BACILLUS AMYLOLIQUEFACIENS UMAS 1002: GENE CHARACTERIZATION AND EXPRESSION IN ESCHERICHIA COLI

Muhammad Suhaib Mat Hussin

Master of Science
(Molecular Biology)
2013
ALPHA-AMYLASE AND ENDOGLUCANASE FROM *Bacillus amyloliquefaciens* UMAS 1002: GENE CHARACTERIZATION AND EXPRESSION IN *Escherichia coli*

Muhammad Suhaib Bin Mat Hussin

A thesis submitted
In fulfilment of the requirements for the degree of Master Science
(Molecular Biology)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2013
APPROVAL SHEET

Name of candidate: Muhammad Suhaib Mat Hussin

Title of thesis: Alpha-amylase and Endoglucanase from *Bacillus amyloliquefaciens* UMAS 1002: Gene Characterization and Expression in *Escherichia coli*

........................................................

(Associate Prof. Dr. Mohd Hasnain Md Hussain)

Supervisor

Department of Molecular Biology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

Date:

........................................................

(Dr. Awang Ahmad Sallchin Awang Hussaini)

Co-Supervisor

Department of Plant Science and Environmental Ecology

Faculty of Resource Science and Technology

Universiti Malaysia Sarawak

Date:
DECLARATION

I, hereby, declare that no portion of the work referred to in this thesis has submitted in support of an application for another degree of qualification of this to any other university or institution of higher learning.

(MUHAMMAD SUHAIB BIN MAT HUSSIN)

Matric No: 08021346
ACKNOWLEDGEMENT

First and foremost, I would to thank Allah the Almighty for the innumerable gifts that He has granted me, for guiding me along in completing this work and for giving me an opportunity to undergo higher education. I hope and wish that this education I have acquired would be beneficial to many in whatever form possible.

I would like to express my total appreciation to the people for their support and for guiding me along in completing this thesis. Very special thanks to my supervisor, Dr Mohd Hasnain Hussain who has been the ideal supervisor. His sage advice, insightful criticisms, and patient encouragement aided the completion of this research in innumerable ways. I am also grateful to my co-supervisor, Dr. Awang Ahmad Sallehin Awang Husaini for his support and guidance from the beginning of this research until its completion.

I would like to express my utmost appreciation to my dearest parents and sibling for their encouragement throughout my whole educational life. I could have not completed my studies without their continuous and immeasurable support.

Finally, I would like to thank all my friends particularly my lab mates and housemates for their help and friendship, who were supportive and patients toward me.
ABSTRACT

α-Amylase and endoglucanase produced by Bacillus amyloliquefaciens strain UMAS 1002, isolated from sago waste was characterized. The genes amyE and engA that encodes α-amylase and endoglucanase respectively was isolated by Polymerase Chain Reaction (PCR) and its DNA sequence was determined. The 1980 bp amyE gene corresponding to 660 amino acids showed 99% homology to the sequence encoding α-amylase from B. subtilis X-23 (GI: 3298505). The α-amylase sequence of UMAS 1002 differs from that of B. subtilis X-23 by 5 amino acids. Whereas the 1497 bp engA gene encoding 499 amino acids residue protein were 100% similar to endoglucanase reported by previous research on the same strain. The molecular weight of α-amylase and endoglucanase from UMAS 1002 were estimated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to be 68 kDa and 42 kDa respectively. Subsequently, amyE and engA genes was individually cloned and expressed in E. coli cells using pET100 expression system. The recombinant α-amylase and endoglucanase extracellular activities were determined and compared to those of the native-type enzymes. Both recombinant proteins from E. coli hosts showed optimum pH and temperature similar to α-amylase and endoglucanase from the native strain. α-amylase was highly active at pH 6 and temperature 60°C. Whereas, the highest activity of endoglucanase was obtained at pH 5 and temperature 70°C.
ABSTRAK

ALPHA-AMYLASE DAN ENDOGLUCANASE DARI *Bacillus amyloliquefaciens* UMAS 1002: PENCIRIAN GEN DAN PENGEKSPRESAN DALAM *Escherichia coli*

α-Amylase dan endoglucanase dari *Bacillus amyloliquefaciens* strain UMAS 1002, dipencilkan daripada sisa sagu telah dicirikan. Gen *amyE* dan *engA* yang masing-masing mengkodkan untuk α-amylase dan endoglucanase telah dipencilkan melalui teknik Tindakbalas-Berantai-Polimerase (PCR) dan jujukan DNA gen-gen tersebut telah ditentukan. Gen *amyE* sepanjang 1980 pasang bes (bp) yang selaras kepada 660 asid amino menunjukan 99% homologi kepada jujukan yang mengkod untuk α-amylase dari *B. subtilis* X-23 (GI: 3298505). Hanya terdapat perbezaan 5 asid amino antara jujukan α-amylase UMAS 1002 dengan jujukan *B. subtilis* X-23. Sementara itu, gen *engA* sepanjang 1497 bp yang mengkod protein dengan 499 asid amino adalah 100% serupa seperti endoglucanase yang dilaporkan dalam kajian sebelum ini melibatkan strain yang sama. Menggunakan gel elektroforesis poliakrilamid sodium dodecil sulfat (SDS-PAGE), dianggarkan berat molekular α-amylase dan endoglucanase dari UMAS 1002 adalah masing-masing seberat 68 kDa dan 42 kDa. Gen *amyE* dan *engA* kemudiannya telah diklon dan diekspres dalam sel *E. coli* menggunakan sistem ekspresi pET100. Aktiviti luar selular α-amylase dan endoglucanase rekombinan telah ditentukan dan dibandingkan dengan enzim asal. Kedua-dua enzim rekombinan dari *E. coli* menunjukan pH dan suhu optimum sama seperti α-amylase dan endoglucanase dari bakteria asal. α-amylase optimum pada pH 6 dan suhu 60°C manakala endoglucanase optimum pada pH 5 dan suhu 70°C.
# CONTENT

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE</td>
<td>i</td>
<td></td>
</tr>
<tr>
<td>APPROVAL SHEET</td>
<td>ii</td>
<td></td>
</tr>
<tr>
<td>DECLARATION</td>
<td>iii</td>
<td></td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>iv</td>
<td></td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
<td></td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>vi</td>
<td></td>
</tr>
<tr>
<td>TABLE AT</td>
<td>vii</td>
<td></td>
</tr>
</tbody>
</table>

## CHAPTER I
**INTRODUCTION**

1.1 Introduction

1.2 Objectives

## CHAPTER II
**LITERATURE REVIEW**

2.1 Starch

2.2 Cellulose

2.3 Amylases

2.3.1 Endo and exoamylase

2.3.2 Debranching enzymes

2.3.3 Transferases

2.3.4 α-Amylase family

2.4 Cellulase

2.5 Microbial degradation of starch

vii
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.6</td>
<td>Taxonomy of <em>Bacillus amyloliquefaciens</em></td>
<td>19</td>
</tr>
<tr>
<td>2.6.1</td>
<td>Characteristics of <em>Bacillus amyloliquefaciens</em></td>
<td>20</td>
</tr>
<tr>
<td>2.6.2</td>
<td>Habitat</td>
<td>20</td>
</tr>
<tr>
<td>2.6.3</td>
<td>Sago starch degrading <em>Bacillus amyloliquefaciens</em> UMAS 1002</td>
<td>21</td>
</tr>
<tr>
<td>2.7</td>
<td>Application of $\alpha$-amylase</td>
<td>23</td>
</tr>
<tr>
<td>2.7.1</td>
<td>Bread and baking industry and as an antistaling agent</td>
<td>23</td>
</tr>
<tr>
<td>2.7.2</td>
<td>Starch liquefaction and saccharification</td>
<td>24</td>
</tr>
<tr>
<td>2.7.3</td>
<td>Textile desizing</td>
<td>24</td>
</tr>
<tr>
<td>2.7.4</td>
<td>Paper industry</td>
<td>25</td>
</tr>
<tr>
<td>2.7.5</td>
<td>Detergent applications</td>
<td>25</td>
</tr>
<tr>
<td>2.8</td>
<td>Applications of cellulases</td>
<td>26</td>
</tr>
<tr>
<td>2.9</td>
<td>Genetic engineering</td>
<td>27</td>
</tr>
</tbody>
</table>

**CHAPTER III MATERIALS AND METHODS**

<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Materials</td>
<td>29</td>
</tr>
<tr>
<td>3.1.1</td>
<td>Luria Bertani (LB) broth</td>
<td>29</td>
</tr>
<tr>
<td>3.1.2</td>
<td>Luria Bertani (LB) agar</td>
<td>29</td>
</tr>
<tr>
<td>3.1.3</td>
<td>Minimum agar</td>
<td>29</td>
</tr>
<tr>
<td>3.1.4</td>
<td>TE buffer</td>
<td>30</td>
</tr>
<tr>
<td>3.1.5</td>
<td>Dinitrosalicylic Acid Reagent (DNS)</td>
<td>30</td>
</tr>
<tr>
<td>3.1.6</td>
<td>Sodium phosphate buffer</td>
<td>30</td>
</tr>
<tr>
<td>3.1.7</td>
<td>Bradford solution</td>
<td>31</td>
</tr>
<tr>
<td>3.1.8</td>
<td>Bacteriological growth media and chemicals</td>
<td>31</td>
</tr>
<tr>
<td>3.1.9</td>
<td>Cloning media and chemicals</td>
<td>31</td>
</tr>
<tr>
<td>3.1.10</td>
<td>Antibiotics</td>
<td>31</td>
</tr>
</tbody>
</table>
3.1.11 PCR primers

3.2 Methods

3.2.1 Bacterial growth and maintenance

3.2.2 Determination of *Bacillus amyloliquefaciens* UMAS 1002 morphological characteristics

3.2.3 Detection of extra-cellular amylase and cellulase activity

3.2.4 DNA manipulations

3.2.4.1 Extraction of total genomic DNA from *B. amyloliquefaciens* UMAS 1002

3.2.5 Cloning preparation

3.2.5.1 Polymerase Chain Reaction (PCR)

3.2.5.2 Agarose gel electrophoresis

3.2.5.3 Preparation of *E. coli* competent cell

3.2.5.4 Transformation and detection of recombinant clones

3.2.5.5 Extraction of DNA plasmid from transformed *E. coli*

3.2.5.6 Cloning and expressions of recombinant clones

3.2.5.7 Nucleotide and amino acid sequences computer analysis

3.2.6 SDS-PAGE protein analysis

3.2.6.1 Preparation of SDS-PAGE Gel

3.2.6.2 Preparation of protein sample

3.2.6.3 SDS-PAGE gel electrophoresis

3.2.7 Enzymes assay

3.2.7.1 Fermentation experimental design

3.2.7.2 Extraction of crude enzyme

3.2.7.3 Temperature optimization
3.2.7.4 pH optimization

3.2.7.5 Dinitrosalicylic Acid (DNS) enzymes assay

3.2.7.5 Calculation of amylase and cellulase activity

3.2.8 Protein assay

CHAPTER IV RESULTS

4.1 Determination of Bacillus amyloliquefaciens UMAS 1002 morphological characteristics

4.2 Detection of B. amyloliquefaciens UMAS 1002 amylase and cellulase extracellular activity

4.3 B. amyloliquefaicens UMAS 1002 total genomic DNA

4.4 Isolation of amylase and cellulase genes from B. amyloliquefaciens UMAS 1002

4.4.1 Nucleotide sequence analysis of α-amylase gene candidate

4.4.2 Nucleotide sequence analysis of engA gene

4.5 Cloning and expression of B. amyloliquefaciens UMAS 1002 amyE and engA genes in E. coli BL21

4.6 Screening of amylolytic and cellulolytic E. coli transformants

4.7 SDS-PAGE protein analysis

4.8 Enzyme analysis

4.8.1 Effect of temperature on α-amylase and cellulase activity

4.8.2 Effect of pH on α-amylase and cellulase activity

4.9 Protein assay
CHAPTER V  DISCUSSION

5.1 Determination of Bacillus amyloliquefaciens UMAS 1002 morphological characteristics 93

5.2 B. amyloliquefaciens UMAS 1002 α-amylase gene (amyE) 93

5.3 α-Amylase from B. amyloliquefaciens UMAS 1002 98

5.4 B. amyloliquefaciens UMAS 1002 endoglucanase gene (engA) 101

5.5 Endoglucanase from B. amyloliquefaciens UMAS 1002 104

5.6 Expression of amyE and engA genes in Escherichia coli strains 106

CHAPTER VI  CONCLUSION 111

REFERENCES 113

APPENDIX

A  Glucose standard curve for reducing sugar determination 132

B  Bradford assay standard curve for protein concentration determination 133

C  B. amyloliquefaciens UMAS 1002 and recombinant E. coli amylase and cellulase enzymatic assay 134

D  B. amyloliquefaciens UMAS 1002 and recombinant E. coli Bradford assay 142
LIST OF FIGURES AND TABLES

Figures

Figure 1.1 Chemical formula of amylose in plant biomass 9
Figure 1.2 Chemical formulae of amylopectin in plant biomass 10
Figure 1.3 Chemical formulae of cellulose in plant biomass 12
Figure 3.1 The cloning strategy for REumas-A and REumas-C using plasmids pET100 43
Figure 4.1 *B. amyloliquefaciens* UMAS 1002 colonies on LB plate and MSA plate 52
Figure 4.2 Extracellular α-amylase and cellulase activity test of *B. amyloliquefaciens* UMAS 1002 53-54
Figure 4.3 Agarose gel (1%) electrophoresis profiles of amplified *B. amyloliquefaciens* UMAS 1002 α-amylase gene. 56
Figure 4.4 Agarose gel (1%) electrophoresis profiles of amplified *B. amyloliquefaciens* UMAS 1002 endoglucanase gene 57
Figure 4.5 Nucleotide sequence of α-amylase gene and deduced amino acid sequence 60-61
Figure 4.6 Topological alignment of α-amylase from *B. amyloliquefaciens* UMAS 1002, *B. subtilis* X-23, *B. subtilis* N7, and *B. licheniformis* 66-67
Figure 4.7 Stereo view of the predicted structure of AmyE protein 68
Figure 4.8 The domain organisation of α-amylase enzyme 70
Figure 4.9 Nucleotide sequence of endo-β-1,4-glucanase gene and deduced amino acid sequences 73-74
Figure 4.10 Topological alignment of endoglucanase from *B. amyloliquefaciens* UMAS 1002, Alkalophilic *Bacillus* Sp., *B. agaradherans*, and *Bacillus* sp. Ksm-635

Figure 4.11 ApET and CpET primer generated PCR product DNA gel electrophoresis

Figure 4.12 REumas-A extracellular α-amylase plate staining test

Figure 4.13 REumas-C extracellular endoglucanase plate staining test

Figure 4.14 α-Amylase SDS PAGE gel electrophoresis

Figure 4.15 Endoglucanase SDS PAGE gel electrophoresis

Figure 4.16 Effect of temperature on *B. amyloliquefaciens* UMAS 1002 and REumas-A α-amylase activity

Figure 4.17 Effect of temperature on *B. amyloliquefaciens* UMAS 1002 and REumas-A α-amylase stability

Figure 4.18 Effect of temperature on *B. amyloliquefaciens* UMAS 1002 and REumas-C cellulase activity

Figure 4.19 Effect of temperature on *B. amyloliquefaciens* UMAS 1002 and REumas-C cellulase stability

Figure 4.20 Effect of pH on *B. amyloliquefaciens* UMAS 1002 and REumas-A α-amylase activity

Figure 4.21 Effect of pH on *B. amyloliquefaciens* UMAS 1002 and REumas-A α-amylase stability

Figure 4.22 Effect of pH on *B. amyloliquefaciens* UMAS 1002 and REumas-C cellulase activity

Figure 4.23 Effect of pH on *B. amyloliquefaciens* UMAS 1002 and REumas-C cellulase stability
Tables

3.1 List of bacterial strains used in this study 32
3.2 List of plasmids used in this study 32-33
3.3 List of primers used for the *B. amyliquefaciens* UMAS 1002 α-amylase and cellulase genes cloning 33
3.4 PCR reaction mixture of 25 µl volume reaction 37
3.5 PCR reaction temperature used for the *B. amyliquefaciens* UMAS 1002 α-amylase and cellulase gene amplification 38
3.6 SDS-PAGE resolving gel mixture of 10 mL final volume 46
3.7 SDS-PAGE stacking gel mixture of 4 mL final volume 46
4.1 Amylase gene candidate (designated pAMY S1) 14 open reading frame presumed to express functioning protein 59
4.2 Comparison of predicted amino acid sequence of *amyE* from *B. amyliquefaciens* strain UMAS 1002 with those of other *Bacillus* strains 62
4.3 Comparison of nucleotide of the cloned PCR fragment to previously published data for *B. amyliquefaciens* and *B. subtilis* 62
4.4 Deduced amino acid composition of cloned *amyE* gene product 63
4.5 Comparison of features of predicted AmyE amino-terminal signal peptide of *B. amyliquefaciens* strain UMAS 1002 with those from the genus of *Bacillus* 65
4.6 Calcium ion sites for *B. subtilis* and corresponded *B. amyliquefaciens* UMAS 1002 71
4.7 Deduced amino acid composition of cloned *amyE* gene product 74
4.8 Comparison of predicted amino acid sequence of engA from

*B. amyloliquefaciens* strain UMAS 1002 with those of other *Bacillus* strains

4.7 Comparison of features of predicted EngA amino-terminal signal peptide of

*B. amyloliquefaciens* strain UMAS 1002 with those from the genus of *Bacillus*
Starch is among the most abundant polysaccharide on earth and a very important source of energy for most organisms. However, to transform starch into a usable energy source, it needs to be hydrolyzed to its monomer, i.e. glucose and the enzymes responsible for this action are the starch-degrading enzymes. Most of the starch-degrading enzymes belong to the α-amylase family. Sago palm are abundantly found in coastal areas of Sarawak, which covers about 13% of the total peat land of the state (Bujang & Ahmad, 2000). According to the previous studies, each single sago plantation is expected to yield 25 tons of sago starch every year. These starch are the main source for the production of syrups, fructose, monosodium glutamate and other simple sugars which are mainly used in the food industry (Bujang et al., 2001). The final waste product in the extraction of starch from sago palm is the starchy fibrous pith residue. Most of the time, this sago wastes are washed off into drains or nearby streams or deposited in the factory's compound due to lack of effective utilization, which could cause serious environmental pollution (Aziz, 2002). Thus ways to minimise environmental pollution from sago waste and finding ways to utilize sago wastes were needed. An attractive and efficient solution is through a biotechnological approach in which microbial strains are employed to degrade the sago waste for production of reducing sugars or this can be fermented to value added products. In a related research, the maximum glucose production by hydrolysis of sago waste using commercial enzymes is being studied.
The economics of production of glucose from sago waste depends on many factors such as its availability, purity and pre-treatment cost. Thus by applying amylase producing microbial strain to the fermentation system in replace of the commercial amylase currently used, the cost to convert sago waste to glucose can be reduced.

Glucose is found in nature in large quantities, primarily in polymeric state as cellulose (β-1-4 linked) and as starch and glycogen (both α-1-4 linked). Cellulose and starch are produced predominantly in higher plants, and glycogen is the main sugar storage polymer in animals (Vihinen & Mantsala, 1989). In the kingdom Plantae, starch is the major carbohydrate reserve in plant tubers and seed endosperm where it is found as granules (Oakes et al., 1991). Starch is made up of amyllopectin and amylose. Amylopectin is a soluble polysaccharide and highly branched polymer of glucose found in plants. The other component is amylose which is a linear polymer consisting of glucose. Starch typically containing several million amyllopectin molecules accompanied by a much larger number of smaller amylose molecules. Whereas cellulose are the main components of primary and secondary cell walls constituting majority of the biomass of hardwoods and herbaceous plants. Each cellulose molecule is an unbranched chain consisting of 1000–1 million D-glucose units, linked together with β-1,4 glycosidic bonds. Enormous amounts of glucose are therefore available to organisms that can hydrolyse glucose polymers efficiently. To exploit the energy and carbon available in cellulose, organisms such as fungi and bacteria produce mixtures of synergistically acting cellulases (Beguin & Anbert, 1993). In animal species, most omnivores and herbivores including human do not produce enzymes that can hydrolyse glucose β-1,4 linkages.
Of the starch-hydrolysing enzymes, the \( \alpha \)-amylases (EC 3.2.1.1) are of special importance as they are responsible for the solubilisation of starch. Starch consists of two glucose polymers: amylose, which is exclusively \( \alpha \)-1-4 linked, and amylopectin which, in addition to the \( \alpha \)-1-4 linkages found in amylose, also contains many \( \alpha \)-1-6 branch points (Guzman-Maldanado & Paredes-Lopez, 1995). The \( \alpha \)-amylases catalyse the hydrolysis of internal \( \alpha \)-1-4 glycosidic linkages and are thus ideally suited to breaking a starch polymer into smaller fragments. They are found in both eubacteria and eukaryota and have a large number of different substrate specificities as well as a huge variation in both temperature and pH optima. The bacterial and fungal \( \alpha \)-amylases, and especially the enzymes from the *Bacillus* species, have found widespread use in industrial processes because of their efficient expression systems have made them attractive for such purposes (Svensson & Sogaard, 1992). Amylases have diverse application in a wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical and sugar industries (Pandey et al., 2000). Among carbohydrolases, amylase is currently enjoying the largest market share of industrial enzymes representing 30% of the world enzyme production (Van-der-Maarel et al., 2002). This is followed by cellulases that are currently hold the second largest market share for industrial enzyme worldwide.

In general, cellulase refers to a class of enzymes that catalyze the hydrolysis of 1-4-\( \beta \)-D glycosidic linkages in cellulose, lichenin and cereal \( \beta \)-D-glucans. And in the most familiar case of cellulase activity, the enzyme complex breaks down cellulose to \( \beta \)-glucose. This type of cellulase is produced mainly by symbiotic bacteria in the ruminating chambers of herbivores. Aside from ruminants, most animals including humans do not produce cellulase in their bodies, and are therefore unable to use most of the energy contained in plant material. According to Henrissat and Bairoch (1993) cellulase enzymes complexes generally consist of
three components: endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase or cellobio-hydrolase (EC 3.2.1.91), and β-glucosidase (EC 3.2.1.21). Cellulases are increasingly being used for a large variety of industrial purposes and consequently substantial effort has gone into their cloning and expression. The main potential applications are in food, animal feed, textile, fuel and chemical industries. Other areas of application include the paper and pulp industry, medical/pharmaceutical industry, waste management and pollution treatment (Pandey et al., 2010). Hydrolysis of cellulosic materials has been intensively investigated since the 1970's and the two most commonly used methods are acid hydrolysis and enzymatic hydrolysis (Sun & Cheng, 2002). Compared with acid hydrolysis, enzymatic hydrolysis has a number of advantages such as a high yield of pure reducing sugars, low environmental impact, and mild reaction conditions (Pan et al., 2005). Since fungi produce large amount of extracellular cellulases, they have been widely studied and utilized for industrial applications. At the same time, there are limited studies on cellulase characterization from bacteria (Jong, 2002) and genetic engineering of these genes offer enormous potential applications in biotechnology and industry.

Gram-positive bacteria of the genus *Bacillus* are important sources of industrial enzymes such as amylase, cellulases and proteases (Bessler et al., 2003). Much of the interest in these bacteria arises from their ability to secrete important enzymes at relatively high concentration (Fogarty and Kelly, 1990). Thus, they are assumed to play a significant role in the biological cycles of carbon and nitrogen. Among the genus *Bacillus*, *B. amyloliquefaciens* and *B. licheniformis* are the two species used most frequently in the commercial production of α-amylase (Pandey et al., 2000). In general, bacterial α-amylase has been classified into two types; one is saccharifying and the other liquefying soluble starch. Production of enzymatic starch hydrolyzates is made by two-steps action of amyloytic enzymes on
gelatinized starch granules at the beginning by liquefying enzymes and next by saccharifying enzymes. α-Amylase of *B. amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus* belong to the latter type.

Researchers in UNIMAS discovered one strain of *Bacillus amyloliquefaciens*, named UMAS 1002 which has the capability to degrade starch as well as cellulose (Apun et al., 2000). Jong, (2002) have isolated and characterized an endoglucanase gene (engA) from the strain that affirmed its cellulolytic capabilities. Subsequently, the engA was cloned and expressed in *Escherichia coli*. Although there are many research being done in isolating and purifying amylase produced by *Bacillus amyloliquefaciens* UMAS 1002 but there is no published information on detailed characterization of the enzyme. Thus, the aim for this research is to characterise the enzyme that is involved in the amylolytic properties of UMAS 1002 strain. In addition, gene manipulation of the amylase gene present a great opportunity that need to be explored. Gene’s manipulations for such enzymes are valuable for application in industrial biotechnology.

The advances in molecular biology, genetics and biochemistry during the past four decades have led to an enormous development in the field of biotechnology. Studies with *E. coli* have played a major role in these developments, and the bacterium has been in the forefront of many technological advances. The α-amylase gene has been expressed from various *Bacillus* species to *E. coli* for hyperproduction of amylase. A few foreign gene products have also been reported to be secreted into the growth medium by *E. coli*. Hmidet et al. (2008) has reported that the α-amylase from a recently identified *B. licheniformis* NH1 isolated from waste water has been cloned and expressed in *E. coli*. Other research on microbial fermentation using native microorganisms indicates that the secretion of the
enzyme can be affected by the carbon catabolite repression regulatory mechanism that is known to reduce enzyme production with the accumulation of the end product. This was supported by Laoide et al. (1989) findings of B. licheniformis α-amylase was affected to catabolite repression by glucose. This microbial short-term can be solve via genetic engineering by transforming the target gene into inducable expression host such as E. coli BL21. The sago waste can be converted into value-added products such as simple sugars using enzymatic reaction, thus bring benefits to the agriculture and the biotechnology industry.
1.2 Objectives

Amylolytic and cellulolytic *B. amyloliquefaciens* UMAS 1002, was isolated from sago waste in Sarawak and earlier studies have shown that it was able to hydrolyse sago waste into reducing sugars in a minimal fermentation medium (Apun et al. 2000). Both amylolytic and cellulolytic properties enable the bacteria strain to utilise sago waste as its carbon source. It was also shown that *B. amyloliquefaciens* UMAS 1002 produced high levels of amylase and cellulase which can hydrolyse the starch and cellulose components of the sago waste. The starch was broken into malto-oligosaccharide, maltose and glucose, while the cellulose was converted into cello-oligosaccharide, cellobiose and glucose. These simple sugars can be fermented to value added products. The research work described in this thesis focused on the characterisation and expression of amylolytic and cellulolytic property of the strain UMAS 1002. There were three main objectives:

1. To isolate amylase gene from *B. amyloliquefaciens* UMAS 1002 using polymerase chain reaction (PCR)-based method.

2. Characterization of *B. amyloliquefaciens* UMAS 1002 amylase and the cellulase genes and the enzymes properties using cloning method.

3. Expression and characterization of the amylase and cellulase genes in *Escherichia coli*.
CHAPTER II

LITERATURE REVIEW

2.1 Starch

Starch containing crops form an important part of the human diet and a large proportion of the food consumed by the world's population originates from them. Besides the use of the starch containing plant parts directly as a food source, starch is harvested and used as such or chemically or enzymatically processed into a variety of different products such as starch hydrolysates, glucose syrups, fructose, starch, maltodextrin derivatives, or cyclodextrins (Pandey et al., 2000). In spite of the large number of plants able to produce starch, only a few plants are important for industrial starch processing.

Plants synthesize starch as a result of photosynthesis, the process during which energy from the sunlight is converted into chemical energy. Starch is synthesized in plastids found in leaves as a storage compound for respiration during dark periods. It is also synthesized in amyloplasts found in tubers, seeds, and roots as a long-term storage compound. In these latter organelles, large amounts of starch accumulate as water-insoluble granules. The shape and diameter of these granules depend on the botanical origin. Starch is a polymer of glucose linked to one another through the C1 oxygen, known as the glycosidic bond. This glycosidic bond is stable at high pH but hydrolyzes at low pH. At the end of the polymeric chain, a latent aldehyde group is present. This group is known as the reducing end.