ENHANCEMENT OF CELLULASE-POOR XYLANASE PRODUCTION BY NATIVE FUNGI *VIA* SOLID STATE FERMENTATION PROCESS

by

TAI WAN YI

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

September 2016

ACKNOWLEDGEMENT

First of all, I would like to express my gratitude to my Supervisor, Dr Lee Chee Keong, for his invaluable guidance and advice throughout this research project. My gratitude extends to my co-supervisor, Prof Darah Ibrahim.

Words fail me to thank both of my parents, who are always there to give their indefinite support and encouragement. Without them, nothing is possible.

Last but not the least, I would like to thank all of my friends and lab-mates who have accompanied me through the thick and thin of this long and tough journey. Special thanks to Mr Azmaizan, Miss Najmah and Mr Ghoni for their technical support, making the experimental proceedings of this research a smooth one.

Tai Wan Yi

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF SYMBOLS AND ABBREVIATIONS

BG	B-glucosidase
CB	Cellobiase
CBH	Cellobiohydrolase
CMC	Carboxymethylcellulose
CMCase	Carboxymethylcelllulase
CSL	Corn Steep Liquor
DNS	Dinitrosalicylic Acid
DoE	Design of Experiment
EG	Endo-glucanase
EMS	Ethyl Methanesulfonate
FPA	Filter Paper Activity
GH	Glycosyl Hydrolase
IUPAC	International Unit of Pure and Applied Chemistry
LB	Lignocellulosic Biomass
M	Molar
nm	Nanometer
OPF	Oil Palm Frond
PDA	Potato Dextrose Agar
SEM	Scanning Electron Microscope
SGB	Second-generation Bioethanol
SmF	Submerged State Fermentation
SSF	Solid State Fermentation

U	Unit
U/g	Unit/gram
U/mgG	Unit/milligram of glucosamine
UV	Ultraviolet
%	Percentage
+	Plus
-	Minus
w/w	Weight per weight
w/v	Weight per volume
v/w	Volume per weight
v/v	Volume per volume
P<	Probability less than
°C	Degree Celsius
β	Beta
α	Alpha

PENINGKATAN PENGELUARAN XILANASE RENDAH SELULASE OLEH KULAT ASLI MELALUI PROSES PEMFERMENTASIAN KEADAAN PEPEJAL

ABSTRAK

Kos tinggi enzim selulase kekal sebagai penghalang terpenting dalam penghasilan bio-etanol daripada biojisim lignosellulosik (LB). Kajian ini bertujuan untuk meningkatkan keupayaan kulat tempatan dalam penghasilan enzimn selulolitik melalui mutagenesis rawak di bawah pemfermentasian keadaan pepejal (PKP) dengan menggunakan pelepah kelapa sawit (PKS). Daripada 95 pencilan yang diasingkan daripada tapak pertanian dan diuji secara kualitatif dan kuantitatif, pencilan kulat filament DWA8 didapati menghasilkan enzim yang terbanyak. Pemeriksaan secara kualitatif menunjukkan bahawa sebanyak 38 kulat pencilan telah menunjukkan halo yang besar dan jelas tetapi hanya 23 daripada pencilan tersebut telah dipilih untuk diteruskan dalam kajian kuantitatif. Bagi analisis kuantitatif enzim, supernatan enzim telah diestrak daripada proses PKP yang menggunakan kepekatan spora sebanyak $1x10^6$ spora/ml dikultur ke atas serbuk PKS selama 7 hari. Akhirnya, DWA8 telah dipilih sebagai pencilan terbaik untuk kajian seterusnya kerana memaparkan aktiviti fpase dan xilanase yang tertinggi, iaitu masing-masing 3.26 U/g dan 5.26 U/g. Pencilan DWA8 kemudian telah dikenalpasti sebagai *Aspergillus niger* berdasarkan morfologi coloni dan pencirian molekul (Genbank no. KP299287). Profil penghasilan enzim telah menunjukkan bahawa hari ke-4 merupakan hari yang paling sesuai untuk PKS kerana aktiviti cmcase, fpase dan xilanase daripada *A. niger* DWA8 adalah amat tinggi pada

masa tersebut, iaitu masing-masing sebanyak 1.13 U/g, 2.55 U/g dan 2.38 U/g. Kaeadah Tindakbalas Permukaan (KTP) telah digunakan untuk mengkaji kesan dua parameter fizikal proses yang telah dikenalpastikan penting oleh cara konvensional demi mengoptimumkan pengeluaran enzim selulase. Kandungan kelembapan sebanyak 75% (b/b) dan jumlah substrat sebanyak 2.5 g telah terbukti memberi kesan yang ketara dalam penghasilan selulase and xilanase. Manakala, tidak ada faktor kimia yang didapati memberi kesan positif melalui KTP. Profil penghasilan enzim yang dijalankan selepas proses pengoptimuman ini telah memilih hari ke-4 sebagai tempoh yang paling sesuai untuk mengekstrak enzim, di mana aktiviti tertinggi fpase sebanyak 2.47 U/g telah diperolehi. Pada masa yang sama, aktiviti cmcase sebanyak 0.74 U/g dan xilanase sebanyak 4.88 U/g telah dilaporkan. Dalam kajian ini, selepas 2 pusingan mutagenesis rawak, rawatan majmuk Ultraviolet (UV) dan Ethyl Methanesulfonate (EMS) telah berjaya menghasilkan mutan *A. niger* dengan peningkatan sebanyak 2.6-kali ganda bagi aktiviti cmcase dan 2.2-kali ganda bagi aktiviti fpase ($p<0.05$) berbanding dengan *A. niger* DWA8 asal. Profil penghasilan enzim dijalankan untuk kedua-dua mutan yang terbaik, aktiviti cmcase tertinggi yang dipaparkan oleh UE2.07 dan EU82 masing-masing adalah 3.4 U/g dan 3.37 U/g; aktiviti fpase tertinggi bagi UE2.07 dan EU82 masing-masing adalah 2.3 U/g dan 2.9 U/g; aktiviti xilanase tertinggi bagi UE2.07 dan EU82 masing-masing adalah 19.3 U/g dan 20.3 U/g. Kesimpulannya Penemuan hasil kajian ini boleh dibangunkan sepenuhnya sebagai skim mutasi untuk mewujudkan kulat berfilamen yang sangat produktif dengan cara yang murah, mudah dan mapan, terutama bagi spesis *Aspergillus* yang dilihat sangat bernilai dalam bidang perindustrian.

ENHANCEMENT OF CELLULASE-POOR XYLANASE PRODUCTION BY NATIVE FUNGI *VIA* SOLID-STATE FERMENTATION PROCESS

ABSRACT

High cost of cellulases remains the most significant barrier to the economical production of bio-ethanol from lignocellulosic biomass (LB). The present study aims at developing a local cellulolytic fungal strain with enhanced cellulolytic ability through random mutagenesis coupled with the feasibility of solid-state fermentation (SSF) by utilizing oil palm frond (OPF) as the substrate. Out of 95 wild isolates isolated from agricultural sites and tested both qualitatively and quantitatively, a native filamentous fungal strain designated DWA8 was found to be the top enzymatic secretor. During qualitative screening, 38 isolates were found to produce distinctive halo but only 23 were chosen to proceed with quantitative screening. For quantitative enzyme analysis, enzyme supernatant was extracted from the SSF process which was conducted using $1x10^6$ spore/mL inoculated onto 5 g of ground OPF, incubated at room temperature for 7 days. DWA8 was found to be the best candidate for further studies, as it produced the highest amount of fpase and xylanase with a considerably high amount of cmcase, which was 3.26 U/g, 5.28 U/g and 0.22 U/g respectively. DWA8 was later being identified as *Aspergillus niger* (Genebank accession no. KP299287) based on colony morphology and molecular characterization. Enzyme profiling has determined the 4^{th} cultivation day as the best cultivation period where the cmcase, fpase and xylanase activities of *A. niger* DWA8 were 1.13 U/g, 2.55 U/g and 2.38 U/g respectively. Next, RSM was employed to study the effects of two physical parameters that were being identified as pivotal using

conventional screening method for optimization purposes. Moisture content of 75% (w/w) and substrate amount of 2.5 g were drawn out to be the optimum condition achieved. Meanwhile, no chemical factor was identified as significant by RSM. An enzyme profiling conducted after optimization process has again identified 4th day as the best cultivation period where 0.74 U/g of cmcase, 2.47 U/g of fpase and 4.88 U/g of xylanase activities had taken place. Later in this work, after two rounds of mutagenesis, the compounded treatment of ultraviolet (UVC) and Ethyl Methanesulfonate (EMS) have generated an *A. niger* mutant with a 2.6-fold increase of cmcase activity and 2.2-fold increase in fpase activity respectively ($p<0.05$) compared with the parental *A. niger* DWA8 strain. During the enzyme profiling of the two best performed doubled mutant, the highest cmcase activity of UE2.07 was 3.4 U/g and of EU82 was 3.37 U/g; the highest fpase activity of UE2.07 was 2.3 U/g and of EU82 was 2.9 U/g; the highest xylanase activity of UE2.07 was 19.3 U/g and of EU82 was 20.3 U/g. This finding is capable to be fully developed as an established mutational scheme to create highly productive filamentous fungus in a cheap, simple and sustainable way, especially for *Aspergillus* species which is viewed as highly industrial valuable.

CHAPTER ONE

INTRODUCTION

1.1 Introduction

As one of the major players in the agricultural sector worldwide, specializing in oil palm, rubber and cocoa export, this county creates a substantial amount of lignocellulosic waste. In the case of the oil palm industry alone, 33 million tons of empty fruit bunch, fiber and shell have been produced annually from the processing of 82 million tons of fresh fruit bunch. Hence, the concept of turning waste to wealth has been gaining interest starting late 1990s. Moreover, the largest portion of total agricultural waste in Malaysia is contributed from oil palm fields. As most of the biomass comes from oil palm plantation, especially oil palm fronds (OPFs) which contain high cellulose content (62%) but low lignin portion (15%) could be a highly potential feedstock for second-generation bio-ethanol generation. However, this cellulosic ethanol technology is still immature in Malaysia, as it requires large capital investment in operating cost, determined by pretreatment and enzymatic hydrolysis processes (Goh *et al.*, 2010).

Solid state fermentation, an old yet emerging fermentation technique, regains new attention as it offers several environmental friendly solutions compared to the commercialized submerged fermentation (SmF). One of these strategies is that SSF uses lignocelluloses as its substrate, providing an alternative avenue and waste management for these residues (Singhania *et al.*, 2009). Fungi need lower moisture, which is around 40-60%, and this becomes another reason for SSF being chosen as the cultivation

method for this research, as the low moisture content offered means that this fermentation type could only be carried out effectively by a limited number of useful microorganisms, primarily yeasts and fungi (Khan and Dwivedi, 2013).

To achieve complete hydrolysis of cellulose in lignocellulosic biomass, a cellulase multi-enzyme protein, which composed of cellobiohydrolase (EC 3.2.1.74), endoglucanase/cellulases (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) is needed (Chand *et al.,* 2005 and He *et al.,* 2009). Apart from cellulose, hemicellulose is another heterogeneous polysaccharides building plant secondary cell wall. Xylan comprises the significant fraction of hemicellulose, and the enzymes responsible for its degradation are the xylanases (Polizeli *et al.,* 2005). In the continuous effort to enhance the amount and quality of these enzymes, random traditional mutagenesis applying physical and chemical agents are claimed to be an age-old yet successful methods, apart from genetic modification and heterogonous expression (He *et al.*, 2009).

1.2 Research Scope

Bearing all this in mind, the first aim of this research focused on the use of soluble and insoluble polysaccharides, carboxymethylcellulose (CMC) and α-cellulose powder to screen a diverse group of indigenous fungi from the natural resources for their capability in producing cellulase and xylanase. Furthermore, the selected candidates were tested for their hydrolysis abilities on the OPF, as its utilization as one type of the agricultural wastes under SSF is still considered rare and primitive. It is certainly a cheap feedstock for second-generation bioethanol production in future. Next, with the

identification of the most potential fungal cellulase producer being confirmed genetically, conditioning parameters and chemical factors that affect the enzyme production were examined to search for the optimum level for the increment of enzyme production. These included moisture content, substrate amount, incubation temperature, spore concentration and media pH. For chemically characterized variables, apart from nutrient supplementations such as the carbon sources and nitrogen sources, some cellulose hydrolysis stimulating elements such as inducers and surfactants were also put under investigation. Finally, the best fungal producer was subjected to randomized mutagenesis since this technique has been assessed and proven successful in the attempt to enhance the desirable attributes of cellulases for the efficient hydrolysis of cellulose. Besides, it is believed that this approach is a better one in maintaining the stability of both mutant and its enzyme after induction procedure.

1.3 Objectives

Hence, the objectives of this study were as follows:

- To isolate and identify a highly potential cellulase-producing wild-type fungal strain through extensive qualitative plate screening
- To optimize the SSF process physio-chemically for the enhancement of cellulase and xylanase production under statistical experimental design
- To develop an over-producing fungal mutant by conventional random mutagenesis using Ultraviolet and Ethyl Methanesulfonate

CHAPTER TWO

LITERATURE REVIEW

Cellulose enzymatic hydrolysis generate reducing sugars including glucose is exclusively specific and selective. Compared to chemical (acid or alkanline) hydrolysis, its utility cost is low as it is usually conducted at mild condition (pH4.8 and temperature 45-50°C). However, its process takes days while it is only in few minutes of time for chemical hydrolysis to complete. Other technical challenge faced by this process is the cost of enzymes and the inhibition of end-product (Sun and Cheng, 2002 and Binod *et al.*, 2011). Consequently, limited success has been achieved in maximizing sugar yields at a very low cost (Yang *et al.*, 2011).

2.1 The Cellulolytic Enzymes

2.1.1 The Enzyme Machinery

Cellulose, being the primary plant cell wall material constitutes the most abundant carbon source on land, whiles its recovery which directly represents carbon cycling as a whole, requires the action of cellulases. These enzymes in turn, are the most diverse ones catalyzing the hydrolysis of β-1,4 glucosidic bond joining two glucose molecules and forming the most recalcitrant natural material, the cellulose molecule (Figure 2.1).Cellulose presented itself in chains orientating in parallel and forming ordered, crystalline domains interspersed by more disordered, amorphous regions (Beguin and Aubert, 1994). Another special feature displayed by this class of enzymes is their ability

to degrade insoluble substrate, which requires the enzymes itself to diffuse into insoluble substrate and move "S" segment of cellulose molecule into its active site, whereas the soluble substrates will diffuse into their respective enzyme host and bind to its active site. Most of these enzymes contain a substrate-cellulose-carbohydrate binding domain (CBM) that is linked to the catalytic domain (CD) by a flexible linker peptide. Cellulase system comprises of two types, with the aerobic utilization of free cellulase non-complexes mechanism *via* a set of individual cellulases, and anaerobic utilization of cellulosomes, large multi-enzyme complexes with multimillion molecular weight. The cellulosome enables the bacteria to attach themselves onto the surface of cellulose with the help of a scaffolding protein, generating tight contact with the substrate which maximizes the uptake of soluble cello-oligossacharides by bacterial cells. The scaffolding subunit of celulosome also contains single CBM coupled with cohesion, binding together with dockerin modules borne by each cellulosomal components (Gusakov, 2011). In the former case, the free cellulase system comprises another three individual components: Endoglucanase (EC 3.2.1.4) that cleave the cellulose molecule randomly, cellobiohydrolase (EC 3.2.1.21) that cleave the cellulose progressively by targeting the chain ends, and finally the β-glucosidase (EC 3.2.1.21) which release free glucose molecules from the cellobiose and also cleave off glucose units from cello-oligosaccharides (Binod *et al.*, 2011; Verardi *et al.*, 2012) (Figure 2.2).

The exocellulases are further divided into two types; the first ones would attacks the reducing ends of cellulose chain while the second type attacks the non-reducing ends.

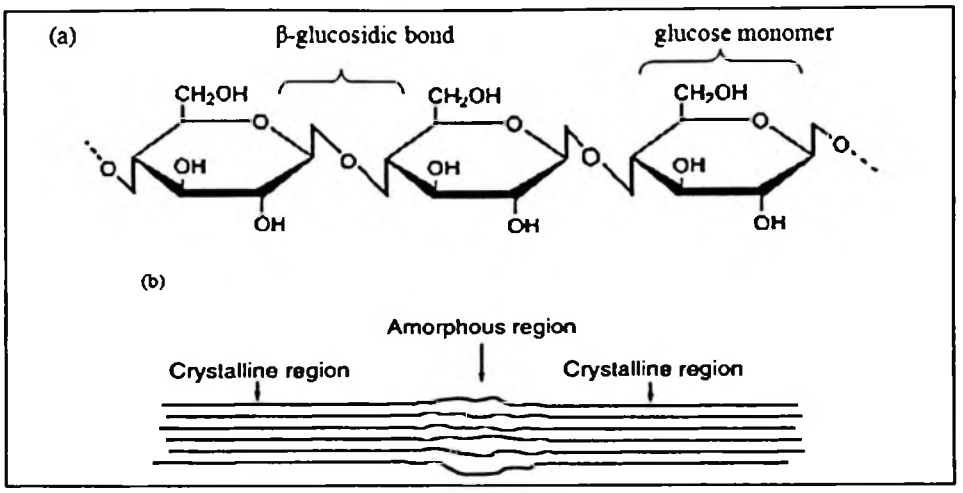

Figure 2.1 Structure of cellulose. (a): β-glucosidic bond and the glucose monomer and (b): Schematic structure of a fibril (Adopted from Begiun and Aubert, 1994)

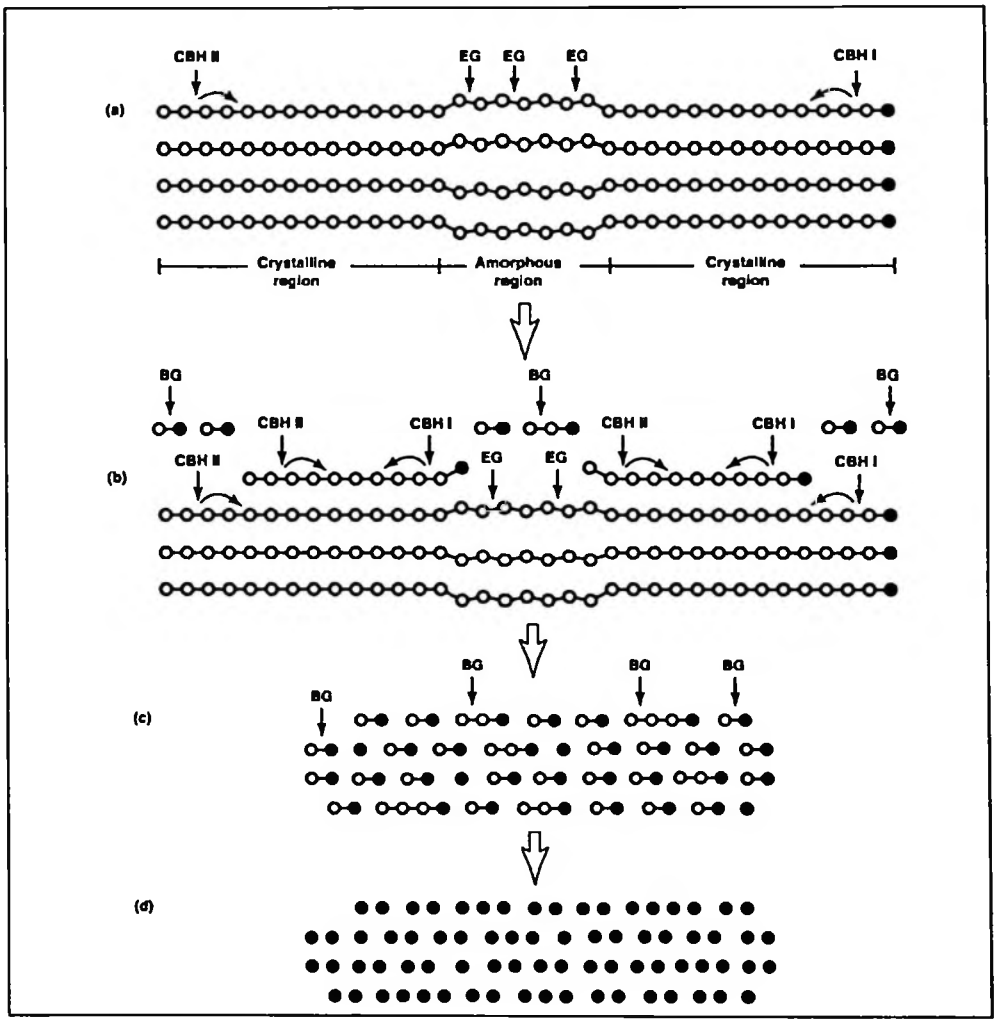

Figure 2.2 Cellulose enzymatic hydrolysis by fungal non-complexes cellulase system. CBH, cellobiohydrolase; EG, endo-glucanase; BG, β-glucosidase; solid circles represent reducing ends. (Adopted from Gusakov, 2011)

The synergism between these three glycosyl hydrolase enzymes carried the whole cellulose digestive mechanism through 2 steps namely primary and secondary hydrolysis. During primary hydrolysis, endoglucanase and cellobiohydrolase release soluble sugars from solid cellulose surface. This depolymerization step happens slowly and it is the rate-limiting step in the whole process. Further hydrolysis of the soluble fractions into ultimately glucose is conducted by β-glucosidase during secondary hydrolysis, which is a much faster process (Binod *et al.*, 2011; Verardi *et al.*, 2012). Apart from this sequential breakdown of linear glucose chain, degradation of the branched chains containing various sugars and functional groups puts hemicellulases into place (Jergensen *et al.*, 2007). Some examples for these ancillary enzymes are glucuronidase, acetylesterase, xylanase, β-xylosidase, galactomannase and glucomannase (Sun and Cheng, 2002).

2.1.2 Classification and Characterization of Cellulases

The availability of the genome sequence data which belongs to several filamentous fungi such as the *Phanerochaete* species, *Aspergillus* species, *Fusarium* species, *Magnaporte* species, *Neurospora* species, *Penicilium* species and *Ustilago* specie has allowed examination of carbohydrate-active enzymes (CAZymes) categorized into different classes and families. CAZymes refer to any enzyme that cleaves, builds and rearranges oligo- and polysaccharides essential for fungal metabolism, which are crucial for biomass degradation (Yang *et al.*, 2011). Glycosyl Hydrolase, such as cellulolytic and hemicellulolytic enzymes are classified with GHs. So far, there have been 118 families

of GHs being identified in all biological systems, with more than 60 of them found in the filamentous fungi mentioned above. Table 2.1 summarized the size of the CAZymes family for the 11 fungal genomes with complete sequences available (Yang *et al.*, 2011), while Table 2.2 showed the nomenclature of cellulolytic enzymes (Kubicek *et al.*, 2009).

Table 2.1 The sizes of the CAZymes family for the 11 filamentous fungal genomes with complete sequences available (Adopted from Yang *et al.*, 2011)

Name	Glycoside Hydrolase	Glycosyl-transferase	Polysaccha-ridelyase	Carbohydrate esterase	Carbohydrate binding module
Aspergillus fumigatus	263	103	13	29	55
Aspergillus niger	248	114	8	25	38
Aspergillus oryzae	303	119	23	30	34
Aspergillus nidulan	252	90	21	33	41
Fusarium graminearum	243	110	20	42	61
Magnaporthe grisea	232	94	5	47	65
Neurospora crassa	174	78	4	22	42
Penicillium chrysogenum	219	102	9	22	49
Phanerochaete chrysosporium	166	57	14	14	N/A
Podospora anserine	230	89	7	49	97
Trichoderma reesei	200	103	3	16	36

Table 2.2 The nomenclature of cellulolytic enzymes (Adopted from Kubicek *et al.*, 2009)

Function	Gene	Protein	GH family
Cellobiohydrolase	*cbhI/cel7a*	CBHI/CEL7A	GH7
	cbhII/cel6	CBH2/CEL6	GH6
Endo-β-1,4-glucanase	*egI1/cel7b*	EG1/CEL7B	GH7
	egI2/cel5a	EG2/CEL5A	GH5
	egI3/cel12a	EG3/CEL12A	GH12
	egI4/cel61a	EG4/CEL61A	GH61
	egI5/cel45a	EF45/CEL45A	GH45
	cel74a	CEL74A	GH74
	cel61b	CEL61B	GH61
	cel5b	CEL5B	GH5
β-glucosidase	*bgI1/cel3a*	BGL1/CEL3A	GH3
	bgI2/cel1a	BGL2/CEL1A	GH1
	cel3b	CEL3B	GH3
	cel3c	CEL3C	GH3
	cel1b	CEL1B	GH1
	cel3d	CEL3D	GH3
	cel3e	CEL3E	GH3

Aerobic *Trichoderma reesei/ Hypocrea jecorina, Phanerochaete chrysosporium,* and *Thermofida fusca* contained multiple cellulase genes that mainly encode a carbohydrate/ cellulose binding domain (CBM), and also several processive cellulase genes. Meanwhile, in anaerobic microbes such as *Clostridium thermocellum, Ruminocccus albus* and *F.flaviflaciens*, apart from these two gene types, they contained scaffolding genes and their multiple cellulase genes encode docerin domains, which are consistent with the presence of cellulosome machinery in anaerobic bacteria capable of degrading cellulosic biomass. Besides, there are few cellulolytic microbes that do not contain either three of the above mentioned genes: *Cytophiga hutchinsonii* and *Fibrobacter succinogenes* that bound tightly to cellulose fiber and *Postia placenta* (aerobic brown rot fungi) that uses peroxide and oxygen radicals to partially depolymerize cellulose fibers, making the chain more accessible to cellulase action. In order to discover more novel class of cellulase genes and the host, metagenomics is the emerging technology which is

used to isolate DNA from various environmental samples such as soil. Although there are continuous screening of DNA libraries and genomic profile of isolated organism, no new cellulase family is being reported recently (Wilson, 2009; Wilson, 2011).

2.1.3 *T. reesei* and Other Potential Fungal Species as the Main Source of Commercial Cellulase Production

Although there have been constant debate regarding higher efficiency and specific activity displayed by anaerobic cellulosomal hydrolysis, bacteria still cannot compete with fungal hypercellulolytic mutants which employ noncomplex system, on the protein expression level (Gusakov, 2011). Many cellulolytic anaerobic bacteria such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activities, but they are incapable to produce enzyme with high titres (Sun and Cheng, 2002). As a result, useful bacterial cellulase domains with high specific activity or temperature resistance are usually expressed in fungal host heterologously. Filamentous fungi are the major source of commercial cellulases, with *T. reesei* being the most studied and engineered fungal species. *T. reesei* RUT C30 is the most powerful strain so far which becomes the reference strain among *T. reesei* high cellulase producers. Much of the 12 β-glucosidases indentified in this fungal mutant are intracellular secreted in small amount, thus requires the extra supply of this enzyme from *Aspergillus* species in order to catalyze complete cellulose saccharification (from cellobiose to glucose) (Gusakov, 2011). Otherwise, accumulation of cellobiose, the glucose dimer, will strongly inhibits the actions of exo- and endoglunases, significantly slowing down the hydrolysis process (Verardi *et al.*, 2012). Apart from this fungal

species, other fungal genera belong *to Aspergillus, Penicilium, Accremonium and Chrysosporium* could serve as its alternatives as they exhibited higher strain productivity, protein production and hydrolytic performance. For example, *Aspergillus* species possessed various genes encoding exo ad endo-glucanases, but receive little attention for deep saccharificationof real biomass. Secondly, high β-glucosidase activity of *Penicilium* species has resulted in 1.5-3.0 fold higher glucose yield compared to *Trichoderma* enzymes. Moreover, the cellulases are less susceptible to inhibitors especially lignin, when it was used to degrade steam-exploded softwood. This property is also showed by *A. cellulolyticus* whereby its laboratory and commercial preparation showed better performance in Avicel than commercial *Trichoderma* cellulase preparations. Besides, cellobiohydrolases from *C. lucknowense* demonstrate comparable performance with *T. reesei* in Avicel hydrolysis, and artificial mixture that contains various cellulases isolated from this particular microorganism displayed notably higher saccharification ability compared to commercial *Trichoderma* preparations (Verardi *et al.*, 2012).

Cellulases secreted by thermophilic microorganisms which could grow in the range of 60-110°C possessed extraordinary stability at extreme pH and high pressure (up to 1000 bar) had made them welcoming for running industrial fermentation. Moreover, they are resistant to high-level of hydrolysis end-product such as glucose and cellobiose, a weak point exhibited by their mesophilic counterparts. Despite all these, these thermophiles require exclusive and expensive media for their cultivation. Their specific growth rates are slow too. Hence, large scale commercial production of thermostable and thermoactive enzymes still remains a challenge (Verardi *et al.*, 2012).

2.2 Methods of Strain/ Enzyme Improvement

2.2.1 Analysis of Genomes, Transcriptomes and Interactomes

A key to lower cellulosic ethanol cost is to reduce enzyme usage and costs, by the improvement of its yield and activity (Kumar *et al.*, 2008; Dashtban *et al.*, 2009; Seidth and Seiboth, 2010 and Yang *et al.*, 2011). This could only be realized by the understanding of the structures and functions of both lignocellulosic material and their degrading enzymes. Comprehensive analysis of genomes, transcriptomes and interactomes *via* high-throughput Next-Generation DNA sequencing techniques has been used to determine the whole genome sequence of several important filamentous fungi such as *Fusarium graminearum, Magnaporthe grisea, Neorospora crassa, Penicillium chryogenum* and *Ustilago maydis*, apart from the more "glamorous" *Aspergillus* and *Trichderma* species. Such genomic data allows the identification and classification of carbohydrate-active enzymes (CAZymes) into different classes based on their amino acid sequence homology. Glycosyl hydrolases such as the cellulase and hemicellulases, the key enzymes catalyzing biomass degradation, are categorized under the GHs, one of the families under CAZymes. To date, 118 families of GHs have been identified in all biological systems, with more than 60 of them found in the filamentous fungi mentioned above (Yang *et al.*, 2011). Besides, comparative genome studies showed that mutation affected genes predominantly associated with nuclear transport, mRNA turnover and vacuolar protein trafficking, making directed strain improvement possible. Furthermore, the availability of large number of fungal and bacterial genomes could supplement screening approaches for novel proteins and potential candidates (Seidl and Seiboth, 2010).

2.2.2 Random Mutagenesis

Classical improvement approaches exploit multigenic complex phenotypes in organisms with poorly defined or monitored genetics. Being one of the examples, forced/ random/ blind mutagenesis induced by physical or chemical agents use methods naïve of host genome sequences or the resulting genetic changes (Crook and Alper, 2013). The term mutation refers to any change in the genetic materials (DNA) that is heritable (Ennis, 2001). Naturally occurring or spontaneous mutagenesis refers to genetic alterations that occur without apparent exposure to any mutagen that damage DNA, and the rate of this highly rare activity can be increased by mutagens which introduce some chemical changes to DNA molecule such as altering bases or breaking the sugar-phosphate backbone. A damaged base or DNA segment is not actually a mutation but a pre-mutational lesion. The formation of a mutation usually depends upon the aberrant operation of some cellular process such as DNA replication or recombination) after encountering a pre-mutational lesion (Ennis, 2001).

Mutagenesis is described as the exposure or treatment of biological material to a mutagen, for instances, a physical or chemical agent that raises the frequency of mutation above the spontaneous rate (Kodym and Afza, 2003). Some of the most important physical mutagens include electromagnetic radiation, such as gamma rays, X-rays, UV light and particle radiation such as fast and thermal neutrons, alpha and beta particles. Many of the early systemic molecular studies of DNA damage and mutagenesis used UV and our understanding of its effects is probably the most extensive (Ennis, 2001). Chemical mutagens include alkylating agents (such as commonly use Ethyl Methanesulfonate (EMS)), intercalating agents (such as ethidium bromide), and

base analogues (such as bromouracil). EMS has been shown to be a very effective and efficient mutagen and has probably become the most popular chemical mutagen (Kodym and Afza, 2003).

Chemical and physical mutagens increase mutational capabilities compared with natural selection. UV irradiation basically disrupts DNA structure while EMS introduces DNA damage, where a dose-specific mutation occurred. To be more specific, UV radiation catalyses the joining of adjacent pyrimidine bases resulting in point mutation, while EMS is a alkylating agent that bonds covalently to DNA and adds the organic group to the bases, resulting in bulky adducts (Ennis, 2001). The frequency and specificities of a mutagen (thus the type of a mutation) applied to the parent cell are the fundamental parameters dictating success in such classical techniques. In *E.coli* system for example, chemical mutagens (e.g. MNNG) have been found to induce changes in a non-random fashion and produce different mutation profile. They are able to delete large (approximately 1kbp) sections of an organism's genome as well as generate mutations at the single base-pair level. Analogous to chemical mutagenesis, UV has its own mutagenic specificity and frequency, but it is slightly broader in its action than other mutagens (Crook and Alper, 2013). It is highly recommended by the literature to change to change the mutagen types as much as possible to avoid the development of resistance, whereby a different mutagen type will introduce mutation via an alternative DNA repair pathway of the host/parent. Generally, characteristics of a mutagen may not be exactly known unless the mutagenesis rate and specificities have been fully characterized for the strain of interest.

Random mutagenesis still remained attractive in the advent of many other advanced techniques such as genetic modification and directed evolution as it generates stable changes in the genomes of host organisms without much prior knowledge of the cellular mechanism or genetic make-up required (Derkx *et al.,* 2014). Being one of the most widely used techniques to generate optimized microorganisms over the last decades, mutagenesis has been applied to improve both monogenic and polygenic traits (Steensels *et al.*, 2014). Hence, for complex phenotypes like the production of cellulolytic enzyme consortium in a high concentration, this technique is especially beneficial in generating mutants that carry multiple mutations under high dosage. Moreover, in recent studies, mutagenesis is often the first step to generate genetic variation in the population, after which genome shuffling of the best-performing mutants is applied, or the mutant population is subjected to directed evolution (Steensels *et al.,* 2014).

The creation of cellulase-hyperproducing *T. reesei,* a carbon catabolite repressor CRE1 mutant, showing resistant to end-product glucose inhibition best symbolized the earliest success of classical mutagenesis (Seidh and Seiboth, 2010). However, this strain improvement technique is limited by the host genetic make-up (Biswas *et al.,* 2014).

2.2.3 Recombinant DNA Engineering

With the rise of modern genetic engineering, glycosyl hydrolase production homologously and heterologously is made possible (Yang *et al.*, 2011). Coupled with gene transformation methods, the identification of a number of positive and negative transcriptional regulators of cellulase gene expression has driven the over-expression of genes involved in desirable pathway, and the homologous gene-knockout (gene deletion)

to block the undesirable pathway (Kumar *et al.*, 2008; Seidh and Seiboth, 2010). For example, the utilization of a strong *T.reesei cbh1* promoter to express thermostable β-glucosidase *ccl3a* from *T. emersonii* in *T. reesei* RUT-30 has promoted a highly thermostable and specific enzyme. Over-expression of *T. reesei* cellobiohydrolase (I,II and III) by this promoter has achieved enzyme yield increment in the range of 1- to 4-fold (Dashtban *et al.*, 2009). On the other hand, a large number of fungal and bacterial genes have been cloned in E. coli, and cellulase genes have been expressed efficiently in *Penicilium crysogenum, T. reesei, Pseudomonas fuorescens* and yeast (Kumar *et al.*, 2008). Recombinant DNA and protein engineering create synthetic designer enzymes. For instance, a heterologously expressed *Neocallimastrix patriciurum* CelD encoding a multi-domain enzyme possessing Endoglucanase, Cellobiohydrolase and xylanase exhibited higher activity on Avicel than *T. reesei.* Besides, *Corynebacterium glutamicum* was metabolically engineered by the cloning of *xylA* and *xylB* genes from *E. coli* to broaden its lignocellulosic substrate utilization (Kumar *et al.*, 2008).Another novel chimeric cellulolytic enzyme was created by fusing Endoglucanase from *Acidothermus cellulolyticus* to Cellobiohydrolase of *T. reesei.* The saccharification was improved (Dashtban *et al.*, 2009).

2.2.4 Fungal Co-Culturing

Apart from recombinant and non-recombinant of genetic methods discussed above, fungal co-culturing offers another way to enhance lignocellulosic hydrolysis and product utilization. This idea resonates around a concept that two or more compatible microorganisms with their ability to secrete different cellulases/ hemicellulases, thus to digest different portion of plant polysaccharides will complement each other during

biomass degradation (Kumar *et al.*, 2008; Dashtban *et al.*, 2009). Many cases of improved hydrolytic activities has been reported under SSF co-cultivation mostly between*T. reesei* and Aspergillus species such as *A. niger, A. phoenicis, A. wentii* and *A. terreus* (Holker *et al.*, 2004; Kumar *et al.*, 2008; Dashtban *et al.*, 2009).

2.3 Solid State Fermentation (SSF)

2.3.1 Physiological Basis of SSF

Solid state fermentation (SSF) refers to cultivation of microbes on solid matrix in the near absence or absence of free water (Singhania *et al.*, 2009). The fermentation process in carried out at average water activities significantly below 1, and water activity is defined when the relative humidity of the gaseous phase is in equilibrium with the solid matrix (Holker and Lenz, 2005). In SSF, the solid matrix could either be the nutrient source or an inert support acts as nutrient carrier for the support of microbial growth and development, with an ample water supply impregnated the solid substrate. By bringing the cultivated microbes in the closest vicinity with the solid substrate, optimal substrate concentration is achieved. This formulation has made SSF resembling natural living condition of filamentous fungi, living in terrestrial habitat and feeding upon solid specific substrate (Tengerdy and Szakacs, 2002; Singhania *et al.*, 2009). Close similarity to fungal metabolism enables SSF to become the alternatives or even the better choice compared to submerged fermentation (SmF), as with faster fungal growth in SSF will lessen the fermentation time and minimize enzyme degradation.

After sporulation, fungal hyphae develop into mycelium carpet, and subsequently followed by the formation of penetrative hyphae structure (Figure 2.3). These formed

the basis of fungal host colonization and penetration for their growth and development. Nutrient availability is supplied by mycelia mat which cover wet solid substrate thoroughly, *via* the secretion and diffusion of hydrolytic enzymes such as cellulases, during the process of degrading (or digesting) lignocellulosic biomass. The metabolic activities are occurred in the liquid void spaces on substrate surface (interface of mycelium and solid bed) and in the substrate pores formed by fungal penetration. Meanwhile, the transport of gaseous products during fungal metabolism may occur in the gaseous void spaces among aerial hyphae. During hydrolysis process or fermentation, oxygen is consumed, while carbon dioxide, water, heat and desirable biochemical products such as active secondary metabolites are being produced. These products are released into solid matrix or liquid filled spaces and being extracted. After a certain period of time, temperature gradient increment due to heat generation during fungal metabolism posed a critical problem for SSF. Heat removal is facilitated by conduction and water evaporation. In the case of latter cooling treatment, the usage of additional liquid will further complicated the complex water balance inherited in SSF system. Water uptake by fungal growth and development, water consumption during hydrolysis reaction and water production during fungal respiration are the biological factors that determined water balance. Besides water balance, pH changes caused by local pH fluctuation owing to carbonic acids release and ammonia exchange is another factor required careful monitoring for efficient enzyme production and action (Holker and Lenz, 2005).

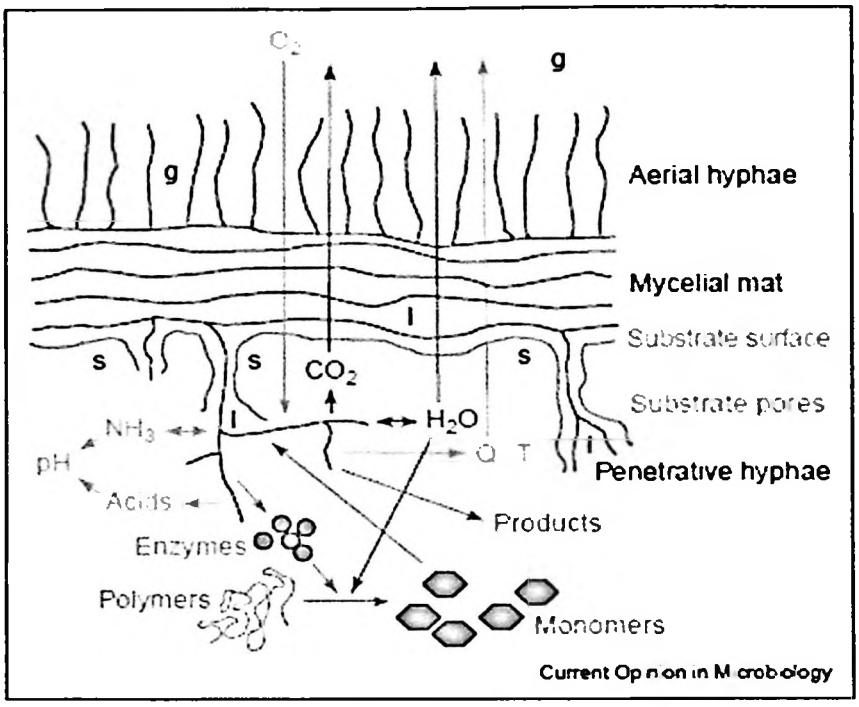

Figure 2.3 An overall biochemical and physical processes involved in solid state fermentation on micro scale (Adopted from Holker and Lenz, 2005). S represented solid subsrate; L represented liquid; g represented gas; Q represented heat development; and T represented temperature.

2.3.2 Molecular Basis of SSF and Its Applications

The most captivating advantages of SSF are the differential physiology (deviation of behaviors) displayed by fungi and other microorganisms cultivated on solid substrate, in relation to the one presented in SmF liquid medium (Singhania *et al.*, 2010). For example, some enzymes that are intracellular in SmF system are extracelluar in SSF system, which is highly desirable for commercial and economical enzyme production (Gonzalez, 2011). Studies have pointed out that environmental stimuli or signals related to solid culture such as the physical barriers of impregnated support, direct contact with air, and water availability in low content, are sensed by fungal mycelium and thus induced SSF-specific genes, such as *glaB* encoding glucoamylase in *Apergillus oryzae* (Holker *et al.*, 2004). The use of concentrated media in SSF (such as starch, malto-

oligosaccharides like maltose) also acts as a strong induction for this particular gene. It was later being found out that *glaB* is regulated transcriptionally by Region A (contains two heat shock element motifs (HSE) and a GC-Box) and Region B with two CAAT sequences of its promoter. As a result, the discovery of various strong promoters have enabled efficient recombinant protein production, whereby *A. oryzae* has been made to produced a 500-fold yield of heterologous protein (calf chymosin) in SSF than it does on SmF (Gonzalez, 2011). On a general aspect, transcriptomic analysis has identified that the upregulated genes in SSF system are located in the pathway of ribosome, DNA replication, oxidative phosphorylation and the TCA cycle. These have been interpreted as that the capacities for protein translation/modification and energy production are much more powerful in the fungus grown on SSF compared to SmF. Protein folding related genes are also upregulated during SSF, suggesting that protein folding and protein glycosylation (post-translational process) are much more efficient under SSF. At the same time, SSF conditions have induced high-level expression of unfolded protein response (UPR) targeted genes, indicating that regulation ability is much more efficient in SSF. This could be possibly caused by extremely high protein synthesis, and also by other stresses such as osmotic, oxidative and nutritional stresses imposed by SSF system (Holker *et al.*, 2004).

A current growth mode depicting fungal microscopic event taken place during SSF proposed that fungal hyphae forms a three-layer of hyphal biomass atop substrate particle, with pores in between (Figure 4.4):

(1) An upper layer with aerial hyphae and air-filled pores

(2) A middle layer with densely packed hyphae and liquid-filled pores

(3) The bottom layer with penetrative hyphal tips projecting into the substrate region

In this model, rapid oxygen diffusion occurred on the uppermost layer but not in the subsequent mycelial layer. Knowing this, *A. oryzae* transformant over-expressed *fhbA* gene encoding a flavohaemoglobin has resulted in higher amylase, protease and glucoamylase activities. Meanwhile, by exploring the dogma suggesting that protein secretion occurs at the apical region of advancing hyphal tips, *A. oryzae* mutants with increased branching produced 50% more amylase, 100% more glucoamylase and 90% more protease in wheat kernel SSF (Gonzalez, 2011).

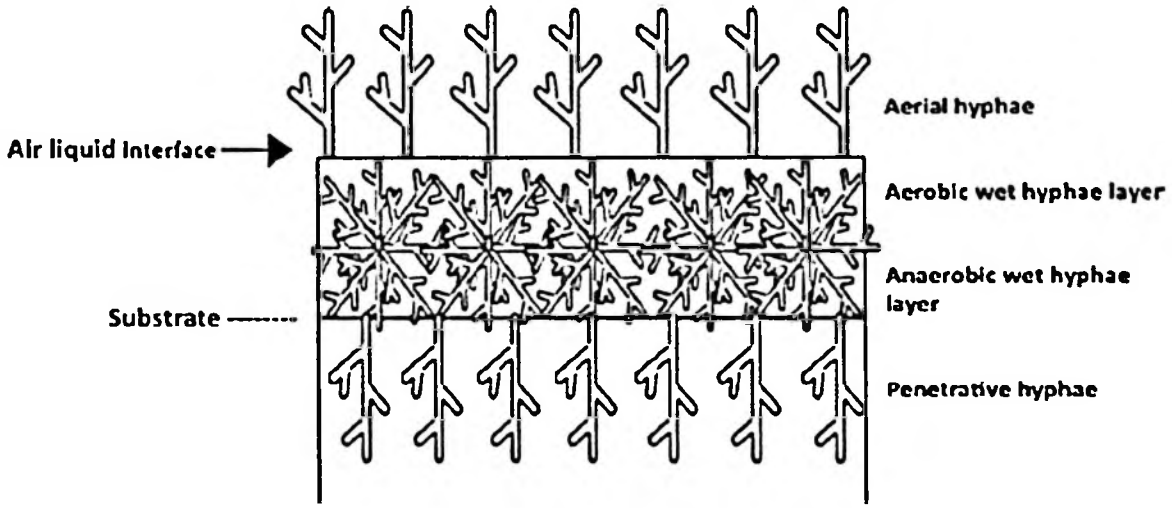

Figure 2.4 Schematic drawing of fungal growth in SSF, showing the three mycelial layers (Adopted from Gonzalez, 2011)

2.3.3 Advantages and Challenges of SSF

Compared to SmF, SSF offers numerous distinctive advantages which includes the economy of space (Kamra and Satyanarayana, 2004), no complex machinery, equipment and control systems (Panagiotou *et al.,* 2003; Topakas *et al.,* 2003), greater compactness of fermentation vessel hence greater product yield (Grajek, 1987; Nighan

and Singh, 1994; Bender *et al.,* 2008), reduced energy demand (Robinson *et al.*, 2001); lower capital and recurring expenditure in industry (Yang *et al.*, 2005); improved downstream processing or product recovery (Ong *et al.,* 2004; Assamoi *et al.,* 2008), absence of foam build-up, improved enzyme stability (Sindhu *et al.,* 2006), lower waste water output and easier control of contamination due to the low moisture content in the system (de Souza *et al.*, 2001; Sanghi *et al.,* 2007). Furthermore, extremely low water activity in SSF could minimize water consumption, and it could be conducted in semi-sterile condition due to restricted growth of contaminating bacteria and fungus.

In the case of enzyme production, it is claimed that the enzymes produced under SSF condition are less prone to substrate inhibition and possessed greater temperature and pH stability. These may due to the reduced water activity of microbes, showed by studies in fungus *A. oryzae,* which accumulates glycerol and arabinitol induced by SSF (Holker and Lenz, 2005). Moreover, the products obtained during SSF process have slightly different or better properties such as more thermo tolerance, higher yield and relatively higher concentration than those obtained in SmF process (Cannel and Moo-Young, 1980; Steinkraus, 1984; Kumar and Lonsane, 1990; Raimbault, 1998; Perez-Guerra *et al.,* 2003). Higher enzyme production has been described for xylanase by *Melanocarpus albomyces* IIS-68 (Jain, 1995), endopolygalacturonase by *Paecilomyces clavisporus* 2A.UMIDA.1 (Souza *et al.,* 2003) and β-galactosidase by *Klyveromyces lactis* (Becerra and Gonzalez, 1996) respectively. In addition, some of the maximum xylanase activities given in the literature are about 1000-4000 U/mL for *T. reesei* and *Schizophyllum commune* in submerged culture and about 20,000 U/g in solid-state culture (Bakir, 2004).

It was reported that the production of α-amylase and amyloglucosidase by *A. niger* was limited/ reduced in SmF compared to SSF (Nandakumar *et al.*, 1999), and that the breakdown of enzymes by contaminating proteases was eight times higher in SmF than in SSF (Viniegra-Gonzalez *et al.,* 2003). The lack of catabolite repression is SSF was also reported by Bakri et al., (2003), whereby xylanase production by *Penicillium canescence* 10-10c was not repressed by high glucose or xylose concentration in SSF. This is also being reported in the production of amylase and tannase by *Bacillus licheniformis* M27 and *A. niger* respectively (Ramesh and Lonsane, 1991; Nabdakumar *et al.,* 1999; Aguilar *et al.,* 2001).

While it is not applicable in SmF, the co-culturing of various fungal species in SSF, by mixing the hypercellulotic mutant with host-specific helper fungus has induced more efficient enzyme system for host cell degradation, which is caused by synergism of individual enzymes, will result in higher volumetric productivity (Gutierrez-Correa *et al.*, 1999). This is being proved by simultaneous saccharification and fermentation that is regulated by joint action of aerobic and facultative anaerobic fungus *via* oxygen manipulation. Nout and Aiddo (2002) have reported that mix cultures in a synergistic way can produce various aromatic components, whereas Fu *et al.,* (2002) have successfully determined 70 volatile compounds in which 29 of them had aroma active properties, when bamboo sprout was processed by undefined mix cultures.

There are a number of desirable products which can only be produced by SSF process. One of them is the production of an active secondary metabolites such as pigments with six different colours ranging from bright yellow to deep red secreted by *Monascus purpureus* during the production of "red rice" (Johns and Stuart, 1991; Juzlava *et al.,*

1996). Another example is the production of fungal spores as biopesticides against plant pathogens such as *Botrytis cinera, Sclerotinia sclerotiorum* (Holker *et al.*, 2004) and *Fusarium oxysporum f.* sp. *Lycopersici* (Pascual *et al.,* 2000). The fungal spores produced under SSF condition are more stable in dry state, more robust and virulent (Viccini *et al.,* 2001). In terms of cost factor, SSF is more favorable over SmF, as the fermentation cost for the production of cellulases was around US$0.20/kg and US$20.00/kg *via* SSF and SmF respectively (Tengerdy, 1996; Wang and Yang, 2007).

On the other hand, efforts are still in progress to validate SSF modeling due to the lack of sufficient process data (Holker and Lenz, 2005). The search for mathematical expression is important to for SSF scale-up as it is use to establish relations between processing parameters, to evaluate and control whole process, making clear depictions of the microbial activity (kinetic pattern and thermodynamic concerns) and solid system (heat and mass transfer) (Singhania *et al.*, 2009). Among the physical and chemical gradients that are built up during the course of SSF, heat generations and water balance will be the most challenging regulating factors. Fungal metabolism results in heat, which is desirable for composting but would probably, destroy the enzymes secreted. To counteract this problem, water evaporation is used and this in turn adding difficulties to the maintenance of water activity in SSF. Neither too high nor too low of moisture content is desirable for SSF, as oxygen penetration will be limited by the former and nutrient accessibility will be limited in the latter scenario, hampering microbial growth. Despite all these, new computer-controlled modeling methods for parameters optimizations and process controlling were developed (Holker and Lenz, 2005; Singhania *et al.*, 2009).