

**ISOLATION AND IDENTIFICATION OF STARCH-DEGRADING BACTERIA
FROM STARCH PROCESSING INDUSTRY**

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The dissertation is dedicated to my father Teh Hock Chuan and my mother Ng Siew Beng, for their partnership in the success of my life.

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ABSTRACT

Cassava starch wastewater and sludge that discarded from the cassava starch processing industries contain high amount of starch and organic materials which causes environmental pollution. Starch-degrading bacteria that isolated from starch wastes are believed to have higher rate of starch degradation and production of α -amylase. The aim of this study was to isolate starch-degrading bacteria from the cassava wastewater and sludge. The isolation was done by serial dilution. Total 17 bacterial isolates were isolated from the collected samples. Among the 17 isolates, one isolate from sludge showed amylolytic activity. The isolate is a Gram-positive bacteria with long rod-shaped under microscopic identification. Cassava wastewater was utilized as substrate in batch fermentation to study the hydrogen production, the growth kinetics, the rate of starch utilisation and the production of reducing sugars of the isolate. There was no hydrogen production from the isolate. The specific growth rate in cassava wastewater medium was 0.04876 h^{-1} . The doubling time and number of cell generation were 14.21 hour and 1.69 respectively. The isolate consumed about 88% of starch at the end of the fermentation. The maximum amylase activity and reducing sugars of the isolate were 41.34 U/mL and 3.207 mg/mL . 16S rRNA gene sequencing and phylogenetic analysis indicated that the isolate is in the same clade as *Bacillus cereus*. The isolate was designated *Bacillus* sp. CS9 and the partial 16S rRNA sequence has been deposited into the GenBank with nucleotide accession number MF138129.

ABSTRAK

Air limbah dan enapcemar kanji ubi kayu yang mangandungi jumlah kanji dan bahan-bahan organik yang tinggi dibuang dari pemrosesan kanji ubi kayu telah menyebabkan pencemaran alam sekitar. Pengasingan bakteria yang mampu degradasi kanji daripada kanji sisa dipercayai mempunyai kadar degradasi yang tinggi dan pengeluaran enzim amilase. Tujuan kajian ini adalah untuk menentukan bakteria yang mempunyai kemampuan degradasi kanji. Pemencilan bakteria dilakukan dengan kaedah pencairan bersiri. Sebanyak 17 bakteria yang dipencilkan daripada sampel yang dikumpulkan. Antara 17 bakteria, hanya bakteria CS9 daripada enapcemar menunjukkan aktiviti amilolitik. Berdasarkan pencirian secara mikroskopik, bakteria tersebut merupakan bakteria Gram-positif dalam bentuk batang. Ubi kayu air sisa digunakan sebagai substrat dalam proses penapaian kumpulan untuk mengkaji pengeluaran hidrogen, kinetik pertumbuhan, kadar penggunaan kanji dan pengeluaran gula isolate. Bakteria tersebut tidak mampu mengeluarkan hidrogen. Kadar pertumbuhan isolate adalah 0.04876 h^{-1} . Kali ganda dan generasi sel adalah 14.21 jam dan 1.69 masing-masing. Kira-kira 88% kanji dalam penapaian kumpulan telah digunakan oleh isolate tersebut. Aktiviti amilase maksimum dan pengeluaran gula adalah 41.34 U/mL dan 3.207 mg/mL masing-masing. Kajian 16S rRNA penjujukan dan analisis filogenetik menunjukkan bahawa isolat adalah dalam klad yang sama dengan *Bacillus cereus*. Bakteria ini dinamakan sebagai *Bacillus* sp. CS9 dan separa 16S rRNA penjujukan disertai dengan nombor nukleotida MF138129 dalam GenBank.

TABLE OF CONTENTS

CHAPTER	TITLE	PAGE
	DECLARATION	ii
	DEDICATION	iii
	ACKNOWLEDGEMENT	iv
	ABSTRACT	v
	ABSTRAK	vi
	TABLE OF CONTENTS	vii
	LIST OF TABLES	xii
	LIST OF FIGURES	xiii
	LIST OF ABBREVIATIONS	xv
	LIST OF SYMBOLS	xvii
	LIST OF APPENDICES	xviii
1	INTRODUCTION	1
	1.1 Background of study	1
	1.2 Problem statement/Significance of study	2
	1.3 Objectives	4
	1.4 Scope of study	4
2	LITERATURE REVIEW	5
	2.1 Starch	5
	2.2 Physiochemical properties of starch	7
	2.2.1 Morphological of starch granules	7

2.2.2	Chemical composition of starch	9
2.2.3	Crystalline structure of starch granules	11
2.2.4	Functional properties of starch	12
2.2.4.1	Swelling and solubility	12
2.2.4.2	Gelatinization and retrogradation	13
2.2.4.3	Rheological properties of starch	14
2.3	Cassava starch processing	15
2.3.1	The introduction of cassava	15
2.3.2	Industrial processing of cassava starch	17
2.3.3	Waste generation from cassava starch processing	18
2.4	Fermentative hydrogen production	19
2.4.1	Biochemistry of dark fermentation	20
2.5	Amylases	22
2.5.1	Alpha-amylases (EC 3.2.1.1)	22
2.5.2	Beta-amylases (EC 3.2.1.2)	23
2.5.3	Gamma-amylases (EC 3.2.1.3)	23
2.6	Microbial amylases	24
2.6.1	Bacterial amylases	24
2.6.2	Fungal amylases	28
2.7	Starch wastes as substrates for amylase production	28
2.8	Industrial application of amylases	29
2.8.1	Starch conversion	29
2.8.2	Baking industry	30
2.8.3	Detergent industry	30
2.8.4	Textile industry	31
2.8.5	Paper industry	31

2.8.6	Production of bioethanol	32
3	METHODOLOGY	33
3.1	Sample Collection	33
3.2	Preparation of enriched starch wastewater media	33
3.3	Isolation of bacteria	35
3.4	Phenotypic identification	36
3.4.1	Biochemical test	36
3.4.2	Gram stain	36
3.5	Glycerol bacterial stock	37
3.6	Hydrogen production assay under batch fermentation	37
3.7	Analytic methods	38
3.7.1	Screening of hydrogen production	38
3.7.2	Measurement of growth kinetics	38
3.7.3	Determination of starch utilization	38
3.7.4	Determination of reducing sugars	39
3.7.5	Measurement of microbial biomass	40
3.8	Molecular identification of bacteria	40
3.8.1	Colony polymerase chain reaction (PCR)	40
3.8.2	Preparation of 1% agarose gel	41
3.8.3	Gel electrophoresis	42
3.8.4	Gene Purification	42
3.8.5	Quantification of purified DNA	43
3.9	Bioinformatics analysis	43
3.9.1	Phylogenetic analysis	43
3.9.2	Nucleotide sequence accession number	44

4	RESULTS AND DISCUSSION	45
	4.1 Isolation of bacteria	45
	4.2 Screening for starch-degrading bacteria	52
	4.3 Microscopic identification of isolate CS9	53
	4.4 Molecular identification of isolate CS9	54
	4.4.1 16S rRNA amplification	54
	4.4.2 Quantitative and purification of gene	55
	4.4.3 Phylogenetic analysis	56
	4.5 Screening for biohydrogen production by <i>Bacillus</i> sp. CS9	58
	4.6 Microbial growth and starch utilisation of <i>Bacillus</i> sp. CS9	60
	4.7 Correlation of reducing sugar production and starch utilisation of <i>Bacillus</i> sp. CS9	62
5	CONCLUSION AND RECOMMENDATIONS	67
	5.1 Conclusion	67
	5.2 Recommendations	68
	REFERENCES	69
	Appendices A-G	83-97

LIST OF TABLES

TABLE NO.	TITLE	PAGE
2.1	Characteristics of starch granules from different botanical source. Adapted from Van de Velde and De Ruyter (2002).	8
2.2	Isolation sites of amylase producing <i>Bacillus</i> spp. and other bacteria from the few previous studies	26
3.1	The formulated media composition of the ESWA	34
3.2	Thermal cycling condition for amplification	41
4.1	The morphological characterisation from each isolates from cassava wastewater and sludge	46
4.2	Growth data in term of microbial biomass of <i>Bacillus</i> sp. CS9 and amylase activity at every sampling interval	65
4.3	Summary of kinetic parameters of <i>Bacillus</i> sp. CS9 in the starch wastewater fermentation medium	66

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE
2.1	Structure of (A) amylose and (B) amylopectin. Adapted from Tester <i>et al.</i> (2004).	10
2.2	A- and B-type polymorphic structures of amylose. Adapted from Hsien-Chih & Sarko (1978).	11
2.3	Industrial processing of cassava roots for the extraction of starch (Nandy, Kaul, & Sekhar, 1995)	17
4.1	The positive starch hydrolysis result from CS9 isolate from sludge on ESWA at 30°C for 24 hours.	46
4.2	The Gram stain of isolate CS9. The bacteria stained purple-blue, indicating the isolate was Gram-positive. The bacteria was long rod shape and arranged in chain.	53
4.3	The DNA fragment of isolate CS9 was showed on at position 1500 bp on the lane 2 after gel electrophoresis. Lane 1 was the 1kb DNA ladder as reference.	55
4.4	The measurement of absorbance on the concentration and purity of isolate CS9 DNA. The ratio 260/280 and 260/230 were 1.90 and 2.01 respectively. The concentration of DNA was 67.8 ng/μL.	56
4.5	Phylogenetic relationship of isolate CS9 with its close relatives based on 16S rRNA gene sequencing. Sequences were aligned using ClustalW and the phylogenetic tree was constructed by neighbour-joining method using MEGA 7. <i>Bacillus</i> sp. CS9 (red box) was assigned with nucleotide accession number MF138129 in GenBank. <i>Pseudomonas putida</i> was selected as an outgroup.	57

4.6	The changes of pH in the fermentation medium at every 8 hour time interval. The initial pH at 0 hour was pH 5.5. pH started to drop slightly at 16 hour (early log phase) and the pH increased after 16 hour. Error bars indicate standard deviation.	59
4.7	Growth profile in terms of microbial biomass of <i>Bacillus</i> sp. CS9 and its starch utilisation.	61
4.8	The oxidation-reduction reaction between aldehyde group of glucose and 3,5-dinitrosalicylic acid to free carbonyl group of gluconic acid and 3-amino-5-nitrosalicylic acid.	63
4.9	Reducing sugar production and starch utilisation of <i>Bacillus</i> sp. CS9	64

LIST OF ABBREVIATION

μg	-	Microgram
μL	-	Microlitre
μm	-	Micrometer
ATP	-	Adenosine triphosphate
bp	-	Base pair
BLAST	-	Basic local alignment search tool
$\text{C}_6\text{H}_{12}\text{O}_6$	-	Glucose
CaCl_2	-	Calcium (II) chloride
$\text{CH}_3\text{CH}_2\text{CH}_2\text{COOH}$	-	Butyric acid
CH_3COOH	-	Acetic acid
cm	-	Centimetre
cm^3	-	Centimetre cube
CO_2	-	Carbon dioxide
DNA	-	Deoxyribonucleic acid
dNTPs	-	Deoxynucleotide triphosphate
EtBr	-	Ethidium bromide
<i>et al.</i>	-	And others
ESWA	-	Enriched starch wastewater agar
$\text{FeCl}_3 \cdot 5\text{H}_2\text{O}$	-	Iron (III) chloride pentahydrate
FHL	-	Formate: hydrogen lyase
g	-	Gram
h	-	Hour
H_2	-	Hydrogen
H_2O	-	Water
kb	-	kilobase

K_2HPO_4	-	Dipotassium phosphate
KH_2PO_4	-	Monopotassium phosphate
L	-	Litre
m^3	-	Meter cube
M	-	Molarity
mg	-	Milligram
$MgSO_4 \cdot 7H_2O$	-	Magnesium sulfate heptahydrate
min	-	Minute
mL	-	Millilitre
mol	-	Mole
NADH	-	Nicotiamide adenine dinucleotide
n	-	Number of cell generation
ng	-	Nanogram
NH_4NO_3	-	Ammonium nitrate
nm	-	nanometer
DNS	-	3,5-dinitrosalicylic acid
PCR	-	Polymerase Chain Reaction
PFL	-	Pyruvate: formate lyase
PFOR	-	Pyruvate: ferredoxin oxidoreductase
rpm	-	Revolutions per minute
rRNA	-	Ribosomal ribonucleic acid
s	-	Second
sp.	-	Species
t_d	-	Doubling time
ton	-	Tonne
U	-	Enzyme unit
UV	-	Ultraviolet

LIST OF SYMBOLS

°C	-	Degree Celsius
%	-	Percentage
∞	-	Infinity
α	-	Alpha
β	-	Beta
®	-	Registered trade-mark
™	-	Trade mark

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A	Determination of starch concentration	83
B	Determination of glucose concentration	85
C	The negative starch hydrolysis results from other isolates except isolate CS9	88
D	16S rRNA gene sequence of <i>Bacillus</i> sp. CS9 in FASTA format	94
E	Dry cell weight of isolate CS9 in the fermentation medium at different incubation time	95
F	Starch utilisation of isolate CS9 in the fermentation medium at different incubation time	96
G	Reducing sugar concentration of isolate CS9 in the fermentation medium at different incubation time	97

CHAPTER 1

INTRODUCTION

1.1 Background of study

Starch is a natural occurring polysaccharide which composed of D-glucan consisting of amylose and amylopectin. The heterogeneity and versatility of starch from agricultural commodities benefits in the food and non-food industries. The extraction and processing of starch from root crops is one of the most significant agro-industry in Asia (Fuglie, Oates, & Xie, 2006). During the processing of starch, large amount of starch wastewater and solid wastes are generated and released into the environment from starch processing industry. Disposal of the starch wastes freely contaminants nearby water sources and the discharged of wastewater and solid contaminants into drainage system causing water blockage and releasing a foul odor (Kolawole, 2014).

Researchers have been investigated on the treatment of starch effluents based on biological processes. The carbohydrate wastes could be utilised for the wastewater treatment to produce high value added product such as α -amylase production. Hydrolysis of starch can be done by starch-degrading microorganisms naturally.

Enzymatic degradation of starch to smaller oligosaccharides such as glucose and maltose by fermentation is usually carried out by α -amylase (Kumar & Das, 2000). Isolation and screening of novel α -amylases producing microorganisms from starch wastes or starchy environment are believed to have higher rate of starch consumption and to produce higher amount of α -amylase (Gopinath *et al.*, 2017). Amylases are the most important industry enzymes constituting about 25% of the enzyme market. The enzyme from microbial sources is preferable because of its plasticity and vast availability (Rana, Walia, & Gaur, 2013). Microbial amylases have more special characteristics than animal and plant enzymes. The enzymes are more stable and active at their optimum temperature. Furthermore, microorganisms can be cultured in large amount in a short time by fermentation having their own biochemical diversity. Microbial enzymes content are easier to manipulate by genetic engineering to derive desired rate of enzyme production (Gurung, Ray, Bose, & Rai, 2013). Starch-degrading enzymes has a great application in biotechnology ranging from food and beverages, fermentation, textile to paper industries due to the great microbial diversity in the environment, optimal growth requirements, simple and cost effective production, accessibility and efficient.

1.2 Problem Statement / Significance of study

Most of the studies focused on the production of microbial α -amylase from soluble starch because soluble starch is easier and faster to degrade by microorganisms. However, soluble starch is more expensive to be used in commercial application and this will increase the expenses on the production of α -amylase. In addition, the reports on the biohydrogen production from facultative anaerobes using starch wastes are somehow restricted. Based on the reviewed literature by Elsharnouby, Hafez, Nakhla, and El Naggar (2013), there was only 20% studies have focused on sustainable feedstock (real waste) for biohydrogen

production. About 80% of the studies on biohydrogen production were done by using pure carbohydrates as substrates.

In Malaysia, there are many cassava processing industries that produce cassava chips. At the same time, the wastes generated from the industries consist of high concentrations of starch and other organic compounds that lead to a detrimental effect of soil and water pollutions. The high load of wastes that being discarded into the rivers, ponds and drainages not only causing blockage of water, but also effect aquatic life. To promote on the sustainability of this industry, the carbohydrate wastes should be handled properly. Hence, starch loaded wastes are converted into value-added product using enzymatic degradation of the starch wastes by starch-degrading bacteria to produce α -amylase.

Moreover, the measurement of hydrogen yield with respect to the bacterial growth kinetics and the consumption of starch were mostly done on mixed consortia. Since the starch waste is hydrolysed to simple sugars for the biohydrogen production, amylolytic enzymes are possible be secreted by the hydrogen producing bacteria to degrade the starch. In other word, there is a possibility certain hydrogen producing bacteria possess the excellent potential to secrete amylase instead of only produce hydrogen gas. Majority of the studies on biohydrogen production do not further investigate the α - amylase production from mixed consortia or pure strain.

This study was focused on the isolation of starch-utilising bacteria from cassava wastewater and sludge. Starch-utilising bacteria were then subjected to screening of starch hydrolysis, amylase activity and potency production of biohydrogen and the sample was identified by molecular technique. The purpose of the study was to highlight the potency of starch-degrading bacteria producing α -amylase at an ambient temperature and in a lower pH using cassava starch wastes as substrate. It was believed that the starch-degrading bacteria that isolated from starchy environment has the better starch utilisation ability and also the potency to produce high amount of α -amylase.

1.3 Objectives of study

- To isolate a novel starch degrading bacteria strain from cassava starch wastewater and sludge
- To identify the novel starch-degrading bacteria strain by molecular techniques
- To investigate the potential ability of the isolated bacteria in the hydrogen production
- To correlate the concentration of starch utilisation with respect to the production of reducing sugar and microbial growth

1.4 Scope of the study

This study focused on the isolation of starch degrading bacteria from cassava wastewater and sludge in an enriched starch wastewater agar. This isolates were screened for its ability to produce extracellular α -amylase using starch hydrolysis test. The positive isolate was further screened for hydrogen production by gas chromatography (GC) equipped with thermal conductivity detector (TCD). Growth kinetics, starch utilisation ability and α -amylase activity of the positive isolate were analysed. Lastly, the isolate was identified by 16S rRNA sequencing and subjected to GenBank.

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