Faculty of Resource Science and Technology

Optimisation of SSF Parameters for Cellulase Production by *Aspergillus niger* via Solid-state Fermentation using Rice Husk as Substrate

Ong Liling

(71266)

Bachelor of Science with Honours

(Resource Biotechnology)

2022

UNIVERSITI MALAYSIA SARAWAK

Grade: ____________

Please tick (√)

Final Year Project Report	✓
Masters	
PhD	

DECLARATION OF ORIGINAL WORK

This declaration is made on the ……29……..day of……June…..2022.

Student's Declaration:

I Ong Liling, 71266, Faculty of Resource Science and Technology hereby declare that the work entitled, Optimisation of SSF Parameters for Cellulase Production by *Aspergillus niger* via Solid-state Fermentation using Rice Husk as Substrate is my original work. I have not copied from any other students' work or from any other sources except where due reference or acknowledgement is made explicitly in the text, nor has any part been written for me by another person.

29/6/2022
Date submitted

Ong Liling (71266)
Name of the student (Matric No.)

Supervisor's Declaration:

I, Rosmawati binti Saat, hereby certify that the work entitled, Optimisation of SSF Parameters for Cellulase Production by *Aspergillus niger* via Solid-state Fermentation using Rice Husk as Substrate was prepared by the above named student, and was submitted to the "FACULTY" as a partial fulfillment for the conferment of Bachelor of Science with Honours (Resource Biotechnology), and the aforementioned work, to the best of my knowledge, is the said student's work

Received for examination by: Dr Rosmawati binti Saat Date: 29 June 2022

I declare this Project/Thesis is classified as (Please tick (√)):

- [ ] **CONFIDENTIAL** (Contains confidential information under the Official Secret Act 1972)*
- [ ] **RESTRICTED** (Contains restricted information as specified by the organisation where research was done)*
- [/] **OPEN ACCESS**

Validation of Project/Thesis

I therefore duly affirmed with free consent and willingness declared that this said Project/Thesis shall be placed officially in the Centre for Academic Information Services with the abide interest and rights as follows:

- This Project/Thesis is the sole legal property of Universiti Malaysia Sarawak (UNIMAS).
- The Centre for Academic Information Services has the lawful right to make copies for the purpose of academic and research only and not for other purpose.
- The Centre for Academic Information Services has the lawful right to digitise the content to for the Local Content Database.
- The Centre for Academic Information Services has the lawful right to make copies of the Project/Thesis for academic exchange between Higher Learning Institute.
- No dispute or any claim shall arise from the student himself / herself neither third party on this Project/Thesis once it becomes sole property of UNIMAS.
- This Project/Thesis or any material, data and information related to it shall not be distributed, published or disclosed to any party by the student except with UNIMAS permission.

Student's signature 29/6/2022 Supervisor's signature: ________________
(29th June 2022)

Current Address:
University of Malaysia, Sarawak, Jalan Datuk Mohammad Musa, 94300 Kota Samarahan, Sarawak

Notes: * If the Project/Thesis is **CONFIDENTIAL** or **RESTRICTED**, please attach together as annexure a letter from the organisation with the period and reasons of confidentiality and restriction.

[The instrument was duly prepared by The Centre for Academic Information Services]

Optimisation of SSF Parameters for Cellulase Production by *Aspergillus niger* via Solid-state Fermentation using Rice Husk as Substrate

Ong Liling (71266)

A project proposal submitted in partial fulfilment of the

Final Year Project 2 (STF3015) course

Supervisor: Dr Rosmawati Saat

Resource Biotechnology Programme

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

2022

DECLARATION

I hereby declare that this thesis is my original work and has not been previously submitted to any institutions. All sources of quotations and citations used in this study have been acknowledged to the authors who own the research journals. The acknowledgement and credits have been with a means of references in this paper.

……………………………………

Ong Liling

Resource Biotechnology Programme

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God for all the blessings throughout my journey in completing this thesis. I am thankful for giving me the knowledge, ability, strength and opportunity to complete this final year project satisfactorily.

Next, I would like to acknowledge and thank my supervisor, Dr Rosmawati Binti Saat, who made this work possible. She was willing to guide and assist me throughout this project study with her patience and knowledge despite how busy she might be. I am sincerely thankful for her support and motivational words as one of her supervisees. Besides that, my sincerest thanks are also dedicated to a postgraduate student Miss Alexandra Cheryl Anak Dennis, for her willingness to guide me whenever I was in need, thanks to you.

I also want to thank all my friends who supported and helped me finish the thesis. My appreciation also extended to all my laboratory mates for constantly sharing knowledge and giving moral support to each other. Last but not least, heartfelt acknowledgement is expressed to my forever love, my family and especially my parents. Without their financial support, guidance, endless love and encouragement, I cannot overcome all the problems and complications along this long journey in my studies. Thank you.

Optimisation of SSF Parameters for Cellulase Production by *Aspergillus niger* via Solid-state Fermentation using Rice Husk as Substrate

Ong Liling

Resource Biotechnology Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak

ABSTRACT

Cellulase enzyme comprises of endoglucanase or carboxymethyl cellulase (CMCase), exoglucanases or cellobiohydrolase and cellobiases or β-D-glucoside glucohydrolase (β-glucosidase). For economical bioconversion of rice husk into cellulase production, it requires high efficient hydrolysis of cellulose to glucose and cellulase via optimisation of solid-state fermentation parameters. This study looks at the optimal initial moisture content, incubation temperature and period for cellulase production by *Aspergillus niger* via SSF using rice husk as substrate. Ground rice husks were pretreated with 2.5 % (w/v) sodium hydroxide solution. *A. niger* was sub-cultured on potato dextrose agar and then incubated at room temperature until fully grown. Solid-state fermentation was carried out with incubation of 5 g of substrate with three plugs of *A. niger* at 25 ℃ – 40 ℃ for two to eight days at 60 % (v/w) to 75 % (v/w) initial moisture content under static condition. Supernatant or crude enzyme extract was successfully obtained and then, the cellulase activity of the crude enzyme was determined using filter paper assay and dinitrosalicylic acid method. Fermented rice husk with *A. niger* recorded the highest cellulase activity at 0.3202 ± 0.0179 U/ml with the optimal condition at 25 ℃, 2 days and 65 % (v/w). These results indicate that rice husk can serve as a cheap and environmentally friendly cellulose source in solving economic issues and enhancing the industrial potential of cellulase production by *A. niger* under solid-state fermentation.

Keywords: *Aspergillus niger*, Cellulase, Rice husk, Solid-state fermentation

ABSTRAK

Enzim selulase terdiri daripada endoglucanase atau carboxymethyl cellulase (CMCase), exoglucanase atau cellobiohydrolase dan cellobiases atau β-D-glucoside glucohydrolase (β-glucosidase). Untuk biokonversi sekam padi yang ekonomik kepada pengeluaran selulase, ia memerlukan hidrolisis selulosa yang tinggi kecekapan kepada glukosa dan selulase melalui pengoptimuman parameter-parameter fermentasi berkeadaan pepejal. Kandungan kelembapan awal, suhu pengeraman dan tempoh yang optimum untuk penghasilan selulase oleh Aspergillus niger *melalui SSF dengan menggunakan sekam padi sebagai substrat dikaji. Sekam padi yang dikisar telah dirawat dengan larutan natrium hidroksida 2.5 % (w/v).* A. niger *disubkultur pada agar kentang dektrose seterusnya dieram pada suhu bilik sehingga dewasa sepenuhnya. Fermentasi berkeadaan pepejal telah dijalankan dengan pengeraman 5 g substrat dengan tiga palam* A. niger *pada 25 ℃ – 40 ℃ selama dua hingga lapan hari dan kandungan kelembapan awal pada* 60 % (v/w) hingga 75 % (v/w) *dalam keadaan statik. Supernatan atau ekstrak enzim mentah telah berjaya diperolehi dan seterusnya, aktiviti selulase enzim mentah tersebut ditentukan melalui kaedah esei kertas penapisan dan asid dinitrosalisilik. Sekam padi yang difermentasi dengan* A. niger *mencatatkan aktiviti selulase enzim paling tinggi iaitu 0.3202 ± 0.0179 U/ml pada keadaan optimum 25 ℃, 2 hari dan 65 % (v/w). Keputusan ini menunjukkan bahawa sekam padi boleh berfungsi sebagai sumber selulosa yang murah dan mesra alam dalam menyelesaikan isu ekonomi sambil meningkatkan potensi industri pengeluaran selulase dengan penggunaan* A. niger *dan fermentasi berkeadaan pepejal.*

Kata kunci: Aspergillus niger, *Selulase, Sekam padi, Fermentasi berkeadaan pepejal*

Table of Contents

List of Tables

List of Figures

List of Abbreviations

A. niger	*Aspergillus niger*
A. flavus	*Aspergillus flavus*
A. ornatus	*Aspergillus ornatus*
A. oryzae	*Aspergillus oryzae*
β-glucosidase	β-D-glucoside glucohydrolase
BGs	β-Glucosidases
BGCs	Biosynthetic gene clusters
CAZymes	Carbohydrate acting enzyme
CBMs	Carbohydrate-binding molecules
CBP	Consolidated bioprocessing
CMC	Carbon methyl cellulose
CMCase	Carboxymethyl cellulase
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats- associated protein 9
ddH_2O	Double distilled water
DNS	Dinitrosalicylic acid
EMC	Existing moisture content

FPA	Filter paper activity
FPase	Filter paper assay
GDP	Gross domestic product
IMC	Initial moisture content
M	Molarity
MB	Megabyte
mg/ml	Microgram per milliliter
ml	Milliliter
mM/ml/min	Millimeter per milliliter per minute
NaOH	Sodium hydroxide
nm	Nanometer
PDA	Potato dextrose agar
OFAT	One-factor-at-a-time
rpm	Revolution per minute
SSF	Solid-state fermentation
SmF	Submerged fermentation
T. reesei	*Trichoderma reesei*

SSL	Self-sufficiency level
S. D.	Standard Deviation
U/ml	Units per milliliter
U/g	Units per gram
U/g DMB	Units per gram of dry mycelial bran
IU/ml	International units per mililiter
v/w	Volume per weight
w/v	Weight per volume
µmol	Micromolar
A_w	Water activity
℃	Degree celcius
%	Percentage
g	Gram
kg	Kilogram
pH	Potential of Hydrogen

CHAPTER 1: INTRODUCTION

Cellulase enzyme comprised of endoglucanase or carboxymethyl cellulase (CMCase), exoglucanases or cellobiohydrolase and cellobiases or β-D-glucoside glucohydrolase (β-glucosidase) has enormous application in the textile, food, pulp and paper industry and animal feed production, thus crucial in the world's industrial enzyme market (Jimat et al., 2015). Since the demand for cellulase production is growing, the emphasis is to produce large amounts and high-quality cellulase production via bioconversion of sustainability and abundant availability at almost zero cost of agro-waste biomass into cellulase by cellulolytic microorganisms through a cost-effective solid-state fermentation (SSF).

Globally, rice (*Oryza sativa*) is the world's second most significant cereal crop sector owing to the daily food intake demand of billions of human beings (Suhot et al., 2021). In 2020, rice and paddy production for Malaysia was 2321636 tonnes which contributed approximately 0.48 million tonnes of rice husk annually (Hazmi et al., 2020; FAOSTAT, 2022). Rice husk consists of 35 % cellulose, 25 % hemicellulose, 20 % lignin and 17 % ash is an abundant lignocellulose biomass agro-waste (Ma'rud et al., 2017). Therefore, rice husks can serve as a cheap and environmentally friendly cellulose source in solving economic issues and enhancing cellulase production of SSF to deal with massive amount of lignocellulose wastes.

According to Anderson et al. (2011), *A. niger* is a fast-growing haploid filamentous fungus with 35 MB in size and eight chromosomes with around 12000 genes in which 57 % of functional genes are used in SSF technology. In addition, *A. niger* is acid-tolerant with a pH range of 1.4 to 9.8, thermo-tolerant between 6 ℃ to 47 ℃, hyphal mode of growth with intense penetration, quick spread or coverage, high tolerance to high osmotic pressure and low water activity (Schuster et al., 2002). Mäkelä et al. (2018) also proved that *A. niger* could efficiently degrade polysaccharides of plant biomass to monomeric sugars, then transport and convert monomeric sugars into energy and biochemical building blocks in catabolic pathways via various extracellular carbohydrate acting enzymes (CAZymes).

During SSF process, a slight change in physiochemical parameters causes significant effects on obtaining favourable microbial growth and the formation of desired products. For example, low water activity influences microbial growth, extends the lag phase, decreases microbes' specific growth rate, resulting in low biomass production (Mitchell et al., 1991). In contrast, high water activity improves bacteria and fungi growth. In 2014, Dutt and Kumar conducted a study on optimisation of cellulase production by *A. niger* (AT-3) and *A. flavus* (AT-2) via SSF using rice straw as substrate with focusing on physiological parameters such as pH and temperature to determine CMCase, FPase, β-glucosidase and fungal protein concentration.

However, there is a lack of approach to fulfil the knowledge gaps regarding economical bioconversion of lignocellulosic waste from rice husk into commercial cellulase production with optimum initial moisture content, incubation period and temperature. Besides, the potential of *A. niger* as low cost, high quality and quantity of lignocellulose degrading enzyme in degrading rice husk into cellulase is still unclear. Therefore, this research focuses on the optimal SSF parameters which are incubation period, incubation temperature and initial moisture content by *A. niger* in producing cellulase using rice husk as substrate.

By using enzyme assays, the optimum initial moisture content, incubation temperature and period can be determined.

Objectives

1. To determine the effectiveness of rise husk as substrate and the potential of *Aspergillus niger* in producing cellulase via SSF.
2. To determine the optimal SSF parameters for *A. niger* in producing high enzyme activity of cellulase by using rice husk as the substrate.

CHAPTER 2: Literature Review

2.1 *Aspergillus niger*

According to de Vries et al. (2017), *A. niger* is a universal filamentous ascomycete fungus under phylum Ascomycota, also commonly defined as sac fungi. A saprotroph with natural habitat in the soil, plant debris, rotting fruit and indoor environment (Upton et al., 2017). Filamentous fungi are the best and used commonly in SSF with specific physiology, enzymological and biochemical properties, especially very optimally active in very low water activity. In addition, *A. niger* is a fast-growing haploid filamentous fungus with 35 MB in size and eight chromosomes with around 12000 genes in which 57 % functional genes (Anderson et al., 2011).

Furthermore, *A. niger* is acid-tolerant within pH 1.4 to 9.8 and thermo-tolerant within 6 ℃ to 47 ℃ (Schuster et al., 2002). With a suitable growth environment, *A. niger* grows and colonises to form vegetative and reproductive hyphae, which have strong power to penetrate solid substrates. Therefore, reproductive hyphae of *A. niger* fungi can utilise nutrients efficiently with quick spread or coverage, high tolerance to and high osmotic and low water activity, thus producing asexually, smooth and colourless conidiophores and dark brown or carbon dark spores (black mould) on the conidial heads (biserite) of reproductive hyphae within the sac (Asci) (Microscope Master, 2021). These carbon dark or dark brown spores protect *A. niger* far away from the sun's radiation to allow thriving in warm areas.

Naturally, *A. niger* can efficiently degrade polysaccharides of plant biomass to monomeric sugars, then transports and convert monomeric sugars into energy and biochemical building blocks in catabolic pathways via various CAZymes (Mäkelä et al., 2018). Throughout regulating transcriptor activators and specific repressors with inducers, *A. niger* is the primary industrial producer enzyme to convert plant-based feedstocks into fermentable sugars (Andersen et al., 2011; Kowalczyk et al., 2014). Furthermore, high transcript levels of CreA and at least partially CreB with glucose transport are necessary for function and stability of CreA to mediate carbon catabolite repression that ensures the preferred carbon source utilisation and optimal match to the available substrate, thus enhancing the repression of gene encoding polysaccharide-degrading enzymes (Ruijter & Visser, 1997; Strauss et al., 1999; Reis et al., 2016).

According to Yu et al. (2021), *A. niger* can isolate and produce various biomolecules as bioactive secondary metabolites by distinct cryptic biosynthetic gene clusters (BGCs). Therefore, *A. niger* is widely applied in waste management, biotransformations, biosynthesis of value-added natural products of nutritional, agrochemical, pharmaceutical and industrial uses, including the production of amylase, acetylesterase, citric acid, fucosidase, proteases, glucosidase, glucose oxidase, phytase, phospholipase, prolyl endopeptidase, mannanase, trehalase, xylanase and extracellular enzymes. According to Duarte and Costaferreira (1994), *A. niger* with endoxylanase isozymes potentially act as industrial bleaching aids to transform lignocellulose in the pulp and paper industry, also degrade phenolic contaminants in wastewater of fermentation broth.

To date, the advancement of the CRISPR–Cas9 system has successfully established *A. niger*'s genome-scale metabolic network to facilitate strain improvement and process optimisation, thus fully exploiting the filamentous fungi industry (Lu et al., 2017). In the future, fine regulation of gene expression, multiple and traceless gene editing of *A. niger* can be further studied to better understand the metabolic model of *A. niger*.

2.2 Cellulase

According to Jimat et al. (2015), most cellulases comprised of endoglucanase or carboxymethyl cellulase (CMCase), exoglucanases or cellobiohydrolase and cellobiases or β-D-glucoside glucohydrolase (β-glucosidase), which can be secreted by protozoans, fungi, bacteria, animals, plants and various microorganisms such as *Aspergillus*, *Cellulomonas*, *Clostridium*, *Trichoderma* and *Thermomonospora* to hydrolyse celluloses. Endoglucanase or CMCase randomly cleaves internal β-1, 4-glucan bonds of cellulose, exposing them for exoglucanases or cellobiohydrolase to hydrolyse nonreducing end of the crystalline cellulosic chain produce cellobiose and cellubiases then further split and convert the disaccharide cellobiose into glucose and completely cellulolysis (Duenas et al., 1995; Schulein, 2000). β-glucosidases absence of CBM hydrolyses the soluble cellobiose and cellodextrins to glucose. In contrast, noncatalytic carbohydrate-binding molecules (CBMs) are functionally unknown at either C- or N-terminus of a catalytic module (Zhang & Zhang, 2013).

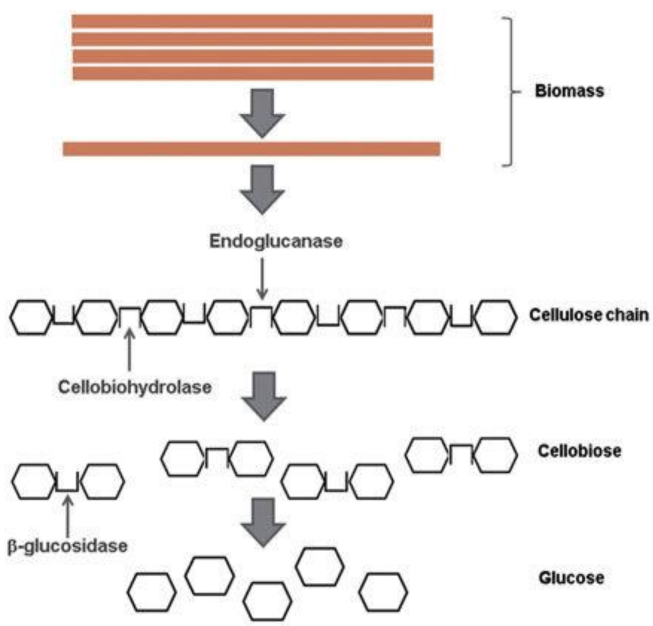

Figure 1. Hydrolysis of cellulose by endoglucanase, cellobiohydrolase and β-Glucosidases (Gomez del Pulgar & Saadeddin, 2014).

There are two strategies of cellulases utilisation that have evolved by cellulolytic microorganisms, which are discrete non-complexed and complexed cellulases to degrade cellulose (Lynd et al., 2005). Commonly, most anaerobic microorganisms produce free cellulases and cellulosomes that attach to the cell surface of microorganisms and may contain CBM attached to a scaffolding protein (Zhang & Zhang, 2013). Moreover, the prominent microorganism employed in cellulase production is shown in Table 1.

Table 1. Major microorganisms are used in cellulase production.

Major group	Microorganism	
	Genus	Species
Fungi	*Aspergillus*	*A. niger*
		A. nidulans
		A. oryzae (recombinant)
	Fusarium	*F. solani*
		F. oxysporum
	Humicola	*H. insolens*
		H. grisea
	Melanocarpus	*M. albomyces*
	Penicillium	*P. brasilianum*
		P. occitanis
		P. decumbans
	Trichoderma	*T. reesei*
		T. longibrachiatum
		T. harzianum
Bacteria	*Acidothermus*	*A. cellulolyticus*
	Bacillus	*Bacillus sp*
		Bacillus subtilis
	Clostridium	*C. acetobutylicum*
		C. thremocellum
	Pseudomonas	*P. cellulosa*
	Rhodothermus	*R. marinus*
Actinomycetes	*Cellulomonas*	*C. fimi*
		C.bioazotea
		C.uda
	Streptomyces	*S. drozdowiczii*
		S. sp
		S. lividans
	Thermononospora	*T. fusca*
		T. curvata

(Sukumaran et al., 2005)

Cellulases are significant in the global industrial enzyme market with a wide application in animal-fed production, food, textile, pulp and paper industries. Cellulases are produced by solid or submerged fermentation with *Trichoderma* and *Aspergillus* species to decrease sugar production costs from lactose to glucose with a bit of sophorose, increase specific activity and better thermostability (Zhang & Zhang, 2013). SmF overrides the repression of cellulase due to the accumulation of reducing sugar (Sukumaran et al., 2005). Therefore, the main technical limitation is increased fermentation times with low productivity. In contrast, SSF is a cost-effective technology to rapidly obtain cellulase production via bioconversion of lignocellulosic biomass employing cellulolytic microorganisms (Pandey, 1992; Pendey et al., 2000; Pendey et al., 2001). According to Tengerdy (1996), the production cost of SSF is 10-fold cheaper than SmF.

High demand for cellulases leads to single-step consolidated bioprocessing (CBP) aids with CBP-enabling microbes, which degrade the hemicellulose and cellulose of plant biomass, then convert the resultant sugars into useful bio-commodities (Lynd et al., 2005; Huang et al., 2013). CBP can increase hydrolysis rate and conversion efficiency, reduce reactor volume, production costs and capital investment compared to dedicated cellulase production (Zhang & Zhang, 2013). In addition, cellulose-adherent cellulolytic microorganisms successfully produce cellulases with nonadherent microbes and contaminations, thus increasing the stability of industrial processes.

2.3 Current and Potential Uses of Rice Husk in Malaysia

According to Suhot et al. (2021), rice (Oryza sativa) is the world's second primary significant cereal crop sector owing to the daily food intake demand. Globally, rice production was 742 million tonnes per year, in which around 148 million tonnes of rice husks were produced, corresponding to 20 % of grain weight (Oliveira et al., 2017). In 2020, rice and paddy production for Malaysia was 2321636 tonnes, of which around 20 % were paddy residues such as rice husk and straw (FAOSTAT, 2022). Since each kg of milled white rice produces around 0.28 kg of rice husk as rice by-products, roughly 0.48 million tonnes of rice husk are produced annually in Malaysia (Hazmi et al., 2020; Rice Knowledge Bank, 2022). Generally, rice husk is a hull that protects seeds or grains with rigid, water-insoluble and abrasive materials with 30 % to 50 % organic carbon and a high level of cellulose–silica structures (Suhot et al., 2021). In addition, rice husk comprises 25 % hemicellulose, 35 % cellulose, 17 % ash and 20 % lignin (Ugheoke & Mamat, 2012; Ma'rud et al., 2017). The proximate analysis and chemical composition of rice husk are shown in Tables 2 and 3, respectively.

Table 2. Proximate analysis of rice husk.

Parameters	%weight
Cellulose	32.67
Hemicellulose	31.68
Lignin	18.81
Ash	11.88
Silica (% of ash)	91.09

(Ma'rud et al., 2017)

Table 3. Chemical composition of rice husk in % of weight (dry basic).

C (%)	H (%)	O (%)	N (%)	S (%)	Ash (%)	HHV (MJ/kg)
40	5	34.8	0.8	0.1	19.5	14.8

(Rice Knowledge Bank, 2022)

Recently, Budget 2022 has been focused on the rice and paddy production to achieve more than 75 % self-sufficiency level (SSL) in 2025 to reduce the country's dependence on rice imports and ensure food security for Malaysians (The Star, 2021). With the increasing demand for rice production, rice husks yield has a significant increase. The burning of agro-wastes releases fumes, ashes and harmful gases to cause air pollution (Athira, Bahurudeen & Appari, 2019). Whilst disposal of agro-wastes by dumping or decomposing naturally in plantation areas is also harmful to the environment pollution and damage to the land (Abu Bakar, Yahya & Neon Gan, 2016). Therefore, effectively convert rice husk into valuable products in forms of energy, economic, technological and environmental balance should be maintained.

Typically, rice husk can be used as extracted silica, biochar or husk itself (Suhot et al., 2021). In addition, rice husk can be utilized as an energy source for steam engines, brick production, rice dryers and gasifiers to power rice mills (Low et al., 2021). High silica level of rice husk ash is a good additive for the concrete and steel industries (Rice Knowledge Bank, 2022). In contrast, the low silica content of rice husk ash can be used as an insulator, activated carbon and soil conditioner. Moreover, rice husk composites are potentially used in the production of automotive, photonics, particleboard, furniture applications and construction materials and furniture applications. Some examples of rice husk usage are shown in Figure 2. Since a whole plant consists of approximately 70 % of the total dry mass of lignocellulose, which comprises lignin, cellulose and hemicellulose, efficient bioconversion of rice husk into glucose and fermentable sugars or other valuable products should be further studied.