process. Firstly, the sample was in liquid phase and wet packing method was preferable in this condition. Secondly, the dialysed crude enzyme is a noncomplex sample which beforehand has undergone precipitation and dialysis. Based on these reasons, running an isocratic elution can easily purify the sample. The column was allowed for complete settlement overnight. Only 1 ml of dialysed enzyme sample loaded on the column while closing the pipe initially. After entering of the sample into the beads, the collection of fraction started while 0.5 M acetate buffer pH 5.4 was carefully added. Fifity tubes of 5 ml fractions were collected and undergone enzyme activity (refer Section 3.15) using microplate reader.

3.19.3 Sephadex G-50 Column Chromatography

Sephadex G-50 column use gel filtration or size exclusion technique. Gel filtration columns are used not only to remove low molecular weight contaminants, such as salt, but also to buffer exchange before and after different chromatography techniques and rapid removal of reagents to terminate a reaction. At the beginning, 10 % Sephadex G-50 was prepared in a beaker using 0.5 M acetate buffer pH 5.4, any impurities was removed and was allowed to settle in the refrigerator overnight. The column chromatography was washed with deionized water 3 times and subsequently with two column volume of 0.5 M acetate buffer pH 5.4.

Next day, the beads was slowly poured into (1.5 × 20 cm; 50 ml) column. Any air bubbles were removed prior to settlement in the refrigerator overnight. Only 1 ml of dialysed enzyme sample loaded on the column while closing the pipe initially. After entering of the sample into the beads, the collection of fraction started while 0.5 M acetate buffer pH 5.4 was carefully added. Fifty fractions with the rate of 5 ml per 30 min continuously collected and were stored in the refrigerator. Enzyme activity (refer Section 3.15) were recorded using microplate reader at 575 nm.

3.19.4 Molecular Weight Determination by SDS-PAGE

Modified method of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of Laemmli (1970) was performed on the partially purified enzyme to determine its molecular weight. Two Falcon tubes were labelled as “Resolving gel” and “Stacking gel”. For resolving gel, the acrylamide solution, 1.5 M Tris buffer pH 8.8 and

water were mixed. To the resolving gel mixture, 100 µl of a 10 % ammonium persulfate (APS) solution was added. The solution was gently mixed, trying not to introduce air. Then, 10 µl TEMED catalyst was added. Once again, solution was gently mixed trying not to introduce air. The tube was immediately covered as the reagent was transferred. A pipette was used to fill space between two plates until the resolving gel solution reached a desired height. Ionized waster was added so that it gently flowed across the surface of the polyacrylamide mixture. The water level ensures that the polyacrylamide gel will have a level surface once it polymerizes. The gel was allowed to polymerize for about 15 to 20 min. The interface between the polyacrylamide and water overlay disappeared temporarily while the gel polymerized. A sharp new interface then formed between the two layers, indicating that polymerization was completed. When polymerization was completed, the water from the top of the resolving gel was removed by tilting the gel to the side and a paper towel was used to wipe out the water.

For the stacking gel, the acrylamide solution, 1.0 M Tris buffer pH 6.6 and water were mixed. Then, 30 µl 10 % APS and 7.5 µl TEMED was added to the stacking gel acrylamide mixtures. The contents were mixed by gently inverting the tube twice. A pipette was used to transfer the stacking gel on top of the resolving gel between the two glass plates. Stacking solution was added until it reached just the top of the plates. Carefully, the comb was lowered into position. The comb was removed and repositioned to remove air bubbles below the comb.

After the gel was polymerized, the comb was removed from the spacer gel. The casting frame was removed from the gel cassette sandwich and the sandwich was placed against the gasket on side of the electrode assembly, with the short plate facing inward. A second gel cassette or a buffer dam was placed against the gasket in the other side of the electrode assembly. Clamps were placed and tighten on the sides of the electrode assembly. The chamber was lowered into electrophoresis tank. The space between the two gels was filled with Tris-glycine running buffer. This forms the upper chamber for electrophoresis. Tris-glycine running buffer was added to the outer (lower) chamber until the level is high enough to cover the platinum wire in the electrode assembly.

The protein sample was retrieved from freezer. Using pipette, the sample was loaded up to 10 µl into well. Then, 5 µl of a protein marker loaded into one lane of the

gel. Sample was loaded slowly and the sample was allowed to settle evenly on the bottom of the well. The tank was connected to the power supply. The tank cover was fitted onto the electrodes protruding up from the electrode assembly. The electrical leads were inserted into the power supply outlets. The black colour wire was connected to black wire; the red colour was connected to red.

The power supply was turned on and the gel was run at a constant voltage of 150 V. The gel was run until the blue dye front nearly reached the bottom of the gel. The power supply was turned off. The gel apparatus was removed from the tank. The clamping frame was opened and the gel cassette sandwich was removed. Carefully, the two plates were apart with spatula. With a separator, the stacking gel was removed from the glass. The gel was placed in a small plastic tray and the tray was labelled. The tray was filled about halfway with deionized water. The gel was placed on a shaker at 50 rpm for 5 min. The water was drained from the tray and 1× TAE buffer was added then was put back on shaker for 30 min. The 1× TAE buffer was drained and deionized water was added then was placed back on shaker for 10 min. For final washing step, the water as drained and deionized water was added then was put back on shaker for 1 h. When individual band were detectable, the result was recorded.

3.20 Characterization of Dialysed Enzyme Fraction

3.20.1 Effect of Incubation Temperature on Dialysed Enzyme Activity

Procedure for protein determination was carried out using method in Section 3.14 and dialysed enzyme activity was carried out using method in Section 3.15. All the steps remain the same except for incubation temperature. Five different temperatures were tested and set to 10, 25, 30, 40 and 50 °C.

3.20.2 Effect of pH Value on Dialysed Enzyme Activity

Procedure for protein determination was carried out using method in Section 3.14 and dialysed enzyme activity was carried out using method in Section 3.15. All the steps remain the same except the pH for 1.0 M sodium acetate buffer was adjusted to get 4 different pH values: 3, 4, 5 and 6.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Introduction

This chapter presents the relevant experimental results and discussions on the findings of this research. The structure of this chapter follows the same structure of the development methodology in Chapter 3. This chapter aimed to clearly show the experimental results starting from the screening process, followed by the analysis, characterization and results verifications. This chapter summarizes the outcome from each activity.

4.2 Screening of Most Potent Isolates

All bacteria from 3 different sources (water, soil and food waste) including identified bacteria were grown with constant temperature and incubation period which were 37 °C and 72 hours. The bacteria had undergone several subscultures to get a single colony and were preserved for further analysis process. Bacterial isolates that were able to grow on these two variables were selected to undergo screening for most potent isolates. The suitable temperature creates ambient condition in which functioning bacteria can grow. The incubation period allows the nutrients in the enriched media to be efficiently consumed by the bacteria and executes enzymatic reaction. The bacteria that were grown in 37 °C production media were classified as mesophile bacteria that can grow in a range of 20 to 40 °C which was a condition that was neither too hot nor too cold. For an instance, bacteria *Bacillus amyloliquefaciens* DL-3 that was isolated from soil showed cellulolytic activity after 72 hours incubation at 37 °C. Enzymatic degradation of rice hull that was used as the substrate succeed in producing pilot-scale

CMCase which was the major cellulase apart from exoglucanase and β-glucosidase (Jo et al., 2008).

The initial 20 bacterial isolates resulted from 3 different sources and 7 identified bacterial isolates were evaluated by using glucose equivalent (reducing sugars). Enzyme activities for both CMCase and avicelase were calculated using enzyme standard curves (refer Appendix E) using DNS method from Miller, 1959. Quantitative approach was carried out to identify the presence of cellulolytic activity as indicated by its ability to produce the highest reducing sugar content which was essential for the preliminary test. From the observation, the enzyme activity was higher in a dark brown coloured than yellowish coloured DNS enzyme assay samples that were measured using microplate reader at 575 nm. In this case, isolates with a higher reading using international unit (U/ml) were considered to be potential producers of cellulases (Shashidhar et al. 2018). Isolates that were successfully tested for both CMCase and avicelase activity were grown on 0.5 g disposed x-ray film substrate as sole carbon source. To put in another way, the M9 media used were absence of any additional carbon source.

The data shown in Table 4.1 indicated mean of enzyme activity with standard deviation (SD) of bacterial isolates. The data also include control which was media without substrate that was grown at 37 °C. From Table 4.1, most bacterial isolates were able to expressed cellulase activity using pretreated x-ray film as sole carbon source. However, poor activity of CMCase were depicted by CL2C and CL4C while for avicelase activity, PS1, CL8A, A6, *P. aerogenosa*, RSS2B, GL5, *K. pneumonia*, RSS4 and A9 produce poor activity which were lower than control. In fact, these bacterial were expressed as the least efficient in producing CMCase and avicelase. This may be due to the selective uptake of nutrients by the bacteria though same substrate was prescribed into the production media. For example, PS1 was selected as the poor producer of avicelase but it was an effective producer of CMCase. Low enzyme activity when grown in pretreated x-ray film as substrate suggested that this carbon source has not given tolerable condition for certain enzymes to further reciprocate.

Deswal et al. (2011) also discussed the poor cellulase production from *Fomitopsis sp*. RCK2010 when grown on *Prosopis juliflora* and corncob which acted as lignocellulosic susbtrate. Unsuitable physical properties of the substrate such as

geometry, particle size and compactness might be the reasons the substrate failed to support enzyme production when grown under solid-state fermentation.

From overall cellulase production, only four bacterial isolates; PS1, *E. faecalis*, CL8A, and *S.typhii* were selected for a further test on x-ray film pretreatment. PS1, CL8A and *S.typhii* were the potential producers of CMCase while *E. faecalis* was the potential producer of avicelase as they showed very good enzyme activities when pretreated x-ray film was used as the substrate. Moreover, CL8A and PS1 were unknown species that were derived from fresh source could be a new source of cellulase enzyme. From observations, same bacterial strain resulted in different result of CMCase and avicelase. Media containing the bacterial strains provide similar nutrients and condition for the enzymes to reciprocate. The different results of cellulase activity may come from mechanism of action of the enzyme itself. For instance CMCase (endoglucanase) designated to be active in random to cleave chain. In the other hand, avicelase (exoglucanase) act on exposed chain. The readily nutrients and condition of the media promote contrasting action to produce different amount of enzymes (Fernandes et al., 2018).

Table 4.1 Screening of initial 27 bacterial isolates for CMCase and avicelase productions using 0.5 g x-ray film substrate

No.	Isolate code	CMCase activity (U/ml)	Avicelase activity (U/ml)
		Mean ± SD	
Bacterial isolates from soil samples			
1	KK2S6A	0.118 ± 0.001	0.161 ± 0.015
2	PS1	0.167 ± 0.014	0.121 ± 0.005
Bacterial isolates from water samples			
1	GL1	0.137 ± 0.002	0.129 ± 0.008
2	GL4	0.151 ± 0.001	0.124 ± 0.014
3	GL5	0.139 ± 0.015	0.112 ± 0.007
4	GL6	0.124 ± 0.009	0.134 ± 0.011
5	GL7	0.132 ± 0.010	0.123 ± 0.009
6	RSS2B	0.123 ± 0.004	0.115 ± 0.021
7	RSS3	0.120 ± 0.005	0.129 ± 0.006
8	RSS4	0.124 ± 0.009	0.107 ± 0.012
9	RSS6	0.150 ± 0.004	0.128 ± 0.013
Bacterial isolates from food samples			
1	A6	0.112 ± 0.008	0.105 ± 0.002
2	A9	0.126 ± 0.009	0.116 ± 0.010

Table 4.1 Continued

No.	Isolate code	CMCase activity (U/ml)	Avicelase activity (U/ml)
		Mean ± SD	
Bacterial isolates from food samples			
3	CL2A Branch	0.156 ± 0.020	0.129 ± 0.011
4	CL2C	0.108 ± 0.006	0.121 ± 0.018
5	CL4B	0.132 ± 0.012	0.135 ± 0.016
6	CL4C	0.105 ± 0.005	0.132 ± 0.009
7	CL8A	0.180 ± 0.016	0.118 ± 0.002
8	CL11	0.134 ± 0.001	0.140 ± 0.013
9	CL14	0.113 ± 0.050	0.128 ± 0.012
Identified bacterial isolates from former researcher			
1	*B. subtilis*	0.139 ± 0.015	0.129 ± 0.005
2	*E. faecalis*	0.160 ± 0.015	0.142 ± 0.008
3	*E. coli*	0.134 ± 0.015	0.121 ± 0.001
4	*K. pneumonia*	0.137 ± 0.012	0.113 ± 0.007
5	*P. aerogenosa*	0.137 ± 0.004	0.107 ± 0.009
6	*S. typhii*	0.143 ± 0.003	0.128 ± 0.009
7	*S. aureus*	0.131 ± 0.002	0.124 ± 0.006
	Control	0.108 ± 0.008	0.120 ± 0.006

**media without substrate was used as control*

4.3 Selection of X-ray Pretreatment Method

In this study, x-ray film waste was pretreated using alkaline solution, NaOH prior to media production and microbial inoculation. This is to ensure other excess materials, such as dye, were washed away and the internal structure of the cellulose was exposed. However, to further explore the potential of x-ray film as the substrate, other physical pretreatment which was heat-fixated of x-ray film were chosen. A secondary check on the pretreatment method could help in choosing most potent isolates that will go through parameter controlling phase.

4.3.1 Heat-Fixated X-ray Film

After screening of 27 initial microorganisms, only 4 most potent microorganisms used for selection of x-ray film pretreatment. PS1, *E. faecalis*, CL8A, and *S.typhii* bacterial strains produced the highest enzyme activity compared to other bacterial strains using 0.5 g x-ray that was pretreated with 2 N NaOH. The next process involved using the four aforementioned isolates. The process used pretreated x-ray film which was boiled until 100 °C for 15 min. The x-ray film was then cooled and 0.5 g of film

was allocated for each 50 ml media and 0.2 g additional substrate specifically to increase enzyme activity, which made a total 0.7 g substrate. This act was common as the possibility of produced higher enzyme concentrations was higher if more soluble carbon source was allocated (Ellilä et al., 2017).

The selected bacteria when grown on media supplemented with heat-fixated x-ray film as the substrate showed poor enzyme activity. The overall results also depicted lower enzyme activity for both CMCase and avicelase than when the media was previously supplemented with 2N NaOH pretreated x-ray film as the substrate. In addition to the alkaline NaOH pretreatment, high temperature was intentionally charged to x-ray film to further break down the cellulose structure to enhance substrate accessibility to enzymes and in turn, release more fermentable sugars (Elumalai et al., 2014).

Table 4.2 depicted the highest CMCase activity was achieved by PS1 and CL8A with 0.121 U/ml while for avicelase, *S. typhii* displayed the highest activity. Overall enzyme activity for CMCase showed comparatively higher result than avicelase which suggested a tendency of compatible relationship between the enzyme and substrate (pretreated x-ray film). However, based on the result, it was concluded that high temperature might not necessarily increase enzyme activity for the substrate due to its well-built cellulose structures (Bhatt et al., 2018). Furthermore, most of the nutritional substance have been ceased and deteriorated due to high heat subjection towards the substrate that changed most of the physical barriers and chemical compositions of the substrate. Subsequently, lower activity was resulted by the enzyme as the changes in substrate turned down the chances of bonding between substrate and enzyme (Singhania, 2009).

In the meantime, only CMCase was selected for the next method which used larger portion of x-ray film (without boiling) as CMCase productions for all isolates are more stable compared to avicelase activity. Only two isolates were selected for the next process which focused on the parameters affecting enzyme activity. The isolates represented microorganisms derived from soil (PS1) and food waste (CL8A), were remotely cultured at the earlier screening process. Table 4.2 presented the data on the CMCase and avicelase activities for all 4 isolates.

Table 4.2 CMCase and avicelase activity under static condition using NaOH and 0.7 g boiled x-ray film

No.	Isolate code	CMCase activity (U/ml)	Avicelase activity (U/ml)
		Mean ± SD	
1	PS1	0.121 ± 0.008#	0.113 ± 0.011
2	*E. faecalis*	0.112 ± 0.005	0.107 ± 0.009
3	CL8A	0.121 ± 0.008#	0.115 ± 0.006
4	*S. typhii*	0.116 ± 0.007	0.123 ± 0.006#
5	Control	0.115 ± 0.008	0.102 ± 0.006

#highest activity

** media without substrate was used as control*

4.4 Identification of PS1 and CL8A Isolates

4.4.1 Identification of Bacterial Isolates by Gram Staining Method

The results from the experiment data indicated that both PS1 and CL8A isolates produced a high value for CMCase activity from the initial 27 different isolates and proceeded to the partial identification process. Both isolates were subjected to microscopic analysis for their characterization and identification by the Gram stain methods. They were examined under immersion lens of a microscope. The gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50–90% of cell envelope), and resulted in stained purple generated by crystal violet, whereas the Gram-negative bacteria have a thinner layer (10% of cell envelope), so it did not retain the purple stain and were counter-stained pink by saffranin. The results of Gram staining method for both PS1 and CL8A isolates are described in Table 4.6. The images of isolates (Figure 7 and Figure 8) under immersion oil lens were attached in Appendix G.

4.4.2 Identification of Bacterial Isolates Using Biolog System

There is a need for a full identification using advanced machine during the inspection and detailing stage. Here, the Biolog System was used as it can specifically identify the name of bacteria with the optimum reading. The Biolog MicroPlate can analyse up to 94 phenotypic tests: 71 carbon source utilization assay (column 1-9) and 23 chemical sensitivity assays. The test panel provides a Phenotypic Fingerprint of a microorganism that can be used to identify it at the species level. The Gen III

Microplate test panel also provides a standardized micro method using 94 biochemical tests to profile and identify a wide range of Gram-negative and Gram-positive bacteria.

The Biolog System (refer Appendix F) for CL8A identified the isolate as *Alcaligenes faecalis* with 87.3 % probability and PS1 as *Providencia rettgeri* with 61.7 %. The optimum temperature for both bacteria was in a range of 30 °C to 37 °C. Both bacteria are motile. Moreover, the *Alcaligenes faecalis* is a non-pathogenic bacterium while *Providencia rettgeri* is pathogenic bacterium. Table 4.3 described the morphological characters of *Providencia rettgeri (P. rettgeri)* and *Alcaligenes faecalis (A. faecalis).*

Table 4.3 Summary of morphological characters of *P. rettgeri* and *A. faecalis*

Colony Morphology	**Observations**	
	P. rettgeri	***A. faecalis***
Gram's reaction	Gram negative	Gram negative
Cell Shape	Rods	Rods
Opacity	Translucent	Opaque
Pigment	Non-pigmented	Non-pigmented
Surface	Smooth	Smooth
Margin	Entire	Entire
Elevation	Convex	Convex
Configuration	Round	Round

4.5 Parameters Controlling CMCase Productivity

Different factors have been considered in providing the optimal conditions for the CMCase activity. In this study, seven different parameters were selected which were incubation condition, carbon source, nitrogen source, initial pH value, amount of substrate, inoculum size and vitamin. While two other variables which were temperature and incubation time, were kept constant at 37 °C and 72 hours respectively. This step was initiatively taken after preliminary test and was decided to be constant throughout the experiment. One factor at a time (OFAT) technique was used to optimize CMCase production using all the factors mentioned earlier.

4.5.1 Effect of Incubation Condition on CMCase Production

In this study, incubation condition was varied by placing the flasks containing production media and pretreated x-ray film in either normal incubator or incubator shaker. The static condition and shaking condition (140 rpm) were used to study the effect of media agitation towards the behaviour of the bacteria and CMCase production (Ellilä et al., 2017). Two isolates were reserved to be further analysed; *P. rettgeri* and *A. faecalis* after preliminary test earlier. A total of 1 g substrate (pretreated x-ray film) was immersed in 50 ml media and incubated at 37 °C for 72 hours.

Figure 4.1 demonstrated the enzyme produced by both bacteria under static and shaking conditions. From the figure, *P. rettgeri* achieved an optimum CMCase activity of 0.135 U/ml under shaking condition and 0.124 U/ml under static condition. While for *A. faecalis*, 0.147 U/ml was obtained under shaking condition and 0.140 U/ml under static condition. It has been shown that, CMCase activity was higher in the presence of 1 g x-ray film when inoculated with *A. faecalis* compared to *P. rettgeri* for both static and shaking conditions. Hence, *A. faecalis* exhibited better performance in consuming substrate to produce enzyme compared to *P. rettgeri*. In this case, shaking the culture provides a vigorous condition, increases surface volume of the substrate and consequently, enzyme activity also increased (Samira et al., 2011). This was also supported by Dar et al. (2013) whose experiment used CMC as the carbon source. The study concluded that the quantity of extracellular enzymes is higher in shaken cultures compared to static cultures. Meanwhile, according to Sulman and Rehman (2013) the enzymes found in the microorganisms can be from both intra and extra-cellular origin, but in the present investigation, extra-cellular form of cellulose was dominant. So, it can be connected by the presence of agitation, enzyme was easily released and bonded with the substrate associated in the production media.

The isolate derived from food waste (*A. faecalis)* was presumably the strongest CMCase producer among other isolates derived from natural sources. The figure depicted CMCase activity under shaking condition (0.147 U/ml) and static condition (0.140 U/ml) which were higher than *P. rettgeri* under both conditions. In the fermentation process, oxygen supply was the limiting factor for cell growth as the oxygen has low solubility in water. Hence, shaking the media can help in removing

generated carbon dioxide, temperature regulation and maintaining moisture level of substrate (Kheiralla et al., 2018).

At the same time, *P. rettgeri* did a poor task to improve enzyme production using x-ray film as substrate. From the figure, the isolate derived from soil produced 0.135 U/ml under shaking and 0.124 U/ml under static condition. The static condition did not help in executing the enzyme and caused accumulation of hydrolysis yield. This has caused limitations in enzyme distribution that indirectly inhibit the production of enzyme involved in metabolic pathway (Roche et al., 2009).

The result in Figure 4.1 suggested that the rest of media culture should be in agitation state for the rest of the experiment. Without a doubt, 1 g x-ray film effectively affected CMCase activity for all selected isolates, hence, providing a suitable amount of nutrition for bacterial grow is essential as it can improve enzyme production. It can be concluded that shaking the culture provided better result compared to leaving it static for CMCase activity produced from 1 g x-ray film.

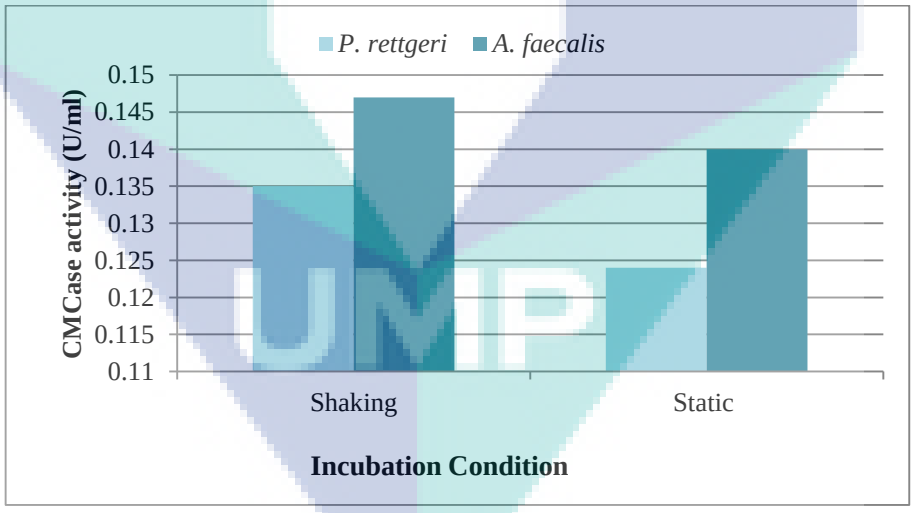


Figure 4.1 Effect of incubation condition on CMCase activity by *P. rettgeri* and *A. faecalis*

4.5.2 Effect of Carbon Source on CMCase Production

Seven different carbon sources have been used, namely carboxymethyl cellulose (CMC), sucrose, starch, yeast extract, maltose, sodium acetate and lactose in addition to 1 gram x-ray substrate were distributed for each flask containing 50 ml media culture. A

total of 0.4 % w/v of carbon sources was used in each flask. After evaluation of CMCase acitivity both bacteria seemed capable of producing CMCase in the presence of additional carbon source.

CMCase activities produced by *P. rettgeri* when supplemented with carboxymethyl cellulose (CMC), sucrose, starch, yeast extract, maltose, sodium acetate and lactose were 0.132, 0.122, 0.251, 0.135, 0.122, 0.123 and 0.118 U/ml respectively. CMCase activity in control flask was 0.105 U/ml. In regard to Figure 4.2, the highest CMCase activity of 0.251 U/ml was recorded for *P. rettgeri* when starch was used as a carbon source. The lowest CMCase activity of 0.118 U/ml was recorded when lactose was used as carbon source.

CMCase activities produced by *A. faecalis* when supplemented with carboxymethyl cellulose (CMC), sucrose, starch, yeast extract, maltose, sodium acetate and lactose were 0.176, 0.464, 0.138, 0.197, 0.158, 0.147 and 1.365 U/ml respectively. CMCase activity in control flask was 0.129 U/ml. On the other hand, the highest CMCase activity was recorded for *A. faecalis* was 1.365 U/ml when lactose was used as a carbon source and the lowest CMCase activity of 0.138 U/ml was recorded when starch was used as carbon source.

Maximum CMCase activity by *P. rettgeri* was achieved when starch was associated in the production media while for *A. faecalis*, maximum enzyme activity was achieved when lactose was used as the additional carbon source alongside the 1 g pretreated x-ray film. One of the reasons was the high sugar concentration in the specific material has provided ideal necessity for growth.

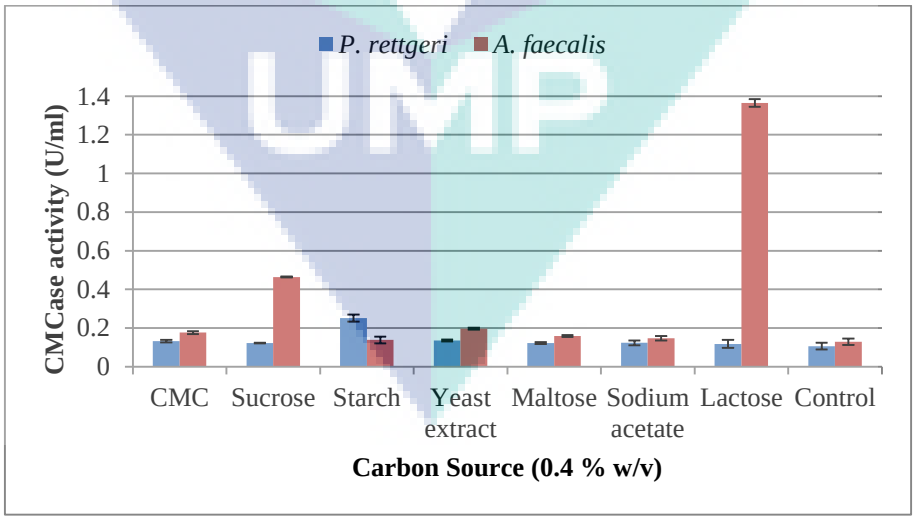
Undeniably, other than carbon source, other factors also affect the enzyme yields and holds an entangled relationship which includes physical and nutritional factors in the fermentation media (Mandal & Ghosh, 2017). Enzyme productivity for other carbon sources was below par as compare to the control might have caused by several factors which were viscosity of the carbon source, nutrient availability and toxicity that may exist. The factors inhibited the enzyme-degrading bacteria to reciprocate the carbon source supply and produce specific enzyme (Ellilä et al., 2017).

The results are on contrary with the result of El-Hadi et al., (2014) which highlighted other carbon source, CMC successfully raised CMCase activity with 1.18

U/ ml. Similarly, the pattern can also be found with 0.1% of different carbon sources like cellobios, sucrose, xylose, CMC, wheat flour and glucose. The maximum result (9.259 U/gds) suggested CMC as the ideal carbon source for CMCase production by *Aspergillus flavus*. This might be due to the CMC that induced the enzyme activity, or may caused by increasing of the penetration rate of CMC through the cell membrane (Narsale et al., 2018)

Previously, Trinh et al. (2013) outlined that the addition of carbon source (sawdust, corncob, sugarcane bagasse, and CMC) slightly increased CMCase production from 32 to 40 %. However, as cellulose is a complex carbohydrate, not all microorganisms are able to use it for energy purpose. When no other carbon source except cellulose was provided in the culture medium, it will take some time for the microorganism to digest the complex carbohydrate. This explained why *Peniophora sp.* NDVN01 strain preferred to consume simple sugar before employing complex polysaccharide cellulose (Sulman & Rehman, 2013).

In general, the CMCase activity produced by *A. faecalis* was higher when grown with the additional carbon sources. This showed that *A. faecalis* was a better CMCase producer compared to *P. rettgeri.* Also, cellulase systems were much more active when grown in the presence of lactose than grown in other carbon source alongside the existing substrate.



**media without carbon source was used as control*

Figure 4.2 Effect of carbon source on CMCase activity by *P. rettgeri* and *A. faecalis*

4.5.3 Effect of Nitrogen Source on CMCase Production

Protein is one of the critical factors for all fully functioning organisms, regardless of their size and complexity. To produce protein, nitrogen sources are needed for the growth of the bacteria. This process also requires amino acid, co-enzymes and nucleic acid supply. To detect the suitable nitrogen source for CMCase production by *P. rettgeri* and *A. faecalis*, the culture media were supplemented with three organic (yeast extract, beef extract and malt extract) and four inorganic (ammonium sulphate, sodium nitrate, potassium nitrate and ammonium chloride) nitrogen sources. Meanwhile, 1 g x-ray substrate and 0.1 % (w/v) of different nitrogen sources was distributed to each flask containing media culture.

CMCase activities produced by *P. rettgeri* when supplemented with beef extract, yeast extract, malt extract, ammonium sulphate, sodium nitrate, ammonium chloride and potassium nitrate were 0.131, 0.130, 0.137, 0.113, 0.136, 0.124 and 0.116 U/ml respectively. CMCase activity in control flask was 0.105 U/ml. As shown in Figure 4.3, the highest CMCase activity reading (0.137U/ml) was recorded for *P. rettgeri* when malt extract used as a nitrogen source while the lowest activity (0.113 U/ml) was recorded when ammonium sulphate was used as a nitrogen source.

CMCase activities produced by *A. faecalis* when supplemented with beef extract, yeast extract, malt extract, ammonium sulphate, sodium nitrate, ammonium chloride and potassium nitrate were 0.132, 0.122, 0.251, 0.135, 0.122, 0.123 and 0.1168 U/ml respectively. CMCase activity in control flask was 0.106 U/ml. On the other hand, the highest CMCase activity reading recorded for *A. faecalis* was 0.485 U/ml when malt extract used as nitrogen source while the lowest activity (0.096 U/ml) was recorded when potassium nitrate used as a nitrogen source.

CMCase production was quite high by *P. rettgeri* in the presence of sodium nitrate, malt extract and beef extract. In contrast, yeast extract, ammonium sulphate, ammonium chloride and potassium nitrate inhibited the CMCase production. The highest CMCase activity was recorded when organic nitrogen source which was malt extract used for both *P. rettgeri* and *A. faecalis*. The lowest CMCase activity was recorded for both *P. rettgeri* and *A. faecalis* when inorganic nitrogen sources were

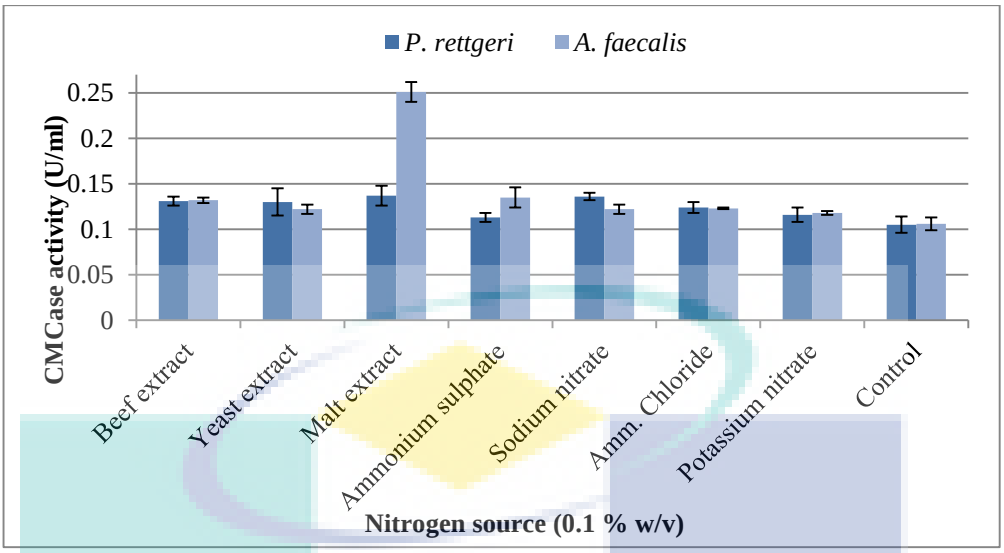
supplied. Both inorganic sources (potassium nitrate and ammonium sulphate) probably contain only the nutrients that satisfy no more than the minimal requirement for growth.

Studies have commonly reported that the presence of organic nitrogen sources highly increased CMCase activity compared to inorganic nitrogen sources (Mahmoud et al., 2014). El-Hadi et al. (2014) used *Aspergillus hortai*s fungal strain and reported similar result where the organic nitrogen sources had an ascending reading compared to other inorganic nitrogen sources. Moreover, (Bai et al., (2017) reported that *Penicillium ochrochloron* ZH1 recorded almost zero enzyme activity when inorganic nitrogen sources (urea) were used as the sole nitrogen source. This result was also in line with Mukherjee et al. (2011) who reported that organic nitrogen source could produce more cellulase.

Li and Gao,(1997) demonstrated the suitable nitrogen sources include peptone, beef extract, yeast extract, ammonium sulphate and casamino acid while no growth was detected when KNO, urea and tryptone were used as nitrogen sources. Vijayaraghavan et al. (2016) described that higher cellulolytic enzyme activity was obtained with peptone (1760 IU/g) compared to yeast extract, casein, gelatine, and urea, while higher level enzyme production was obtained with sodium dihydrogen phosphate (1190 IU/g) compared to other inorganic salts.

Furthermore, (Aggarwal et al., 2017) elaborated that the presence of additional nitrogen sources along with the nitrogenous compounds in the substrate could promote enhanced growth and consequent enzyme production. The result recorded 12.0 ± 0.07 U/gds, which was the highest level of CMCase production by the fungus in the presence of ammonium nitrate.

In summary, media supplemented with malt extract as the nitrogen source produced higher CMCase activity and *A. faecalis* was obviously produced more stable amount of CMCase than *P. rettgeri*.



**media without nitrogen source was used as control*

Figure 4.3 Effect of nitrogen source on CMCase activity by *P. rettgeri* and *A. faecalis*

4.5.4 Effect of Initial pH Value on CMCase Production

A wide range of pH values have been tested for CMCase production by *P. rettgeri* and *A. faecalis* to examine the role of pH in more details. Both isolates were inoculated into media supplemented with 1 g x-ray as substrate at pH values between 3 to 9, which was estimated to be the most suitable pH range. Control for initial pH for both *P. rettgeri* and *A. faecalis* was pH 7 media, similar to the pH of M9 media.

As shown in Figure 4.4, the maximum CMCase activity recorded for *P. rettgeri* when the culture medium was adjusted to pH 8 was 0.125 U/ml, while the minimum CMCase activity recorded when the culture medium was adjusted to pH 9 was 0.096 U/ml. In the meantime, the maximum CMCase activity recorded for *A. faecalis* when the culture medium was adjusted to pH 9 was 0.129 U/ml and the minimum CMCase activity recorded when the culture medium was adjusted to pH 5 was 0.107 U/ml.

Instead of using a buffer, the pH of modified minimal media (M9) was adjusted by using 0.1N hydrochloric acid (HCl) and 1N sodium hydroxide (NaOH). The usage of two chemicals gives the possibility of altering the pH as long as the concentration involved is relatively low. However, certain steps should be considered such as not using excessive amount of NaOH to correct the addition of HCl and vice versa. This is

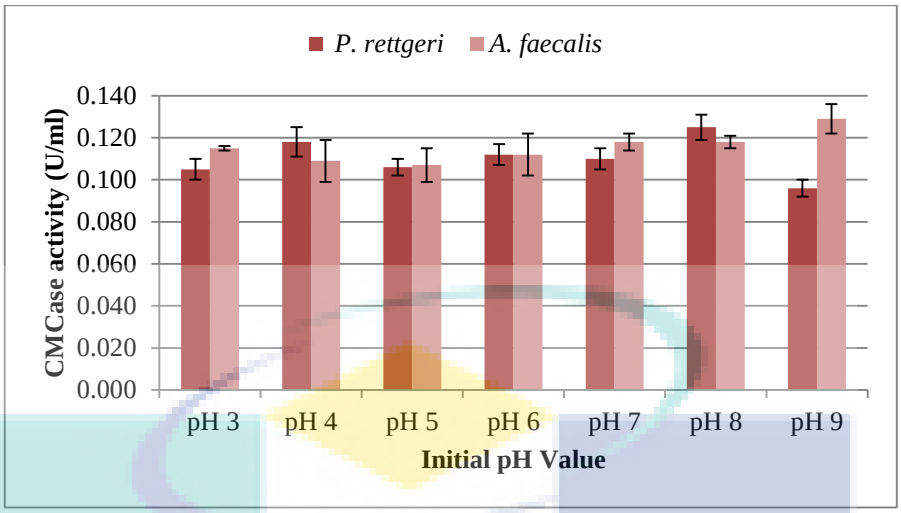
partly because the growth of microorganism can be affected by salt formation from the uncontrolled amount of the chemicals.

One factor at a time (OFAT) is an approach which allows one parameter to be examined at a time for a particular purpose, for example; only the pH at the beginning of the media culture is measured carefully. The isolate is then left to mingle and absorb the nutrients throughout the culture period. Thus, the compatibility of pH condition for the same isolates throughout the whole culture period is only decided after the culture has been well developed. During the enzyme activity measurement, the readings for each pH reflect how well the isolates react to the pH conditions. In this phase, as long as the condition demonstrates a dynamic and balance interaction, the isolates will have higher chances of producing the enzymes.

Any bacteria that can grow in acidic condition are considered as acidophiles. On the other hand, most bacteria are considered as neutrophiles and are stable at near-neutral pH. Alkaliphile bacteria or prokaryotes can survive in extreme pH between 7 to 12 and show optimal growth at pH 9. *P. rettgeri* and *A. faecalis* were both alkaliphiles which preferred high pH values, specifically pH 8 and pH 9. It has been shown that the unknown bacterial isolates can grow immensely at high pH while other bacteria species can only survive in the maximum pH of 7 (Padan et al. 2005).

When pH level increases, the hydrogen bonds holding together strands of DNA will break up which lead to shifting of macromolecules. Previous studies using *A. niger* reported that the optimum pH for cellulase production was sustained between the pH 6.0 and 7.0. The common range of growing CMCase production by *A. hortai* in liquid state fermentation was also evaluated and the bacteria can hold on to pH value ranging from 4.5 to 8 (El-Hadi et al., 2014).

In summary, CMCase activity produced by *A. faecalis was* relatively higher than *P. rettgeri* which suggests better CMCase productivity and stability. CMCase activity produced by *A. faecalis* was optimum in pH 9 medium.



**pH 7 media was used as the control*

Figure 4.4 Effect of initial pH value on CMCase activity by *P. rettgeri* and *A. faecalis*

4.5.5 Effect of Amount of Substrate on CMCase Production

As shown in 3.13.4, in addition to using a pretreated x-ray film as a substrate, 7 different amounts of substrate were used. Media containing 1 g of substrate was used as a control for *P. rettgeri* and *A. faecalis*, similar to amount of substrate in substrate pretreatment stage.

Figure 4.5 showed that the maximum CMCase activity recorded for *P. rettgeri* when the culture medium was supplemented with 2.5 g of substrate with 0.181 U/ml and the minimum CMCase activity recorded when the culture medium was supplemented with 0.2 g of substrate with 0.124 U/ml.

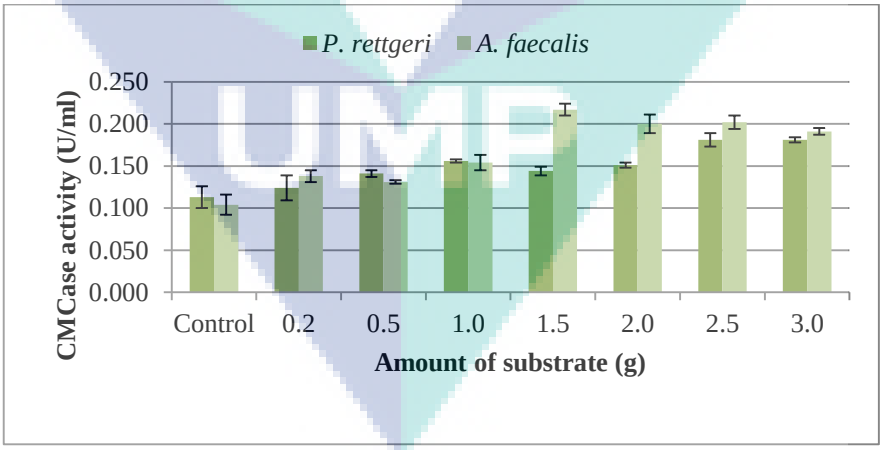
The amount of substrate is closely related to the increase in growth pattern. It can be observed that the growths are in rather stable state at a lower concentration. The reaction with enzyme can occur freely as the substrates were well accommodated. However, once the growth reaches an optimal level, the addition of any substrates cannot be tolerated as the entire binding site has been occupied. In the same way, the pattern can be seen with CMCase activity in Figure 4.4 at 0.2 to 1.5 g of substrate the activity was elevated until at one point, the activity slowly subsided, signaling a lost in site engagement of the substrates.

On the other hand, the maximum CMCase activity recorded for *A. faecalis* when the culture medium was supplemented with 1.5 g of substrate at 0.217 U/ml while the

minimum CMCase activity recorded when the culture medium was supplemented with 0.5 g of substrate is 0.131U/ml. As shown in the results, 1.5 g and 2.5 g exhibited the highest CMCase activity for PS1 and CL8A, respectively. Both isolates favoured the substrate which is the source of cellulose. However, only certain amount of substrate has shown optimum activity. After optimum level at 1.5 g, the enzyme production started to reduce probably due to catabolic repression (Sarkar & Aikat, 2014)

Without any modification of substrates or component available, the ability of enzyme complexes to hydrolyze the substrate will deteriorate (Mansfield et al., 1999). An x-ray film was used as substrate which acts as feeder to the microbe in the media. However, to react effectively, the pretreatment needs to take place before the cellulose are hydrolysed. According to Ge et al. (2011), the pretreatment breaks down the crystalline structure. However, different isolates need different amounts of substrate to actively contribute to CMCase production.

The volume of microorganisms was not corresponding to enzyme productivity as only small volume of inoculum needed for the enzyme to have higher yield. In some of the substrates, there is a poor accessibility of the nutrients due to the existence of obstinate complexes that hinder and limit the degradative enzymes to use and assimilate nutrients from the media (Salim et al., 2017).



**media without substrate was used as the control*

Figure 4.5 Effect of amount of substrate on CMCase activity by *P. rettgeri* and *A. faecalis*

4.5.6 Effect of Inoculum Size on CMCase Production

Four different inoculum sizes have been tested for CMCase production by *P. rettgeri* and *A. faecalis*. Both isolates were inoculated into media supplemented with 1 g x-ray as substrate for each 2, 5, 7 and 10 ml inoculum sizes.

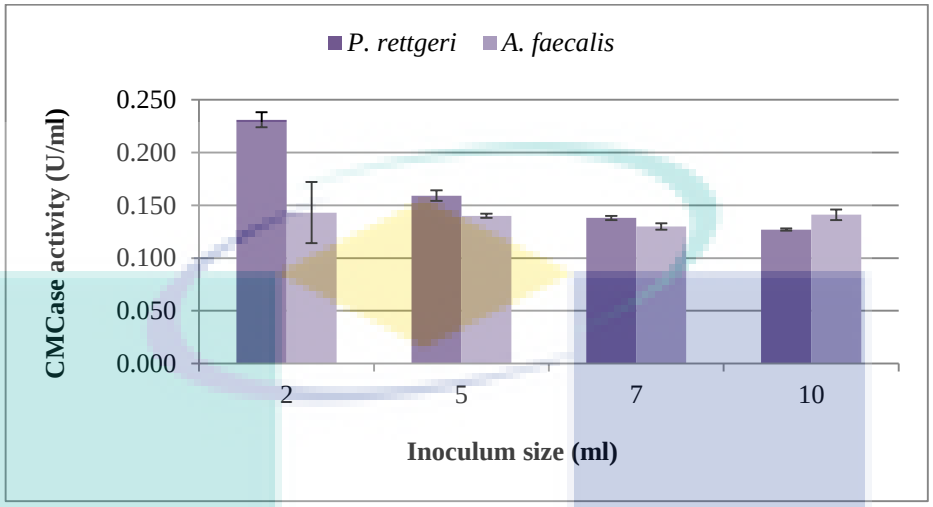
As shown in Figure 4.6, the maximum CMCase activity recorded (0.231 U/ml) for PS1 isolate when the culture medium was supplemented with 2 ml inoculum and minimum CMCase activity was recorded (0.127 U/ml) when the culture medium was supplemented with 10 ml inoculum size. From the result of *P. rettgeri*, there was a clear declining pattern of enzyme activity starting from 2 ml of inoculum size, the enzyme activity decreased drastically at about 5 ml inoculum size. The competition to grow can hinder the enzyme production especially oxygen and other nutrients.

On the other hand, the maximum CMCase activity for *A. faecalis* was 0.143 U/ml which was recorded when the culture medium was supplemented with 2 ml inoculum size and the minimum CMCase activity (0.130 U/ml) was recorded when the culture medium was supplemented with 7 ml inoculum size. This indicated that the enzyme activity of *A. faecalis* was similar to *P. rettgeri*. However, at 10 ml, the activity abruptly increased. This can be explained through the necessity of microbial growth related to the duration of the lag phase where microorganisms are adjusting to their environment.

At the same time, microbial growth can also be affected by the decrease of the digestion rates in presence of pretreatment by-products. Hence, an appropriate period of adaption for the microbial ecosystems is required to enhance the development of specific microbial populations, such as those fermenting organic molecules to methane in the presence of by-products (Monlau et al., 2014). A study by Palsaniya et al. (2012) exemplified protease production which has a rather prolonged lag-phase, however, the study observed that with the addition of bacteria, at certain point, microbial growth has increased higher compared to before the addition of bacteria.

Based on the results, only 2 ml inoculum was needed for both *P. rettgeri* and *A. faecalis* to achieve the highest CMCase activity. In short, though both isolates'

inoculum size was similar during the highest enzyme activity, CMCase produced *A. faecalis* was more stable when incubated with 2 ml inoculum size.



**media containing 5 ml inoculum was used as the control*

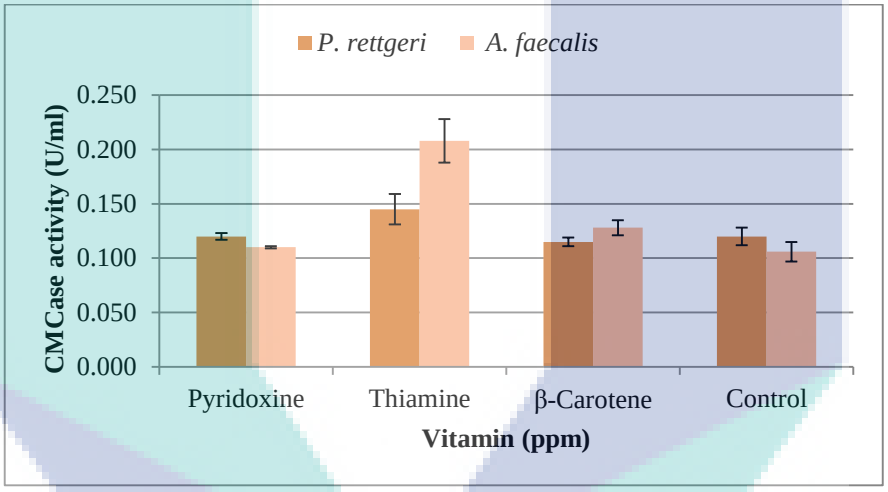
Figure 4.6 Effect of inoculum size on CMCase activity by *P. rettgeri* and *A. faecalis*

4.5.7 Effect of Vitamin on CMCase Production

Vitamin is an additional factor which supports enzyme production by accelerating chemical reaction. This leads to enzymes' dependency on this substance for their survival. To detect the suitable vitamin for CMCase production by *P. rettgeri* and *A. faecalis*, the culture medium was supplemented with three different vitamins, pyridoxine, thiamine, and β-Carotene. One gram x-ray substrate and 100 ppm of different vitamins were separately distributed into each flask containing the media culture.

Figure 4.7 illustrated that the maximum CMCase activity recorded for *P. rettgeri* was 0.145 U/ml when the culture medium was supplemented with thiamine while the minimum CMCase activity recorded when the culture medium was supplemented with β-Carotene was 0.115 U/ml. On the other hand, the maximum CMCase activity recorded for CL8A isolate when the culture medium was supplemented with thiamine size was 0.208 U/ml while the minimum CMCase activity recorded when the culture medium was supplemented with pyridoxine was 0.110 U/ml.

The results showed that both *P. rettgeri* and *A. faecalis* took up thiamine as their strongest vitamin supply. While vitamin supplement to microbial isolates was rarely induced, vitamin B-complex could be used to determine the productivity of thermostable amylases from *Bacillus* sp. (Rodrı, 2006). In addition, Gerken et al., (2013) reported that thiamine used in biofuels production with the use of *Chlorella vulgaris* and other microalgae in the form of thiamine-HCl. Moreover, despite the rare utilization of vitamin in enzyme production from microorganism, there seemed to be a direct relationship between vitamins and coenzymes, for instance, as reported by Shimizu, (2008), coenzymes help enzymes to bind onto substrates. In the meantime, some coenzymes are directly synthesized from vitamins.



**media without vitamin was used as the control*

Figure 4.7 Effect of vitamin on CMCase activity by *P. rettgeri* and *A. faecalis*

4.6 Optimum Parameters for CMCase Production

The degradation of cellulose was studied at different initial pH value, amount of substrate, carbon source, nitrogen source, inoculum size, vitamin and incubation condition. While two other variables which were temperature and incubation time, were kept constant at 37 °C and 72 hours respectively.

Table 4.4 and Figure 4.8 presented the parameters of the highest CMCase activities for *P. rettgeri* and *A. faecalis*. Ultimately, using all the preferred optimum ingredients and amount for each parameter, a larger scale production took place right

after that. CMCase production by *P. rettgeri* was carried out in shaking condition, using starch as carbon source, malt extract as nitrogen source, initial pH at 8, 2.5 g amount of substrate, 2 ml inoculum size and thiamine as vitamin resulted in 0.934 U/ml. While CMCase production by *A. faecalis* was carried out in shaking condition, using lactose as carbon source, malt extract as nitrogen source, initial pH at 9, 1.5 g amount of substrate, 2 ml inoculum size and thiamine as vitamin resulted in 4.559 U/ml which was significantly higher than *P. rettgeri*. Thus, *A. faecalis* has been further investigated by purifying the resulted enzyme fraction. Only supernatant was used in the ammonium sulphate precipitation and dialysis process.

According to Figure 4.8, *A. faecalis* favoured the x-ray film substrate as the sole carbon source more than *P. rettgeri*. Both microorganisms used were the result of unknown bacteria isolation from selective sources in the early stage of the study. Thus, only bacteria were used to be directly interacted with media supplemented with nutrients. Notably, bacteria can extracellularly secrete cellulose in addition to the suitable carbon source, nitrogen source, pH and temperature. These physical variable factors can affect mycelial growth and production (Vimal et al., 2016). Ponnambalam et al. (2011) also pointed out that the presence of a strong carbon source may accelerate growth and metabolic necessity to produce more cellulase enzyme.

Studies have found that cellulase production is dependent on the nature of the carbon source used in culture media. In this regard, choosing the cheapest and appropriate substrate is significant for the successful production of enzymes. It has been confirmed that the strain could grow in the substrate used (Sadhu et al., 2013). According to Imran et al. (2016), this is due to the less complex and consequently, easy assimilation of the cellulose material by the isolated microbe. As a result, despite the fact that carboxymethyl cellulase already exhibited an extensive role in the yield and production of the enzyme, agricultural wastes definitely present a cheaper carbon source that can be utilized for enzyme production.

Optimum summary of *A. faecalis* and *P. rettgeri* showed highest CMCase activity after all the factors needed were studied. In this case, x-ray film was used as the primary carbon source and also additional carbon source was added. Study of the

substrate proves that x-ray film that was used as substrate can effectively help in cellulose degradation by the microorganisms to produce CMCase.

Table 4.4 Parameters optimization summary for *P. rettgeri* and *A. faecalis*

No.	Parameters	Bacterial isolate	
		P.rettgeri	***A. faecalis***
1	Carbon sources (g)	Starch	Lactose
2	Nitrogen source (g)	Malt extract	Malt extract
3	Initial pH value	8	9
4	Substrate concentration (g)	2.5	1.5
5	Inoculum sizes (ml)	2	2
6	Vitamin (ppm)	Thiamine	Thiamine
7	Incubation condition	Shaking	Shaking
Enzyme activity (U/ml)		0.934± 0.020	4.559 ± 0.018
Protein content (mg/ml)		0.895 ± 0.016	2.878 ± 0.092

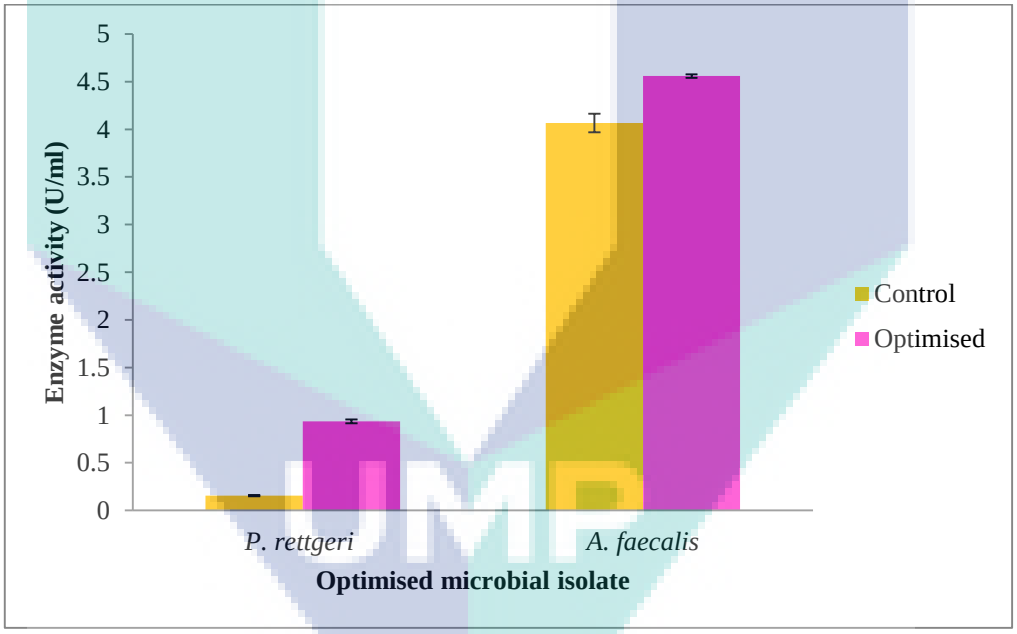


Figure 4.8 CMCase activities for *P.rettgeri* and *A. faecalis* at optimum parameters conditions

4.7 Partial Purification of CMCase

A. faecalis was produced on a large scale to carry out ammonium sulphate precipitation after optimization of 6 different parameters. Initially, the fermented broth was centrifuged at 5000 rpm for 8 min at 4 °C to obtain the supernatant. In next step, the supernatant was saturated to 60 % using ammonium sulphate crystals. After

overnight saturation, the formed precipitates were collected by centrifugation at 12000 rpm, 15 min at 4 °C re-dissolved with 0.5 M acetate buffer (pH 5.4) and dialysed for 3 hours to remove the residual ammonium sulphate. One millilitre of the dialysed 60 % ammonium sulphate fraction was applied to DEAE-cellulose column equilibrated with 0.5 M acetate buffer (pH 5.4). The enzyme fractions were eluted in the same buffer with a linear gradient of 5 ml for 50 tubes.

The result of the previous process showed a clearer peak of the CMCase needed to be acquired. The step from DEAE-cellulose column was repeated for G-50 Sephadex column. One millilitre of the fractions was collected from DEAE-cellulose column applied to Sephadex G-50 column equilibrated with 0.5 M acetate buffer (pH 5.4). The enzyme fractions were eluted in the same buffer with a linear gradient of 5 ml for 50 tubes. The fraction displayed a maximum CMCase activity which was further subjected to SDS-PAGE.

4.7.1 Precipitation Using Ammonium Sulphate and Dialysis

The results from previous parameters optimization in Section 4.6 showed that the *A. faecalis* produced more CMCase compared to *P. rettgeri*. The CMCase activity of the optimum parameter resulted in 4.559 U/ml. Thus, precipitation using ammonium sulphate has been carried out to remove impurities and for precipitating the cell. Four different saturations of ammonium sulphate were prepared; 50, 60, 70 and 80 % where 60 % ammonium sulphate had the highest CMCase activity 1.73 U/ml which exhibited a greater ability to precipitate protein. The other saturation results were shown in Table 4.5.

The dialysis bag or semi-permeable membrane was used as it created equilibrium between the liquid in the dialysis bag (16 000 MWCO) and surrounding (sucrose). Applying the exact percentage of ammonium sulphate allowed the enzyme activities to achieve the optimum rate due to the active flow of enzyme to and from the sample inside the dialysis bag. For this reason, 60 % ammonium sulphate was selected and brought to dialysis process. The result indicated 2.496 U/ml enzyme activity of dialysed crude enzyme.

Table 4.5 Enzyme activity of CMCase at different saturations of ammonium sulphate and dialysis

Type of Purification	CMCase activity (U/ml)
50 % of $(NH_4)2SO_4$	1.51
60 % of $(NH_4)2SO_4$	1.733
70 % of $(NH_4)2SO_4$	0.467
80 % of $(NH_4)2SO_4$	0.789
Dialysis	2.496

4.7.2 DEAE-Cellulose Column Chromatography

The dialysis was followed by a purification process, specifically the DEAE-cellulose column chromatography. This method was used to separate protein by eluting the dialysed crude enzyme in a column. DEAE-cellulose is a type of weak-anion exchange chromatography. The enzyme activities and protein contents of the 50 fractions are demonstrated in Figure 4.9.

Initially, only 1 ml of the dialysed crude enzyme was subjected to the column with the series addition of 0.5 M pH 5.4 acetate buffers. Five ml of dripping were eluted periodically to collect 50 fractions in separate tubes. The graph illustrated the lack of consistency of molecules concentration that was eluted within the 50 fractions. Nevertheless, one peak clearly displayed a high enzyme activity at 17^{th} with a reading 1.243 U/ml. The peak may indicate a presence of target protein. In the meantime, the results of the protein contents (mg/ml) exhibited distinct protein contents at 3^{rd}, 43^{rd} and 46^{th} peaks with 0.214 mg/ml, 0.202 mg/ml and 0.206 mg/ml, respectively. The high reading of protein concentration may be a false reading of the troublesome contaminants which was there all along during wash phase. The optimum separation of the target enzyme may be unnecessarily interrupted by the contaminants.

Subsequently, all the resulted fractions from DEAE-cellulose (fraction 1-50) were collected and dialysed. Then, the dialysed sample was loaded (1 ml) onto Sephadex G-50 column afterward.

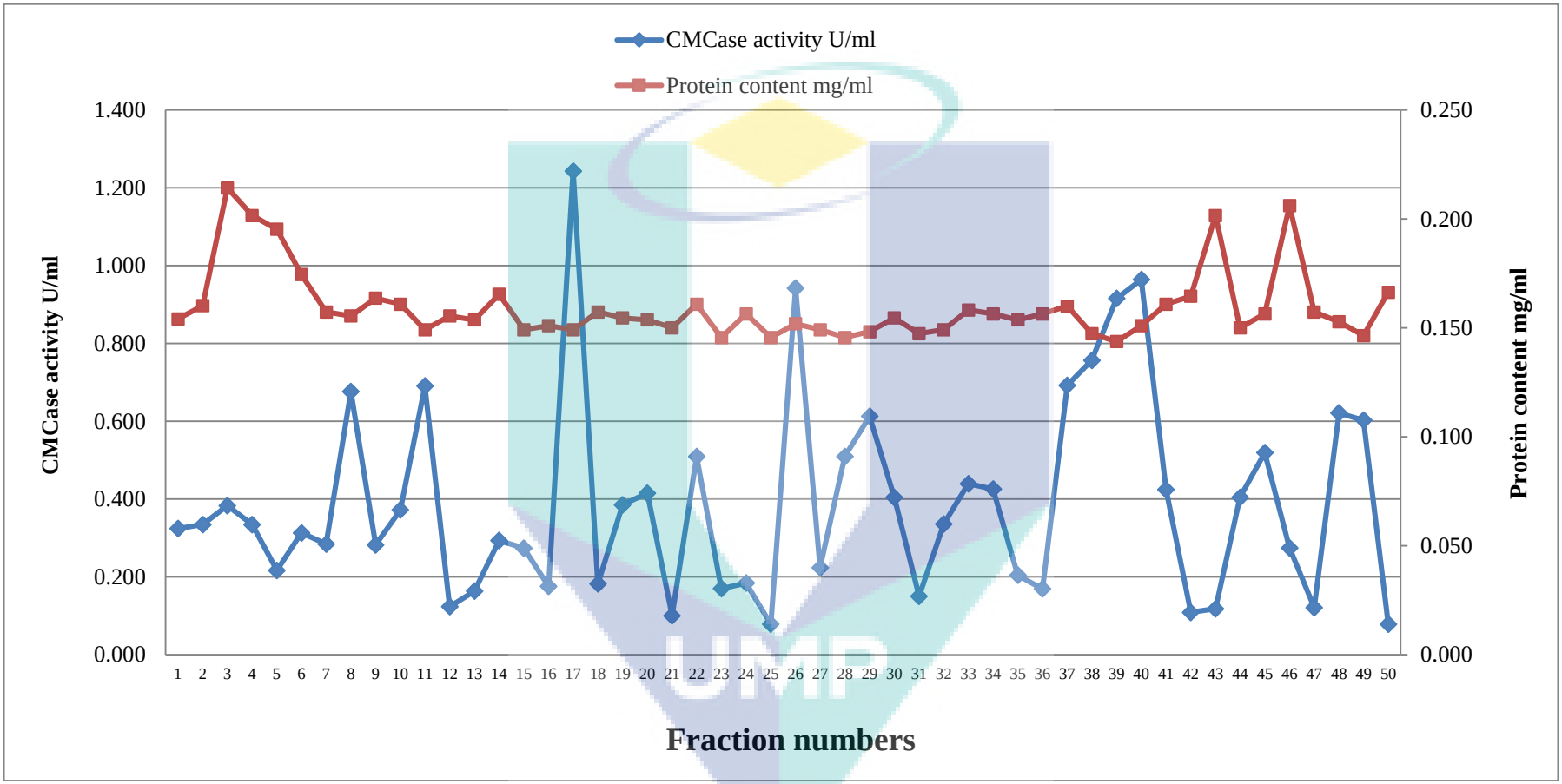


Figure 4.9 CMCase activity and protein content of 50 DEAE-cellulose fractions. This process was carried out according to method in Section 3.19.2

4.7.3 Sephadex G-50 Column Chromatography

Sephadex is a dry powder which can expand its size when excess water is added to form beads for the gel filtration column. Sephadex G-50 is a gel filtration or size exclusion chromatography. Size fractionation is a good final step for purification. Fifty fractions were eluted from time to time with the series addition of 0.5 M pH 5.4 acetate buffers until all 50 fractions were collected.

As shown in Figure 4.10, the 31st fraction recorded the highest peak which illustrated an active CMCase activity with a reading of 1.038 U/ml. The 31st elution was gradually separated which may indicated a smaller size of target enzyme as it travels through the column slowly. Besides that, the protein content result revealed two distinct peaks at 37th and 39th with readings of 0.948 mg/ml and 1.064 mg/ml, respectively while the separation of molecules in the sample mixture of target enzyme was reflected through molecular weight distribution.

The enzyme activity of the target fraction was 0.30 U/ml and protein content was 0.78 mg/ml with the final preparation recorded an activity recovery of 6.58 % and fold purification of approximately 0.24. The selected buffer, 0.5 M pH 5.4 acetate should not cause inactivation or precipitation but maintains the biomolecules stability and target proteins activity. Earlier, similar buffer was used to wash the column and for the elution. As a matter of fact, a single buffer was used throughout the entire separation process. An important point to be highlighted was the interactions of solute molecules that should be considered before the elution process. The usage of different pH of buffer will caused the differences in ionic strength of the eluting buffer. In order to unbind the desired enzyme, different salt concentration gradient needed to cause the release of the solute molecules. This explained the poor yield was resulted at 6.58 % which was caused by inappropriate charge which can reversibly bind to desired enzyme and increase the overall result if proper ionic strength for the sample elution was used.

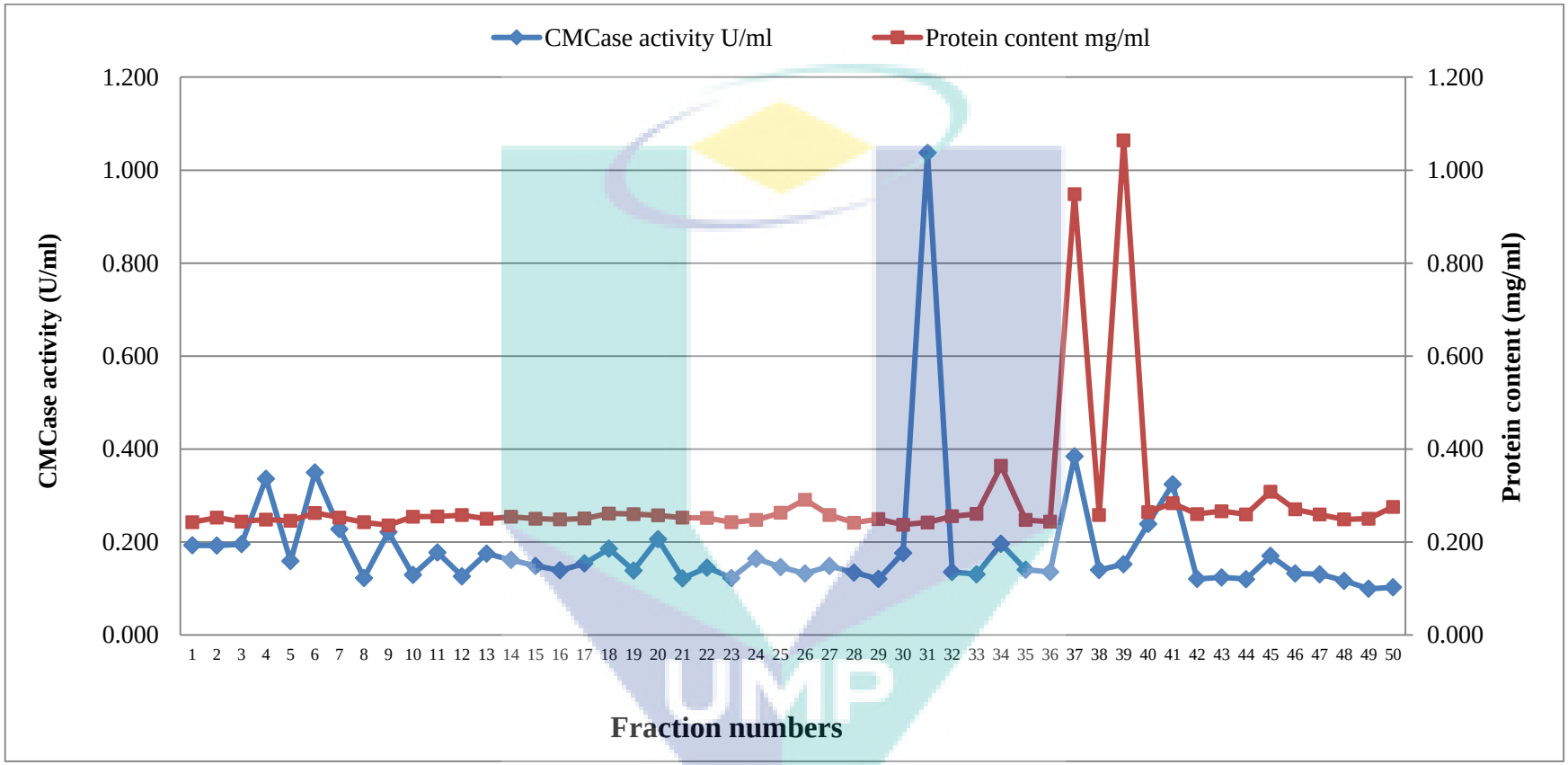


Figure 4.10 CMCase activity and protein content of 50 Sephadex G-50 fractions. This process was carried out according to method in Section 3.19.3

The summary of the purification of CMCase was presented in Table 4.6. The cellulase enzyme produced by the *Alcaligenes faecalis* was purified to 0.24 fold with the yield of 6.58 %. Annamalai et al. (2012) on the other hand, produced 8.66 fold purification and 11.55 % yield of *B. licheniformis* AU01.

Other than that, results by Potprommanee et al., (2017) demonstrated that after DEAE-cellulose ion exchange chromatography, the purification was approximately 5.12 fold with a recovery yield of 10.14 %. Gaur and Tiwari (2015) also used similar type of ion-exchange chromatography to produce culture supernatant containing cellulase enzyme. The final result for the cellulase produced by *Bacillus sp*. was 28.8 % overall recovery which was significantly higher than overall recovery by *A. faecalis*.

Table 4.6 Purification profile of CMCase from *A. faecalis*

No.	Purification sample	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Protein fold	Yield %
1	**Crude enzyme**	4.56	2.88	1.58	1.00	100.00
2	**A. sulphate (60 %)**	2.50	5.95	0.42	0.27	54.80
3	**DEAE**	0.63	0.43	1.47	0.93	13.80
4	**Sephadex**	0.30	0.78	0.38	0.24	6.58

4.7.4 Molecular Weight and Purification Profile of CMCase

The homogeneity of the concentrated enzyme fraction and its molecular mass were analysed by SDS-PAGE according to Laemmli (1970). The enzyme fraction (10 µl) from the Sephadex G-50 purification process was subjected to right lane as in Figure 4.11. The left lane represented protein marker which was simultaneously loaded onto the gel and was run at constant voltage of 150 V.

The bands that emerged on the resolving gel showed different molecular mass of 198, 98, 62, 49, 38, 28, 17, 14, 6 and 3 kDa. More importantly, a single band presented molecular weight result for the CMCase produced by *Alcaligenes faecalis* can be visibly observed. A band was detected at approximate 60 kDa molecular mass after comparing it with standard commercialized protein, Plus2 Pre-stained Protein Standard Protocol. The finding was in accordance to (Bai et al., 2017), which explained cellulases produced by most of the microorganism with molecular mass ranging from 20 to 60 kDa.

A similar result was reported for the molecular weight of the purified CMCase produced by *B. subtilis* subsp. *subtilis* A-53 with approximately 56 kDa, based on its mobility calculated with standard calibration proteins. The strain was derived from sea water due to wide range in environments (Kim et al., 2009). The present finding supported that molecular weight of endoglucanase varies with different bacteria (Trinh et al., 2013).

In other case, 45 kDa of partially purified CMCase produced by *Aspergillus oryzae* was revealed using the same method (SDS-PAGE). Carbon source that was used in the study was cheese whey which has been used widely in cellulase production for the affordability (Youssef, 2011). Using different substrates (commercial substrates, sawdust and medicinal herb waste), *Paenibacillus chitinolyticus* CKS1 that was isolated from forest soil was confirmed as the most potent cellulase producer. The strain was identified based on 16S rRNA gene sequence. Renaturing SDS-PAGE and zymogram analysis resulted in two bands of cellulases at 70 and 45 kDa (Mihajlovski et al, 2015).

The obscurity of the protein band could be caused the low concentration of target protein and the smearing of protein band may have caused by the contamination of the sample. In this case, the protein sample may be contaminated by lipids or fats. In addition, choosing an incorrect buffer or temperature could cause the enzyme to function improperly and cause smearing (Annamalai et al., 2012).

On a contrary, the band of desired enzyme may be present but in very small concentration. The absence of visible band on the corresponding marker band indicated little concentration of other individual bands. Thus, the indefinite band caused a slight hindrance in validation process of the enzyme's size. Hence, the enzyme needs to be concentrated in order to produce more visible band. Centrifugal filter should be one of the concentration method options without causing protein denaturation and losing any protein. In fact, volume of the protein while without concentrating the buffer by using appropriate cut-off filter.

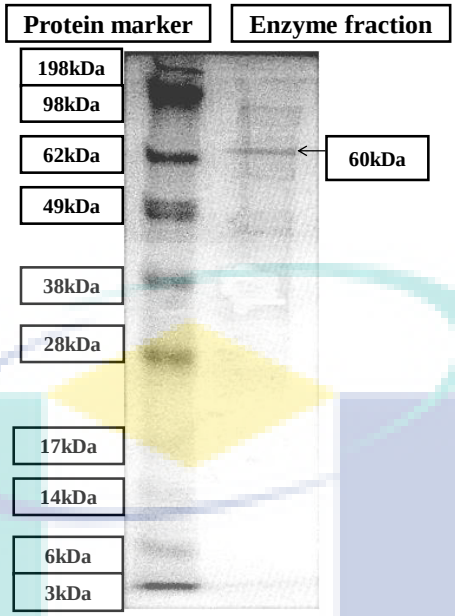


Figure 4.11 SDS-PAGE (6.58 %) of CMCase from *Alcaligenes faecalis* illustrated purified band of CMCase

4.8 Characterization of Enzyme Fraction

The characterization of enzyme is needed to obtain the exact point where the enzyme can be most exploited and most stable. This includes the mechanism that catalyses the enzyme production. In this section, temperature and pH value for optimum enzyme production have been studied.

4.8.1 Effect of Temperature on Dialysed Enzyme Activity

Figure 4.12 exhibited that the highest CMCase activity reading recorded for *A. faecalis* isolate at 25 °C was 2.815 U/ml. The enzyme was stable at the ambient surrounding around 25 °C to 37 °C and the enzymes tend to gain kinetic energy as the temperature increased. This also increased the enzyme activity. The activity continued until it reached a point where enzyme denatures. This can be dependent on the structure of the enzyme. This result parallel to Dar et al. (2013) which reported that the enzyme retained more than 70 % of its maximum activity after 30 min exposure to temperatures ranging from 25 °C to 37 °C.

It was found that activity of the CMCase increased rapidly when the temperature was raised from 20 to 30 °C. However, an increase in temperature beyond 50 °C resulted in a sharp decline in the activity. This was because the increasing temperature beyond the optimum value which causes a decrease in the catalytic rate of the enzyme due to its denaturation (Aggarwal et al. 2017). On the contrary, thermostable enzymes constituted enzymes that can withstand high temperature, which was achieved by cellulase activity that has been optimum at 50 °C and decreased rapidly with increasing temperature above 60 °C (Annamalai et al. 2014). Similarly, Mahmoud et al. (2014) explained that maximum enzymatic activity was obtained at incubation temperature of 50 °C while the thermal stability of the cellulase enzyme was up to 55 °C and reduction in the activity reached 15 % when the temperature reached 60 °C.

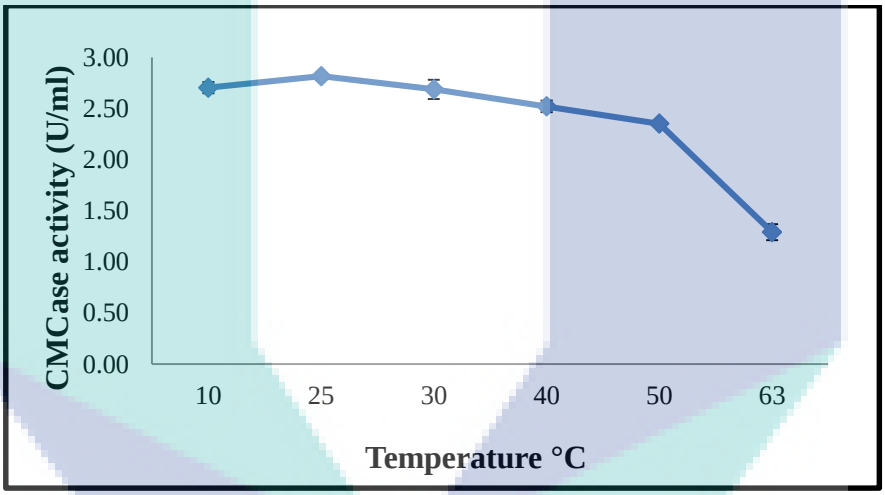


Figure 4.12 Effect of temperature on dialysed enzyme activity

4.8.2 Effect of pH Value on Dialysed Enzyme Activity

The reaction was performed in different pH range to find the optimum pH of purified CMCase; pH range from 3 to 6 was tested to find the suitable pH values for enzyme activity. Figure 4.13 exhibited CMCase produced by *A. faecalis* was most stable at pH 6. The CMCase activity was observed to be high at pH 6. This indicated that the cellulose content was readily available for assay hence the high activity. At pH 4, there was a slight increasing in the reading but the highest was recorded at pH 6. This may be attributed to the changes of the enzyme shape which created the best condition for the enzyme to bind to the active site.

In this study, the enzyme showed its optimal activity at the pH range of 3 to 6. This was comparable to the result of Dar et al. (2013) where CMCase activity achieved a stable form at pH 4 to 7, as well as Trinh et al. (2013) which showed a stable enzyme activity in the production of *Peniophora* sp. was recorded at pH 7. Consequently, the enzyme has been completely stable over the range of pH 5.0-7.0 at 40 °C for 24. Analysis by Vimal et al. (2016) on other types of cellulase confirmed that pH of 4 to 9 contributed to enzyme stability. Previously, Gaur and Tiwari (2015) studied the enzyme activity pH ranging from 4 to 10. In the study, the optimum enzyme activity was found at pH 7 and after the characterization was carried out, the enzyme achieved stability at pH 7.5.

The downside of choosing a narrow range of pH, the complete ability of enzyme hydrolysis cannot be traced. The enzyme preference may include alkaline condition which portrayed by purified cellulase from *B. licheniformis* AU01. The enzyme achieved optimum stability at pH 9 (Annamalai et al., 2012).

It was apparent in a research by Potprommanee et al. (2017) that the CMCase activity by Geobacillus sp. HTA426 was observed to be minimum at pH 10 in pH ranging from of 3 to 10. Right after pH 7, the stability dropped till pH 10. The increase in pH affected the charges on the amino acids within the active site, for instance, the enzyme was not able to form enzyme-substrate complex contributing to the decrease in enzyme activity.

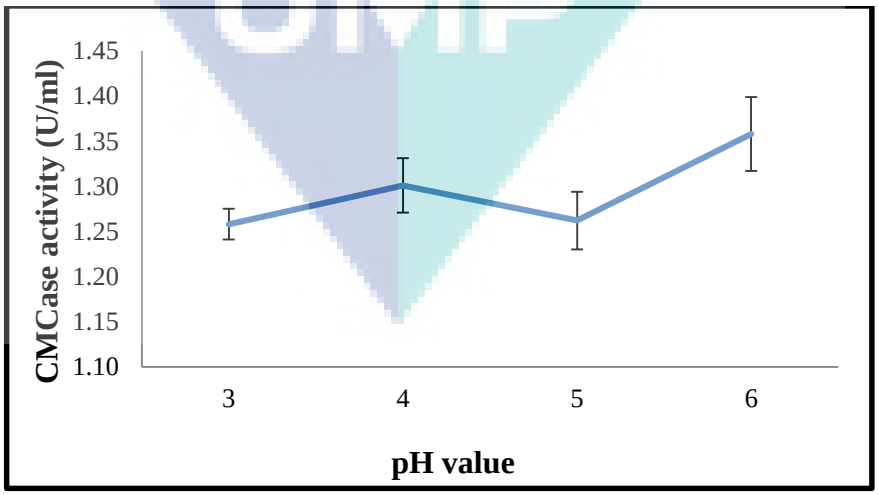


Figure 4.13 Effect of pH on dialysed enzyme activity

CHAPTER 5

CONCLUSION

5.1 Introduction

This project lastly defined the abilities of microorganism to produce partially purified enzyme. In this chapter, the conclusion and future work was successfully discussed.

5.2 Conclusion

In conclusion the objectives of the study have been achieved which is x-ray film waste as substrate helped in hydrolysis of simple sugar, thus indirectly produce refine cellulase which is carboxymethyl cellulase. More importantly, out of 27 initial isolates that have been derived from wastes (water, soil and food), 2 isolates showed a good potential as cellulose-degrading microorganisms and were able to degrade x-ray film and produce CMCase. Both inducers were identified as Gram negative bacteria namely *Alcaligenes faecalis* (CL8A isolate) and *Providencia rettgeri* (PS1 isolate). Characterization of CMCase assay resulted in 60 kDa molecular weight 7 % yield.

In this study, CMCase production using CL8A isolate was the best in shaking condition, at initial pH 9 supplemented with lactose (carbon source), malt extract (nitrogen source) and thiamine (vitamin), in addition of 1.5 g x-ray film substrate and 2 ml bacterial inoculum. Whereas, CMCase could be extensively harnessed using PS1 isolate at initial pH 8 supplemented with starch (carbon source), malt extract (nitrogen source), thiamine (vitamin) and 2 ml bacterial isolate.

Characterization of the partially purified enzyme involved temperature and pH value. Among the various conditions, the enzyme was pointed out to be stable at pH 5 and 25 °C. Bacterial isolates derived from food source was the best source of CMCase production which proposes a renewable sources being exploited. Cellulase has a lot of industrial applications including production of food and medicines and help to breakdown the waste plants materials to clean up the environment. The most refine cellulase in the study was CMCase which is used in textile and detergent industry.

The results presented here show that *Alcaligenes faecalis* is promising for future biotechnological applications, since it is able to produce CMCase enzyme (endoglucanase) in appreciable amounts when grown on a low-cost residue such as disposed x-ray film.

5.3 Recommendations

In this study, a few improvements can be implemented to ensure a firm results in the future research. The points are as follow:

i. In this study, only bacterial isolates were obtained from the existing culture and also isolation process from soil, food and water. Fungi have been confirmed as a powerful cellulosic-degrading microorganism which could replace bacteria in the future.

ii. The recovery of cellulosic material can also be applied to non-hazardous waste using this method imprint. Hence, the constructed method will be time, energy and cost wise.

iii. The cellulose percent of x-ray film can be estimated using gas chromatography analysis to have a better review of the components in x-ray film sheet.

iv. Scanning electron microscope (SEM) can be done to reveal structural changes before and after pretreatment process.

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APPENDIX A
M9 MINIMAL SALTS (2×) MANUAL SHEET

GIBCO
invitrogen cell culture

M9 Minimal Salts (2X)

Gibco® M9 Minimal Salts (2X) solution is used in the preparation of M9 minimal media. The composition of the M9 Minimal Salts include buffering agents, a nitrogen source and necessary ions critical to the completion of M9 minimal media. M9 minimal media also requires a carbon source to support microbial growth. As a complete media, M9 minimal media is typically used to define the nutritional needs of bacteria such as *Escherichia coli* (*E. coli*). Auxotrophs, organisms having unique nutritional requirements due to mutation(s), will not be able to grow in M9 minimal media without additional supplements such as amino acids or other required nutrients.

Description	Cat. No.	Size
M9 Minimal Salts (2X)	A13744-01	1000 mL

Intended Use

For research use only. CAUTION: Not intended for human or animal diagnostic or therapeutic uses.

Storage and Handling

15°C to 30°C

Shelf Life

12 months

Medium Preparation

M9 Minimal Salts (2X) solution (see formulation below) is not a complete medium and requires dilution and supplementation of a carbon source and other nutrients to propagate microbial growth.

Disodium Phosphate Heptahydrate.....25.6 g/L
Monopotassium Phosphate.....6 g/L
Sodium Chloride.....1 g/L
Ammonium Chloride.....2 g/L
Final pH (2X solution): 6.6-7.0 at 25°C

M9 Minimal Complete Medium Preparation: The following preparation is a guide for use. Additional supplementation may be required depending on the nutritional needs of the microbe.

1. Aseptically add 500 mL/L M9 Minimal Salts (2X) medium to a sterile container.
2. Aseptically add the following sterile solutions to the container:
   a. 20 mL of 20% D-Glucose solution
   b. 2 mL of 1.0 M $MgSO_4$ solution
   c. 0.1 mL of 1.0 M $CaCl_2$ solution
3. Adjust to volume to 1000mL with sterile H_2O and mix until homogeneous.

Note: Additional supplements may include: casamino acids, unnatural amino acids, heavy isotope labeled amino acids, trace metals, thiamine, antibiotics, etc.

Note: Different carbon sources and pH adjustment can also be used to complete M9 minimal media. If necessary, sterile filtration may be used to sterilize the final formulation.

Use

Procedure

Consult appropriate references for recommended test procedures. *(See References Section)*

Expected Results

Growth should be evident by the appearance of turbidity.

User Quality Control

Prepare complete M9 minimal medium as described in the **Medium Preparation** section. Inoculate and incubate at 33 to 37°C for 18 to 48 hours.

Organism	Inoculum CFU	Recovery
E. coli (BL21 (DE3))	30-300	Good to excellent

Related Products

LB Broth, (10855)

Terrific Broth, (A13743)

Technical Support

For additional product and technical information, such as Material Safety Data Sheets (MSDS), Certificate of Analysis, etc, please visit our website at www.invitrogen.com. For further assistance, please email our Technical Support team at techsupport@invitrogen.com.

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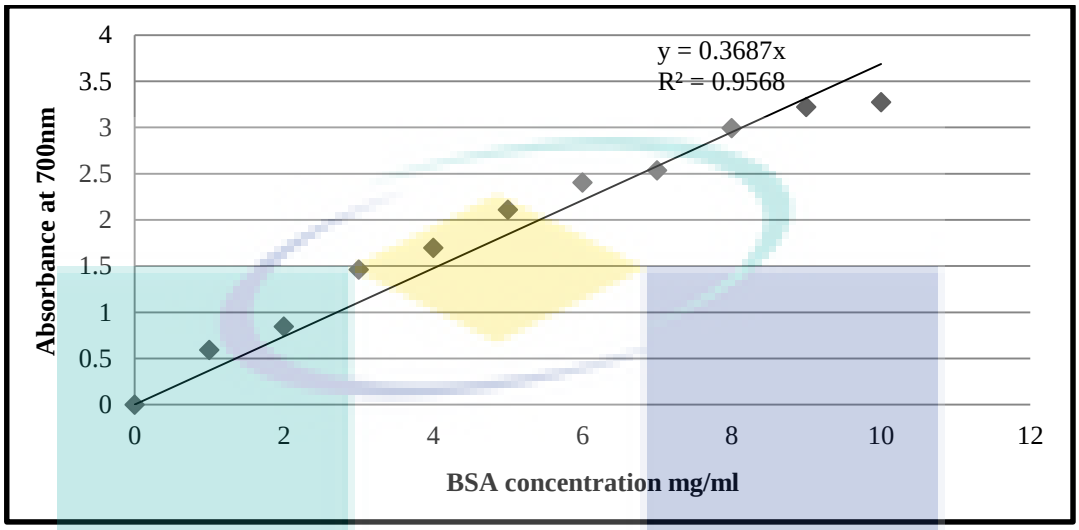
August 2011 Form No.6068

APPENDIX B
DATA FOR PROTEIN STANDARD CURVE

Table 1 Data for protein standard curve

Tube	BSA (ml)	dH2O (ml)	BSA Concentration (mg/ml)	Absorbance at 700 nm	
				Mean	± SD
1	0.0	1.0	0.0	0	0
2	0.1	0.9	1.0	0.484	0.027
3	0.2	0.8	2.0	0.807	0.045
4	0.3	0.7	3.0	0.991	0.049
5	0.4	0.6	4.0	1.194	0.0007
6	0.5	0.5	5.0	1.304	0.04
7	0.6	0.4	6.0	1.539	0.004
8	0.7	0.3	7.0	1.664	0.182
9	0.8	0.2	8.0	1.803	0.245
10	0.9	0.1	9.0	2.001	0.001
11	1.0	0.0	10.0	2.098	0.037

APPENDIX C
STANDARD CURVE OF PROTEIN



(values in mean ± SD, n = 3)

Figure 2 Standard curve for protein content (mg/ml)

Figure 3 Colour changes of Lowry protein determination sample from colourless to blue/dark blue

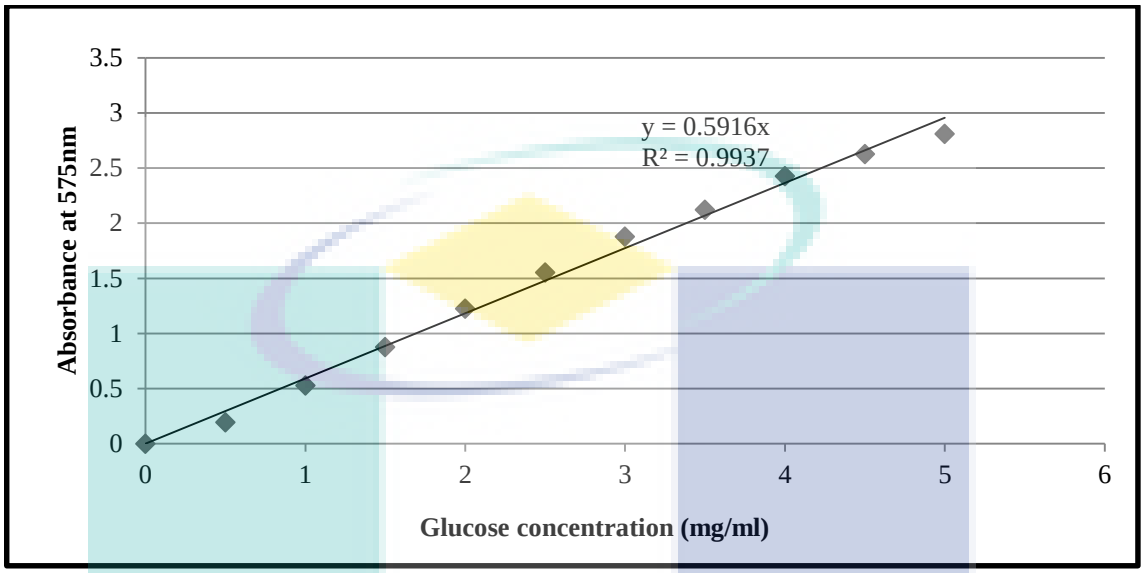
APPENDIX D
DATA FOR ENZYME STANDARD CURVE

Table 2 Data for enzyme standard curve

Tube	Glucose (ml)	dH_2O (ml)	Glucose Concentration (mg/ml)	Absorbance at 575 nm		Standard Factor (SF)
				Mean	± SD	
1	0.0	3.0	0.0	0	0	0
2	0.1	2.9	0.5	0.193	0.006	2.591
3	0.2	2.8	1.0	0.528	0.011	1.894
4	0.3	2.7	1.5	0.878	0.004	1.708
5	0.4	2.6	2.0	1.225	0.056	1.633
6	0.5	2.5	2.5	1.553	0.363	1.331
7	0.6	2.4	3.0	1.878	0.148	1.597
8	0.7	2.3	3.5	2.122	0.047	1.649
9	0.8	2.2	4.0	2.429	0.021	1.647
10	0.9	2.1	4.5	2.628	0.006	1.712
11	1.0	2.0	5.0	2.813	0.004	1.777
AVERAGE STANDARD FACTOR (ASF)						1.594

Standard Factor (SF) = $\frac{\text{Concentration of standard (mg/ml)}}{\text{Absorbance}}$ (1)

APPENDIX E
STANDARD CURVE OF ENZYME



(values in mean ± SD, n = 3)

Figure 4 Standard curve for enzyme assay (mg/ml)

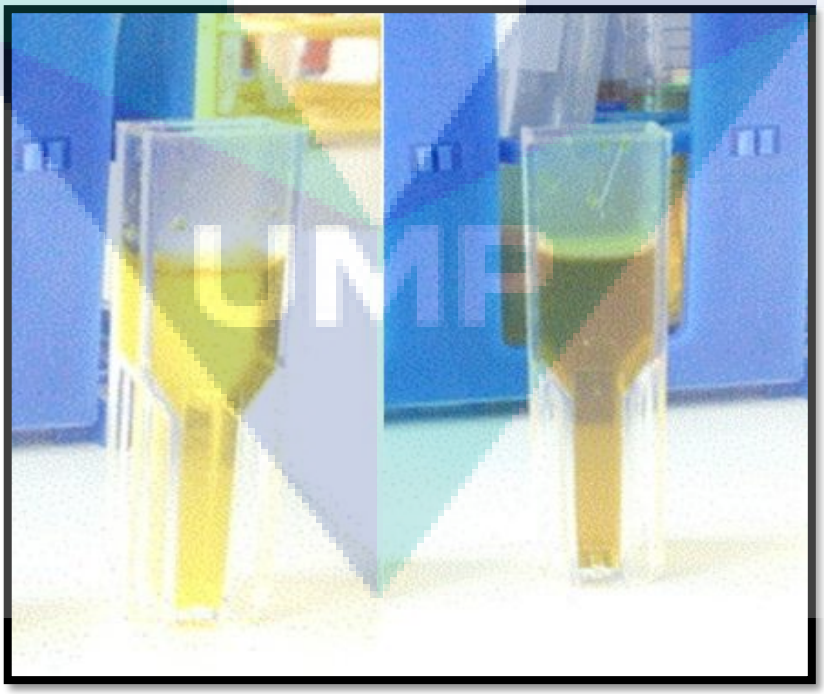


Figure 5 Colour changes of DNS enzyme assay sample from yellow to dark brown

APPENDIX F
BIOLOG SYSTEM RESULTS

To :	NOOR AFIFAH FAUZI	Attn :	-
Address :	FAKULTI SAINS & TEKNOLOGI INDUSTRI, UNIVERSITI MALAYSIA PAHANG		
c.c. :	-	Page :	3 pages
Fax No : -	Tel No :		Sample Lab No: 2015/562

Sample description : 2 bacteria culture on plate

Sample marking : Refer below

Date of sample received : 22-10-2015

Date reported : 03-11-2015

RESULTS:

Parameter : Identification of microorganism

No	Sample Marking	Result		Test Method
		ID	Probability	
1	CL8A	*Alcaligenes faecalis ss faecalis*	87.3 %	Gen III MicroPlate IFA (Protocol A) Biolog Microbial Identification System
2	PSI	*Providencia rettgeri*	61.7 %	Gen III MicroPlate IFB (Protocol B) Biolog Microbial Identification System

The certificate shall not be reproduced except in full without the written approval of the laboratory.

The above analysis is based on the sample submitted by the customer.

.....

2015/562
Page 1 of 3

A. INTRODUCTION

Biolog MicoPlate analyzes a microorganism in 94 phenotypic tests: 71 carbon source utilization assay (column 1-9) and 23 chemical sensitivity assays (column 10-12). The test panel provides a Phenotypic Fingerprint of the microorganism that can be used to identify it at the species level. All necessary nutrients and biochemical are prefilled and dried into 96 wells of the MicroPlate.

Tetrazolium redox dyes are used to colorimetrically indicate utilization of the carbon sources or resistance to inhibitory chemicals. All of wells start out colourless when inoculated. During incubation there is increased respiration in the wells where cells can utilize a carbon source and/or grow. Increased respiration causes reduction of the tetrazolium redox dye, forming purple colour.

Negative wells remain colourless, as does the negative control well (A1) with no carbon source. There is also a positive control well (A10) used as reference for the chemical sensitivity assai in coloumn 10-12. After incubation, the phenotypic fingerprint of purple wells is compare to Biolog's Database. If a match is found, a species level identification of the isolate is made.

MicroPlate used in this analysis: Gen III MicroPlate, IFA (Protocol A)

The Gen III MicroPlate test panel provides a standardized micromethod using 94 biochemical tests to profile and identify a broad range of Gram-negative and Gram-positive bacteria.

B. RESULT

Sample Marking	:	CL8A
ID	:	*Alcaligenes faecalis ss faecalis*
ID details	:	Probability: 87.3 % Incubation Hours: 24 hours Refer attachment for following supportive document • Positive and negative graphic • Positive and negative numerical • OD reading at 490nm (colour) and 750nm (turbidity)
Sample Marking	:	PSI
ID	:	*Providencia rettgeri*
ID details	:	Probability: 61.7 % Incubation Hours: 24 hours Refer attachment for following supportive document • Positive and negative graphic • Positive and negative numerical • OD reading at 490nm (colour) and 750nm (turbidity)

C. INTERPRETATION

- Final identification results are displayed in the ID Box. Entry #1 is the best match selected from the database.
- PROB - Allows you to compare Biolog's IDs to other systems that use this type of calculation. If there is a "No ID" result, the PROB value is not displayed.
- SIM - Similarity index value used to assess how well a sample is identified. A value of 1 is a perfect match. A value of zero equals no match.
- DIST - Indicates the approximate number of mismatches between your MicroPlate results and the database pattern for that species.
- The black + and - signs show mismatches between your sample and the database record.
  All + your pattern is giving fewer positive reactions than the species you're comparing it to.

All - your pattern is giving more positive reactions than the species you're comparing it to.

- Pos/Neg Graphic - displays the threshold values for calling positive, negative, and borderline. It also displays the major mismatches between the #1 ranked species and your MicroPlate.
- Pos/Neg Numerical - displays the data values for the wavelengths read or default values for manual read.
- ODs - displays the raw OD values for the wavelengths read
- False positive – strong reducing bacteria and/or capsule producing bacteria have to change to other protocol. Negative control well (A1) become too dark.

APPENDIX G
GRAM STAINING RESULTS

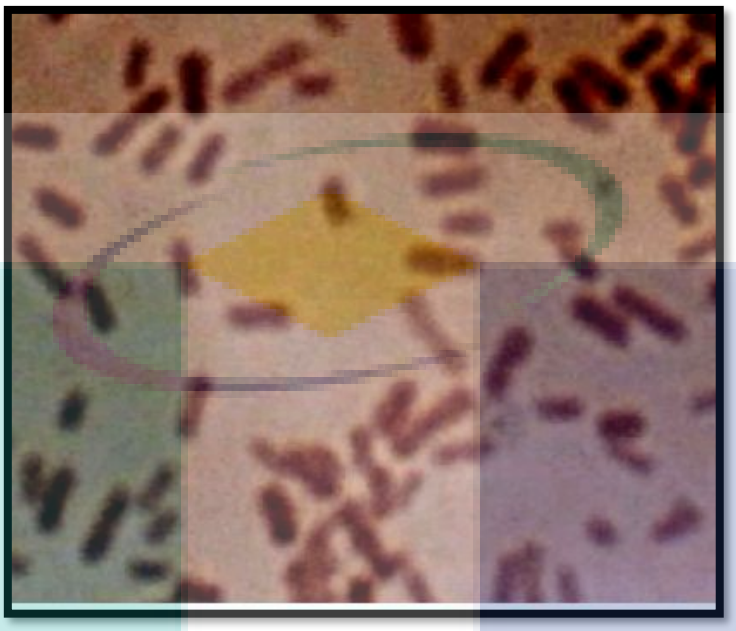


Figure 7 *Alcaligenes faecalis* (CL8A isolate) observed under immersion oil 1000× lens microscope

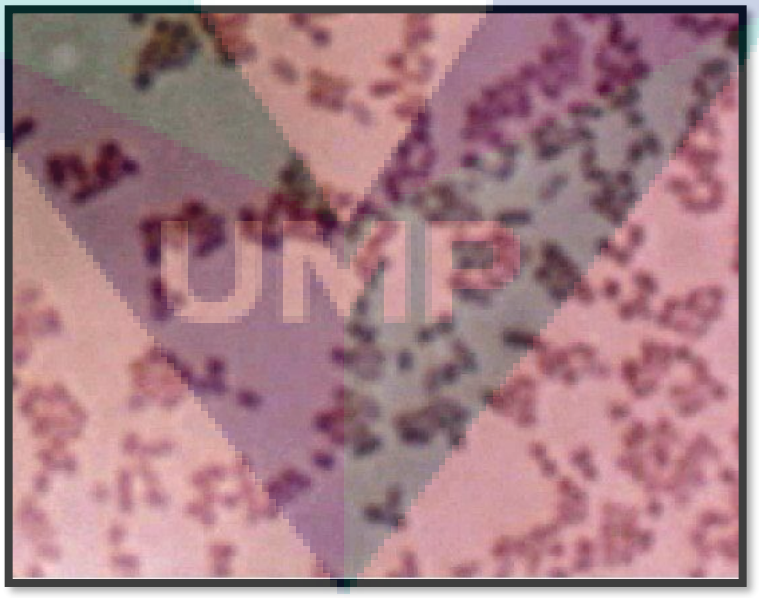


Figure 8 *Providencia rettgeri* (PS1 isolate) observed under immersion oil 1000× lens microscope

APPENDIX H
STUDENT'S ACHIEVEMENTS

List of publications:

1) Essam A. Makky & Noor Afifah Fauzi (2014). Avicelase enzyme from sawdust : isolation , production and optimization, *Journal of Global* ***Biosciences,*** 3(1), 299–303.
2) Noor Afifah Fauzi, Siti Hajar Mohd Rasdi, Essam A. Makky & Mohd Hasbi Ab Rahim (2016) Bioremediation of disposed X-ray film for enzymes production. *Global Journal of Advanced Research*, 3(2), 101-106.

List of award:

1) Microbial Cellulases From Disposed Xray Film Management, Creation, Innovation, Technology & Research Exposition (CITREX) 2016, **(Silver award)**