CHARACTERISATION OF MANNANASE FROM *Pontibacillus* sp. CL43

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This thesis is dedicated to my beloved parents for their fully support and encouragement.

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ABSTRACT

Lignocellulosic biomass is a renewable feedstock for biofuel production. Pretreatment is performed to destroy the biomass recalcitrant structure subsequently allow enzymes to hydrolyse cellulose and hemicellulose for sugars production. These sugars could be used for biofuel production. In chemical pretreatment of lignocellulosic biomass, metal salts (KCl, $CaCl_2$ and $MgCl_2$) and surfactants (Tween 20, Tween 80 and Triton X-100) are used. These compounds retain in the biomass after the treatment and affect the performance of enzymes in following hydrolysis process. Hence, enzymes with metal- and surfactant-tolerance characteristics are preferable in biofuel production. This project focused on characterising *Pontibacillus* sp. CL43 and its mannanase which is responsible for degradation of mannan, a component present in lignocellulosic biomass. Strain CL43 is a Gram positive bacterium with rod-shaped, endospore-forming, catalase- and oxidase-positive. Strain CL43 could hydrolyse bile esculin, casein, starch, xylan, Tween 20, Tween 40, Tween 60 and Tween 80. Strain CL43 was susceptible to ampicillin, carbenicillin, doxycycline, gentamicin, minocycline, neomycin, oxacillin, penicillin G, piperacillin, polymyxin B, rifampicin, streptomycin and tetracycline. Mannanase of strain CL43 exhibited optimal activity at 60 °C, pH 7 and 2% (w/v) NaCl. Mannanase activity was found to be stable in the presence of metal ions (Ca^{2+}, Cd^{2+}, Mn^{2+}, K^{+}, Mg^{2+} and Co^{2+} with relative activity ranged from 87-128%) and surfactants (Tween 20, Tween 40, Tween 60, Tween 80 and Triton X-100 with relative activity ranged from 88-107%). Mannanase of strain CL43 with good tolerance to these metal ions and surfactants indicated it is a good candidate to be used for lignocellulosic biomass hydrolysis.

ABSTRAK

Biomas lignoselulosa adalah sumber bahan mentah yang boleh diperbaharui yang digunakan untuk penghasilan biofuel. Pra-rawatan dijalankan untuk memecahkan struktur biomas yang rekalsitran kemudiannya membolehkan enzim untuk menghidrolisis selulosa dan hemiselulosa bagi penghasilan gula. Gula jenis ini boleh digunakan untuk penghasilan biofuel. Di dalam pra-rawatan kimia biomas lignoselulosa, garam logam (KCl, $CaCl_2$ dan $MgCl_2$) dan surfaktan (Tween 20, Tween 80 dan Triton X-100) telah digunakan. Kompaun ini kekal dalam biomas lignoselulosa selepas pra-rawatan dan ianya dapat mempengaruhi prestasi enzim dalam proses hidrolisis yang berikutnya. Oleh itu, enzim yang mempunyai ciri-ciri logam- dan surfaktan-toleransi lebih disukai dalam penghasilan biofuel. Projek ini difokuskan pada pencirian *Pontibacillus* sp. CL43 dan mannanase yang bertanggungjawab untuk mendegradasi mannan, dimana ianya merupakan salah satu komponen yang terdapat dalam biomas lignoselulosa. Strain CL43 adalah Gram positif bakteria yang berbentuk *rod*, penghasilan endospora, katalase- dan oksidase-positif. Strain CL43 boleh menghidrolisis esculin, kasein, kanji, xilan, Tween 20, Tween 40, Tween 60 dan Tween 80. Strain CL43 sensitif kepada ampicillin, carbenicillin, doxycycline, gentamicin, minocycline, neomycin, oxacillin, penicillin G, piperacillin, polymyxin B, rifampicin, streptomycin and tetracycline. Mannanase daripada strain CL43 mempamerkan aktiviti optimum pada 60 °C, pH 7 dan 2% (w/v) NaCl. Aktiviti mannanase didapati stabil dengan kehadiran ion logam (Ca^{2+}, Cd^{2+}, Mn^{2+}, K^{+}, Co^{2+} dan Mg^{2+} dengan aktiviti relatif 87-128%) dan surfaktan (Tween 20, Tween 40, Tween 60, Tween 80 dan Triton X-100 dengan aktiviti relatif 88-107%). Mannanase daripada strain CL43 menunjukkan toleransi yang baik terhadap ion logam dan surfaktan. Oleh yang demikian, enzim ini boleh menjadi calon yang baik bagi penghidrolisisan biomas lignoselulosa.

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LIST OF ABBREVIATIONS

Al^{3+}	-	Aluminium (III) ion
$Al_2(SO_4)_3$	-	Aluminium (III) sulfate
ASW	-	Artificial seawater
Ca^{2+}	-	Calcium (II) ion
$CaCl_2$	-	Calcium (II) chloride
CAZy	-	Carbohydrate-active enzymes
Cd^{2+}	-	Cadmium (II) ion
$Cd(NO_3)_2$	-	Cadmium (II) nitrate
CE	-	Carbohydrate esterase
Co^{2+}	-	Cobalt (II) ion
$CoCl_2$	-	Cobalt (II) chloride
Cu^{2+}	-	Copper (II) ion
$CuSO_4$	-	Copper (II) sulfate
DMSO	-	Dimethylsulfoxide
DNS	-	Dinitrosalicylic acid
et al		et alia
Fe^{2+}	-	Ferum (II) ion
$FeSO_4$	-	Ferum (II) sulfate
GH	-	Glycoside hydrolase
H^+	-	Hydrogen ion
H_2O_2	-	Hydrogen peroxide
K^+	-	Potassium ion
KCl	-	Potassium chloride
LBG	-	Locust bean gum
MA	-	Marine agar
Mg^{2+}	-	Magnesium (II) ion
$MgCl_2$	-	Magnesium (II) chloride
Mn^{2+}	-	Manganese (II) ion
$MnCl_2$	-	Manganese (II) chloride
Na^+	-	Sodium ion

NaCl	-	Sodium chloride
Ni^{2+}	-	Nickel (II) ion
$NiSO_4$	-	Nickel (II) sulfate
OD	-	Optical density
OF	-	Oxidative fermentative
SDS	-	Sodium dodecyl sulfate
-SH groups	-	Sulfhydryl groups
sp.	-	Species (singular)
spp.	-	Species (plural)
Zn^{2+}	-	Zinc (II) ion
$ZnSO_4$	-	Zinc (II) sulfate

LIST OF SYMBOLS

α	-	Alpha
β	-	Beta
°C	-	Degree Celsius
g	-	Gram
h	-	Hour
kPa	-	Kilo Pascal
mL	-	Millilitre
mm	-	Millimetre
mM	-	Millimolar
M	-	Molar
nm	-	Nanometre
n	-	Number
%	-	Percentage
®	-	Registered trademark
rpm	-	Revolutions per minute
U	-	Unit
v/v	-	Volume per volume
w/v	-	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Background

Owing to the increasing demand of fossil fuels, global warming and emission of greenhouse gases, it is important to produce biofuels to maintain sustainable environment (Saini, Saini and Tewari, 2015). First generation biofuels are produced directly from food crops such as corn, sugarcane, soybean and others. While second generation biofuels are produced from non-edible biomass such as lignocellulosic biomass and do not compete with food crops production (Amoozegar et al., 2019). Bioethanol produced from lignocellulosic biomass is considered as a renewable and sustainable biofuel (Wang et al., 2012). Bioethanol production also reduces the environmental pollution arising from disposal of lignocellulosic waste (El-Naggar, Deraz and Khalil, 2014).

Lignocellulosic materials mainly composed of cellulose, hemicellulose and lignin components (Isikgor and Becer, 2015; Zhao et al., 2011a). Cellulose is the major component of lignocellulose biomass (Isikgor et al., 2015). Cellulose determines the structure and framework of cell wall. Hemicellulose acts as linkage that non-covalent bonded to cellulose and covalent bonded to lignin (Michelin et al., 2015). Hardwood hemicelluloses contain mostly xylans, whereas softwood hemicelluloses contain mostly glucomannans (Saha, 2003). Lignin protects plant from pathogen attack and environmental stress (Li, Pu and Ragauskas, 2016). Cross linking of lignin provides mechanical support for upward growth of plant (Chen, 2014).

Mannan is the important part of hemicellulose in plant cell walls and could be hydrolysed by mannanase (Mizutani et al., 2012). Mannanase could be produced from microorganisms, animals and plants (Luo et al., 2012). Fungal mannanase was found maximally actives in acidic to neutral pH range (4-7) whereas bacterial mannanase is found to be active at neutral to alkaline pH (6-12). For example, pH optimum of mannanase from *Bacillus nealsonii* PN11 and *Bacillus* sp. N16-5 are at pH 8 and 9.6 respectively. Besides, majority of mannanases show the thermo-tolerant property with optimum activity in the temperature range of 40-65 °C (Srivastava and Kapoor, 2017). Several halo-tolerant mannanase were also reported in the case of *Bacillus* sp. strain NN, *Bacillus* sp. HJ14, *Pantoea agglomerans* A021, *Enterobacter* sp. strain N18, *Sphingomonas* sp. JB13, *Scopulariopsis candida strains* LMK004 and LMK008 (Amoozegar et al., 2019; You et al., 2016; Zhang et al., 2016; Zhou et al., 2012). Searching for new manannase is an important aspect because manannase with additional features such as tolerant to metal ions and surfactants could make this enzyme more applicable in industries.

Microbial mannanase is widely used because of it is relatively low cost in production and easily controllable condition (El-refai et al., 2014). Mannanase is widely applied in various biotechnology industries such as bleaching of pulp and paper, laundry detergent, instant coffee processing and biofuel industries (Mou et al., 2011). This is the first report on mannanase characterisation for genus *Pontibacillus*.

1.2 Problem Statement

Fossil fuel is major source of energy and its demand gradually increase until may not meet global demand in the future. Burning of fossil fuel also causes global warming and environmental pollution (Amelio et al., 2016). Bioethanol produced from lignocellulosic biomass is considered as a renewable and sustainable biofuel to meet the increasing demand (Wang et al., 2012). Lignocellulosic biomass normally undergoes suitable pretreatment to remove the lignin and reduce crystallinity of cellulose (Baruah et al., 2018). Chemicals that are commonly used for the pretreatment are metal salts (KCl, $CaCl_2$ and $MgCl_2$) and surfactants (Tween 20, Tween 80 and Triton X-100) (Putro et al., 2016). However, these chemical residues remained in pre-treated lignocellulosic biomass, which affect the following enzymatic hydrolysis process. Therefore, investigation of enzyme with extra features including metal- and surfactant-tolerant is important to ensure activation of enzyme in the hydrolysis process. Mangroves are salt-tolerant forest ecosystems that are situated between terrestrial and marine environments where seawater is mixed with freshwater (Wu et al., 2018). The swampy and saline environments make it possible for obtaining microorganisms and their enzymes with unique properties (Gao et al., 2010). Therefore, the aim of this study is to characterise *Pontibacillus* sp. CL43 isolated from mangrove sediment and its mannanase which is one of key enzymes in lignocellulosic biomass degradation.

1.3 Research Objectives

The objectives of the research are:

(a) To characterise the *Pontibacillus* sp. CL43 phenotypically

(b) To determine the effect of temperature, pH and salinity on activity and stability of mannanase

(c) To assess the stability of mannanase in presence of metal ions, organic solvents and detergents

1.4 Scope of Study

The isolated bacterium *Pontibacillus* sp. CL43 was streaked on marine agar and the mannanase activity was screened using qualitative methods. Bacterial phenotypes were studied based on its morphology, biochemical tests, antibiotic-resistant tests, physiology and API kit. Next, activity and stability of mannanase were determined at different temperature, pH and salinity. Lastly, stability of mannanase was assessed in presence of metal ions, organic solvents and detergents.

1.5 Significance of Study

Lignocellulose wastes are generated in large amounts every year, including leaves, stalks, stems, wheat and others. Disposal of these wastes to the landfill causes environmental problems. However, they can be used to produce some value added products. For example, lignocellulose wastes could be converted to bioethanol. Mannan is the major component of hemicellulose. Mannanase is one of the hemicellulases that degrades mannan into smaller molecules (Cheng et al., 2016). This enzyme is widely used in various biotechnological applications, such as bioethanol production, coffee extraction and paper processing (Xia et al., 2016). So far, no mannanase from members of *Pontibacillus* has been reported. In this project, characteristics of mannanase from *Pontibacillus* sp. CL43 were investigated for its potential application such as saccharification process for bioethanol production in future.

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