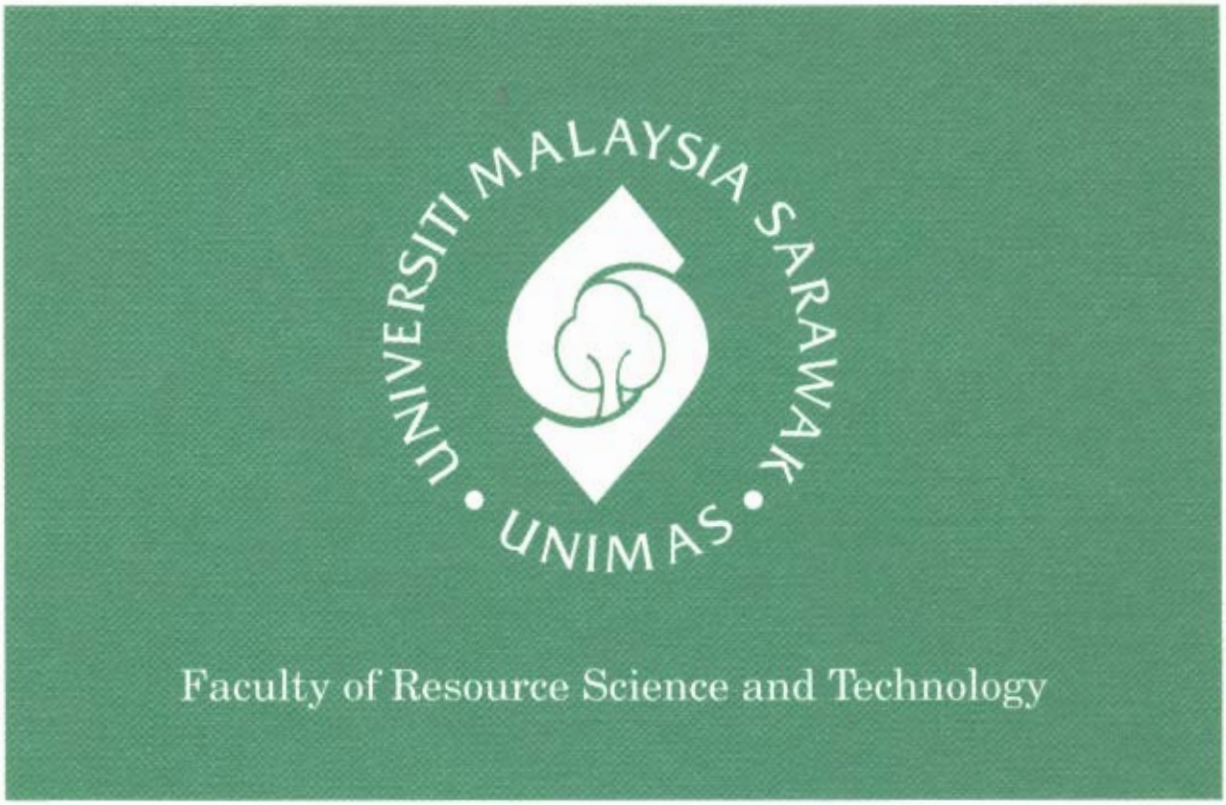

CHARACTERIZATION OF AMYLASE GENE FROM *BACILLUS AMYLOLIQUEFACIENS*

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Bachelor of Science with Honours
(Resource Biotechnology)
2005

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Characterization Of Amylase Gene From *Bacillus Amyloliquefaciens*

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ABSTRACT

An attempt of this study is to identify and characterize amylase gene of *Bacillus amyloliquefaciens*. By understanding the genes this will be essential in order to manipulate them in the nearest future especially in Sago industries. Using lysozyme, an enzymatic lysis method to extract the genomic DNA, it gave a satisfactory result when observed in 1% Agarose Gel Electrophoresis. The molecular analysis involves two different methods; Polymerase Chain Reaction (PCR) and partial digestion of the genomic DNA. In former procedure, desired bands were gel excised and introduced into the ligation reaction and then transformed into *E.coli* XL-1 Blue. After transformation, white colonies where observed on the LB-Agar Plates. The isolated plasmid from the white colonies was treated with restriction enzyme, *Eco*RI. However, confirmation test failed to detect the presence of inserted DNA fragment. For the latter procedure, genomic DNA was partially digest using *Hin*dIII and 2.5 Kbp fragment was selected. Yet the DNA could not be cloned due to time constraint.

Keywords: *Bacillus amyloliquefaciens*, amylolytic, amylase.

ABSTRAK

Percubaan kajian ini adalah untuk mengesan and mengcirikan gen amylase dari bakteria Bacillus amyloliquefaciens. Dengan memahami dan memanipulasikan gen- gen ini, adalah merupakan satu kepentingan dalam masa hadapan terutama dalam industri Sagu.
*Melalui kaedah lysozyme, iaitu cara penglysisan melalui enzim, genom DNA telah dapat diperolehi dengan jayanya bila diperhatikan dalam elektroforesis gel agarose sebanyak 1%. Analisis molekular melibatkan dua cara iaitu, tindak balas rantai polimer (PCR) dan pemotongan tertentu genom DNA. Kaedah yang pertama, band yang diingini telah di potong dan diperkenalkan kepada E.coli XL-1 Blue selepas proses ligasi. Selepas transformasi itu, terdapat beberapa coloni putih diperhatikan pada agar LB. Plasmid tadi telah dirawat dengan enzim Eco*R1. *Tetapi ujian pengesan menunjukkan tiada fragmen yang dihendaki hadir. Untuk kaedah kedua, genom DNA yang telah di potong secara rambang mengunakan Hin*dIII *dan saiz fragmen 2.5 Kbp telah dipilih. Langkah pengklonan tidak dapat disempurnakan kerana kesuntukan masa.*

Kata kunci: Bacillus amyloliquefaciens, amilolitik, amylase.

1.0 INTRODUCTION

Sago is considered to be one of the most abundant plant product and major source of energy in the human diet (Bujang *et al.*, 2001). Natural sago stands covers millions of hectares that stretch over Malaysia, Indonesia, Thailand and other island in the South Pacific Region (Flores, 2000). Sarawak produces 50% out of 90% production of sago starch from sago palm that grows well in swampy lowlands with minimal care. Basically, there are two main species of sago palm; the thorny (*Metroxylon rumphii*) and the thornless (*Mertroxylon sagu*) (Jeffery, 1986). Sago starch is an important industrial where it can be developed for diverse production especially in the manufacture of sodium glutamate. It also can be applied in local food industries like glucose, high fructose syrup, making noodle, caramel, sago pearl, crackers, bread thickeners, stabilizers and texturisers. Other industries involve are paper, textiles, plastic, alcohol, adhesive and paper glue (Bujang *et al.*, 2000).

Sago pith waste or sago *hampas* is one of the waste product produce in sago processing, where due to the study, it shows that sago pith waste contain mainly starch (41.7% to 65.0%), fiber (14%), protein, sugar and fair amount of mineral (Wina *et al.*, 1986). Dried *hampas* also contained a high proportion of starch of fermentable carbohydrate, up to 66% on average. Starch is a primary storage polysaccharides found in higher plants. There are 2 components of starch; amylose and amylopectin. Amylose is a linear polymer composed of α(1-4)-linked glucose units. It lengths are quiet heterogenous but in average of 1,000 glucose residue. Amylopectin is branched polymer of glucose with α (1-4) linkage along the main backbone and α (1-6) linkages forming the branch points.

According to Doelle, (1998) and RunChang *et al.* (1999), this residual waste is commonly found in a bulk and dumped at the sago factory and usually it has no commercial uses apart from being utilized as animal feed mainly for pig and poultry (Yeong and Ali,

1982), as soil conditioner (Bintoro and Sianapar, 1993), particle board (Haryanto *et al.*, 1991) and mushroom cultivation (Vikineswary, 1997). However, by utilizing microbial degradation method, sago *hampas* can be converted to more valuable product or commercial uses. Starch–degrading enzymes have different action pattern and have been characterized by a number of different criteria. There are; endoamylase that cleaves internal α (1-4) or α (1-6) linkages and exoamylase that cleave α (1-4) glucosidic linkages starting from the free non-reducing ends and continue down the chain.

Here the study was conducted to characterize and sequence the amylase gene from *B.amyloliquefaciens*. This includes characterizing the types, size of the nucleotides sequence, configuration of alpha or beta, pH optimum, thermal stability and ion calcium requirement. Thus, applying the knowledge of genetic expression of this bacterium for commercial use. It is also to continue the effort of the previous study, therefore this project describes the work on isolation and characterization of amylase gene from this bacterium involving 2 methods; Polymerase Chain Reaction using degenerated primers and partial digestion of genomic DNA of *B.amyloliquefaciens* including genetic and molecular studies such as isolation of genomic DNA, restriction digestion, ligation, transformation, cloning and Polymerase Chain Reaction (PCR).

2.0 LITERATURE REVIEW.

2.1 *Bacillus Amyloliquefaciens.*

B.amyloliquefaciens is an aerobic Gram-positive bacterium. This is indicated by blue-violet appearance when gram stain procedure is being applied. It also rod shape, motile, catalase positive and form endospore that usually form an oval shaped spore in the center. The production of endospore is subsequently adopted as a key characteristic in the classification and identification of members of the genes (Harwood *et al.*, 1990). Usually this bacterium inhibit in soils or rotting plant materials.

A strain of *Bacillus spp* has been successfully screened and isolated; *Bacillus amyloliquefaciens* UMAS 1002 (Apun *et al.*, 2000). This bacterium is capable of degrading both cellulose and starch from the sago pith waste because it contained amylolytic and celluloytic enzyme properties. Most of *Bacillus spp* are non pathogenic (except *B.anthracis*). It has a history of safe use in both food and industry and recognized as safe by US Food and Drug Administration (Arbige *et al.*, 1993). Besides that, this bacterium is easy to manipulate by genetics, good secretors of proteins and metabolites and simple to cultivate. The members of these genera are known to secrete a large amount of extra cellular enzymes; amylase and protease. *B.amyloliquefaciens* secretes high levels of α-amylase and protease (Ingle *et al.*, 1976). However, amylase enzyme is the main focus in this study.

2.2 Amylase Enzyme.

Enzymes are proteins that function as organic catalysts in cells (Thiel *et al.*, 1993). One of the important is amylase; starch degrading enzyme. It is an endoamylases that hydrolyses internal α-1,4-linkages in amylose and amylopectin producing linear branched saccharides with various lengths. The enzyme, amylase can be divided into two groups, first exoamylases; glucoamylases and β-amylases and second is endoamylases; α-amylases and pullulanase. Most enzymes are produced in large scale and are commercially available especially in syrups industries. For example in 1992, market values for amylases used for above application exceed $100 M (Ferrari *et al.*, 1993). Therefore, the ability to exploit the genetics of *B.amyloliquefaciens* makes the organism amenable to commercial use with the knowledge of genetics expression. Therefore the characterization of amylase gene is now being studied moleculary in *B.amyloliquefaciens* UMAS 1002.

3.0 OBJECTIVES

The main aim of this study is to gain amylase gene. Therefore, the objectives are as follow:

1) To extract amylase genes from *B.amyloliquefaciens* and amplify the gene after cloning in host vector.
2) To carried out sequencing and characterization of the amylase gene of *B. amyloliquefaciens*.
3) To clone amylase gene isolated from *B.amyloliquefaciens* into expression vector for enzyme analysis.

4.0 MATERIALS METHODS

4.1 Confirmation Test.

4.1.1 Media.

The confirmation on amylolytic activity was done using biochemical test; starch hydrolysis test. The bacterium, *B.amyloliquefaciens* from the stock culture that grown in Luria Broth (LB) was sub-cultured into another media that contain 2g agar, 0.2g yeast extract, 0.1g KH_2PO_4, 0.5g $MgSO_4$ and 0.5g soluble starch in 100ml of distilled water (Apun *et al.*, 2000). Then the culture was incubated at 30 ± 2^0C for overnight. To visualize the hydrolysis zones, the plate was flooded with an aqueous solution of Lugol's iodine, which the degradation of starch resulted in the formation of clear zone around the colonies (Hyun and Zeikus, 1985).

4.2 Molecular Analysis.

4.2.1 METHOD I: Designing Primers For Polymerase Chain Reaction (PCR).

4.2.1.1 Genomic Extraction.

A 15 ml fresh overnight cultured in McCartney bottle was centrifuged at 13,000 rpm (KUBOTA 7820) for 15 min to collect the cell pellets. Then the cell pellets was resuspended with 5ml of TE Buffer. The cell was frozen overnight in –20^0C freezer. The next day, an amount of 0.5ml 250mM Tris [pH 8.0] and 10mg/ml of Lysozyme were added to the frozen suspension and was thaw at room temperature on ice. Next, 1ml Lysis Buffer was added and placed in 50^0C water bath with gentle shaking for 1 hour. Approximately, 6ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 13,000rpm for 15 min. The DNA aqueous, the top layer was transferred into a new bottle by avoiding the interface. 0.1 volume of 3M Natrium acetate (NaOAC) and 2 volumes of cold 95% ethanol was added

and mixed. The suspension was centrifuged at 13,00 rpm for 15 min and the DNA pellet was taken out. The pellet was air-dried and dissolved in 2ml TE Buffer. Next, RNase A (10mg/ml) was added and the DNA sample was concentrated by incubates the samples at 37^0C for 10 min. After that, 0.1 volumes of Natrium acetate and 2 volumes of ice-cold absolute ethanol was added, mixed gently and incubated at -20^0C for 1 hour. The sample was centrifuged at 13,000rpm for 15 min. Next, 70% of ethanol were added and centrifuged again as before. The supernatant was discarded and the DNA pellet was let to dry before 50µl of TE Buffer was added. Finally, 5µl of genomic DNA was analyzed in 1% of agarose gel electrophoresis.

4.2.1.2 Degenerated Primers.

The generated primers were constructed base on the sequences of amylase gene from three different species of *Bacillus*. They are *B. licheniformis*, *B. stearothermophilus* and *B.amyloliquefaciens*. These amino acids were chosen because of the high homology sequence. According to Yuuki *et al.*, 1985, the amino acid sequences of *B. licheniformis* α-amylase had 65.4% and 80.3% homology with *B. stearothermophilus* and *B. amyloliquefaciens* α-amylases, respectively. A computer program aligned the sequence so those highly conserved regions are apparent. Each of the primer was ranged in between 18-24 nucleotides long. The 4-5 nucleotides at the 3'end of each primer (forward and reverse) must match all two or three of the *Bacillus* sequence exactly. However if mismatch happen throughout the rest of the primers, the primers were designed to match the mismatch so that the sequence are matched. The melting temperature (Tm) of each primer (the dissociation temperature of the primers) within the range of 52^0C – 62^0C . Therefore Tm estimated as Tm = 4^0(G+C) + 2^0(A+T). The percentage of G+C within each of the primer should be about 50% where the G+C content is approximately the same for each of the primers.

4.2.1.3 Polymerase Chain Reactions (PCR).

The PCR reaction had been performed using DNA as a template in Peltire Thermal Cycler (PTC–200 DNA Engine Version 4.0). Two sets of primer consist of forward and reverse primer were used to fish out the amylase genes from *B.amyloliquefaciens*. The first set was *AmSp1* and *AmSp2* (degenerated primer) while the second set of primer was *Amy1* and *Amy2* (degenerated primer), refer to Appendix B. The PCR reagents were obtained from Promega and the mixtures were as follow.

Table 1: The composition of PCR reaction mixture for primer sets *AmSp1/AmSp2* and *Amy1/Amy2*.

Reagents	**Primers *AmSp1/AmSp2***	**Primers *Amy1/Amy2***
10X PCR Buffer without magnesium	2.5µl	2.5µl
10mM dNTPs	0.5µl	0.5µl
25mM $MgCl_2$	4.5µl	4.5µl
25pmol/µl of each primers	1.0µl	1.0µl
Taq DNA Polymerase (5u/µl)	0.5µl	0.25µl/0.5µl
DNA template	2.0µl	2.0µl
Sterilized ultra pure water	13.0µl	13.25µl/13.0µl
Total volume	**25µl**	**25µl**

The parameters for PCR method was indicated by the table below.

i) ***AmSp1/AmSp2* primers**

Table 2: The parameters for PCR method for *AmSp1/AmSp2* primers.

Steps		**Temperature (°C)**	**Time (min)**
Step 1	Initial Denaturation	94	3
Step 2	Denaturation	94	1
Step 3	Annealing	53	1
Step 4	Elongation	72	2
Step 5	Step return to step 2 for 35 cycles.		
Step 6	Final Extention	72	5

ii) *Amy1/Amy2* primers

Table 3: The parameters for PCR method for *Amy1/Amy2* primers.

Steps		**Temperature (°C)**	**Time (min)**
Step 1	Initial Denaturation	94	2
Step 2	Denaturation	94	1
Step 3	Annealing	50	11/2
Step 4	Elongation	72	2
Step 5	Step return to step 2 for 35 cycles.		
Step 6	Final Extention	72	5

Finally, 5µl of PCR product has been analyzed in 1% agarose gel electrophoresis that contains 1µl/ml of Ethidium Bromide.

4.2.1.4 DNA Extraction From Agarose Gel.

DNA Extraction Kit K513 Fermentas was used to extract the DNA from agarose. The methods were undertaken following manufacturer's recommendations.

4.2.1.5 Transformation And Ligation Product Into *E.coli*

The PCR fragment was ligated to cloning vector, pGEM-TEasy with the total reaction as follow.

Table 4: Reaction mixture for ligation process.

Reagent	Volume
2X Rapid Ligation Buffer, T4 DNA Ligase	5.0µl
pGEM-T Easy Vector (50ng)	1.0µl
PCR product	3.0µl
T4 DNA Ligase	1.0µl
Deionized Water	-
Total	**10.0µl**

Then, the ligation product was transformed into *E. coli* XL-1 Blue. The transformation reaction was spread over LB- Ampicillin plates with isopropyl-β-D-thiogalastopyranoside (IPTG) (25 mg/ml water) and X-Gal. Next the plate was incubated overnight at 37^0C to observe white colonies. The existences of white colonies indicate that the bacteria carry recombinant plasmid. The colonies were then growth in LB-Amp.

4.2.1.6 Alkaline Lysis Plasmid Miniprep.

A 1.5 ml of cell overnight cultured of *E. coli* XL-1 Blue that contain insert of PCR product in pGEM-TEasy was transferred into an Eppendorf tube and centrifuged at 13,000 rpm (Centrifuge 5415 C) for 1 min to collect the cell pellets. The supernatant was discarded and 1.5ml from the same culture was added to the tube. The tube was centrifuged again and the supernatant was discarded. Next, 100µl of iced-cold Solution I (GTE solution) was added and mixed gently to dissolve the pellet. The suspension was incubated on ice for 10 min. Following incubation, 200µl of fresh prepared Solution II (Lysis solution-do not exceed 5 min incubation) and 150µl of ice-cold Solution III (Potassium acetate, acetic acid and sterile water) was added and mixed gently. Again, the suspension was incubated on ice for 10min and centrifuged at 13,000rpm for 5 min at 4^0C. Then, the upper layer of supernatant was transferred to fresh tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The mixture was mixed gently and was centrifuged at 13,000rpm for 2 min at 4^0C. Again, the upper layer of supernatant was transferred to fresh tube. After that, 2 volume of absolute ethanol was added and the tube was centrifuged at 13,000rpm for 2 min at 4^0C and then the supernatant was discarded. The pellet was washed with 500µl of ice-cold 70% (v/v) ethanol and centrifuged at 13,000rpm for 2 min at 4^0C. Then the supernatant was discarded and air-dry the pellet. Finally, the pellet was resuspended in 30µl of TE Buffer pH 8.0 containing RNase A (10µg/ml). A 5µl of the mixture then were analyzed in 1% agarose gel electrophoresis.

4.2.1.7 Restriction Digestion Of Plasmid.

A total volume of 10.0µl restriction enzyme digest mixture was prepared. The mixture contained.

Table 5: The composition of restriction digest of plasmid that contain desire fragment of PCR product

Reagents	**Volume**
Plasmid (contain desire insert)	5.0µl
10X Buffer	1.0µl
Bovine Serum Albumin Acetylated (BSA)	0.1µl
Enzyme *Eco*RI	0.5µl
Distilled deionized water (ddH_2O)	3.4µl
Total Volume	**10.0µl**

The mixture was mixed by gently pippetting and incubated at 37^0C for 4 hour. The enzyme had been inactivated by adding 4 µl of 6X Loading Dye to the mixture after incubating. Finally, 5µl of this mixture was analyzed in 1% of agarose gel electrophoresis. The pattern and length was compared and checked to the early PCR fragment to confirm the desired fragment whether it has been cloned into the host vector before the fragment is send for sequencing analysis.

4.2.2 METHOD I: Partial Digestion And Cloning Of Chromosomal DNA Into *E.coli.*

4.2.2.1 Genomic DNA Extraction.

The methodology is the same as method described in **4.2.1.1.**

4.2.2.2 Partial Digestion Of Genomic DNA

An analytical scale of restriction enzyme; *Hin*dIII was performed with the reagent obtained from Promega. The restriction mixture was prepared on ice as follow.

Table 6: The composition of the partial restriction digest enzyme mixture with *Hin*dIII enzyme.

Reagents	**Volume**
10X Buffer	12.0µl
Extracted DNA	93.6µl
Distilled deionized water (ddH_2O)	-
Bovine Serum Albumin Acetylated (BSA)	2.4µl
Enzyme *Hin*dIII	12.0µl
Total	**120µl**

The tube was incubated at 37^0C and for every 15min; 10µl of the mixture was pipetted out into new fresh Eppendorf tube. Each tube therefore contained 10µl for each period of time: 0 min, 1 min, 30 min, 45 min, 60 min, 75 min, 90 min, 105 min, 120 min, 150 min, 180 min and overnight incubation. Then, the enzyme was inactivated by adding 4µl of 6X Loading Dye to the mixture. Lastly, 5µl of this mixture from each tube were analyzed in 1%

agarose gel electrophoresis to observe desire cut of fragment in the approximately between ~2.0Kbp-1.0Kbp.

4.2.2.3 Calcium Chloride ($CaCl_2$) Bacterial Competent Cells Preparation.

E. coli XL-1 Blue [rec A1 end A1 gyr A 96 thi-1 hsd R17 sup E44 rel A1 lac [F' pro AB lac $I^qZ\nabla$ M15 Tn5 (Tet)] was obtained from stock culture and inoculated into 5ml of Luria Broth (LB) before incubated overnight at 37°C in shaking at 200rpm. The next day, 5ml of the culture was added into 50 ml fresh LB Broth and incubated at 37°C for ~2 hours with shaking at 200 pm to give an OD_{600} reading reach about 0.5. The culture was then cooled on ice for 10-20 min and centrifuged at 3500rpm at 4°C for 5 min. After centrifugation, the supernatant was discarded and the cells were washed by gently resuspending them in 25ml iced –cold 100mM $CaCl_2$. The cells were kept on ice for 10 min. 2. ml of cold, sterile 100mM $CaCl_2$ was added and the cells were further centrifuged at the same condition. To prepare the stocks, 20% (v/v) pure glycerol were added to cell suspension and mixed well. Aliquots (200µl) was transferred into Eppendorf tubes and then snap-frozen in liquid nitrogen before being stored at –80°C freezer. Other *E. coli* strain competent cell, BL-21 [(DE 3) p *Lys* S strain F' *omp* T *hsd* S_B (r^{-B} M^{-B}) *gal dcm* (DE 3) pLys] was also prepared using the same method.

4.2.2.4 Alkaline Lysis Plasmid Miniprep.

E.coli XL-1 Blue that contains plasmid vector pET -41a-c (+) after transformation was inoculated into 5ml of Luria Broth (LB) and incubated overnight at 37°C. The next day, 1.5ml of culture was pipetted into Eppendorf tube. The cells were pelleted and the supernatant was discarded. Next, 100µl of iced-cold Solution I (GTE solution) was added and mixed gently to

dissolve the pellet. The suspension was incubated on ice for 10 min. Following incubation, 200µl of fresh prepared Solution II (Lysis solution-do not exceed 5min incubation) and 150µl of ice-cold Solution III (Potassium acetate, acetic acid and sterile water) was added and mixed gently. Again, the suspension incubated on ice for 10 min and centrifuged at 13,000rpm for 5 min at 4^0C. Then, the upper layer of supernatant was transferred to fresh tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The mixture was mixed gently and was centrifuged at 13,000rpm for 2 min at 4^0C. Again, the upper layer of supernatant was transferred to fresh tube. After that, 2 volume of absolute ethanol was added and the tube was centrifuged at 13,000rpm for 2 min at 4^0C and then the supernatant was discarded. The pellet was washed with 500µl of ice-cold 70% (v/v) ethanol and centrifuged at 13,000rpm for 2 min at 4^0C. Then the supernatant was discarded and air-dry the pellet. Finally, the pellet was resuspended in 30µl of TE Buffer pH 8.0 containing RNase A (10µg/ml). A 5µl of the mixture then were analyzed in 1% agarose gel electrophoresis. Then the vector was treated with the same enzyme that used to cut genomic DNA of *B. amyloliquefaciens* to produce sticky ends.

4.2.2.5 Cloning Partially Digested Fragment From *B.amyloliquefaciens*.

The vector fragment of pET -41a-c (+) was ligated to the partial digestion of the DNA with the desired size fragment by using T4 DNA Ligase. The restriction mixture was prepared as follow.

Table 7: The ligation composition of the chromosomal DNA and plasmid DNA.

Reagents	**Volume**
Digested Chromosomal DNA	8µl
Digested Plasmid DNA	8µl
10X Ligase Buffer	2µl
T4 DNA Ligase	2µl
Total	**20µl**

Then it was transformed into *E.coli* BL-21. The ligation product was spread over agar plates that contain LB, soluble starch with isopropyl-β-D-thiogalactopyranoside (IPTG) (25mg/ml water). Next, the plate was incubated overnight at 37^0C. Clear zone visualized around the colonies after flooding the plates with Lugol's iodine shows the degradation of starch.

5.0 RESULTS AND DISCUSSION.

5.1 Confirmation Test.

The result of biochemical test showed that the bacterial stock was confirmed *B. amyloliquefaciens*. This is because the bacterium colonies that appeared as white creamy colonies, circular shape on Luria Bertani plate agar that is the same as detected from present study to detect and confirmed that the bacterium was B. amyloliquefaciens. This was also supported by observation of halo or clear area when the plate was flooded with Lugol's iodine in minimal media containing soluble starch. The ability to degrade soluble starch is the detection of amylolytic activity.

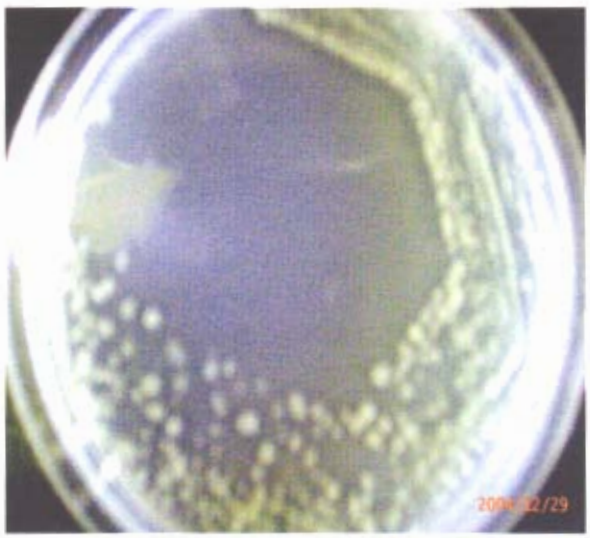

Figure 1: Colonies of *B.amyloliquefaciens* on minimal media with soluble starch.

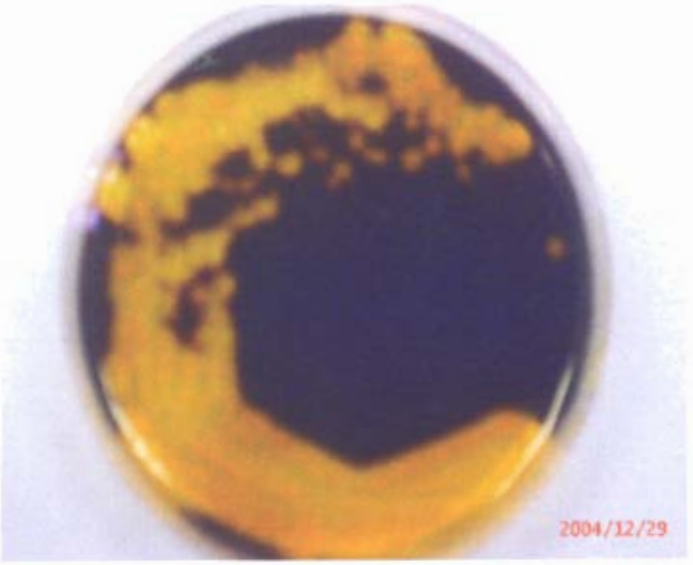

Figure 2: Observation of halo or clear zone that indicates the bacterium undergoes amylolytic activity when the minimal media with soluble starch was flooded with Lugol's iodine.

5.2 Molecular Analysis.

The molecular analysis technique was initiated by isolating the genomic DNA from *B. amyloliquefaciens*, the organism of interest and a good quality of DNA is important to give an effective molecular result. The DNA isolation for this bacterial species was relatively tough because as a gram-positive bacterium, *B. amyloliquefaciens* have a cell envelope that is multi-layered in structure. The layered consist of the protoplast membrane and proteinacious outer layer that called the S-Layer, in some species (Sleytr and Messner, 1983). Furthermore, it has been detected that not all method is suitable to isolate a high molecular weight of DNA from this *Bacillus sp* which require a cell rupture at the first stage. Therefore enzymic lysis by using lysozyme was the best method that gives a satisfactory result as a fine bright band were observed under visualization by electrophoresis using 1% agarose (**Figure 3**).

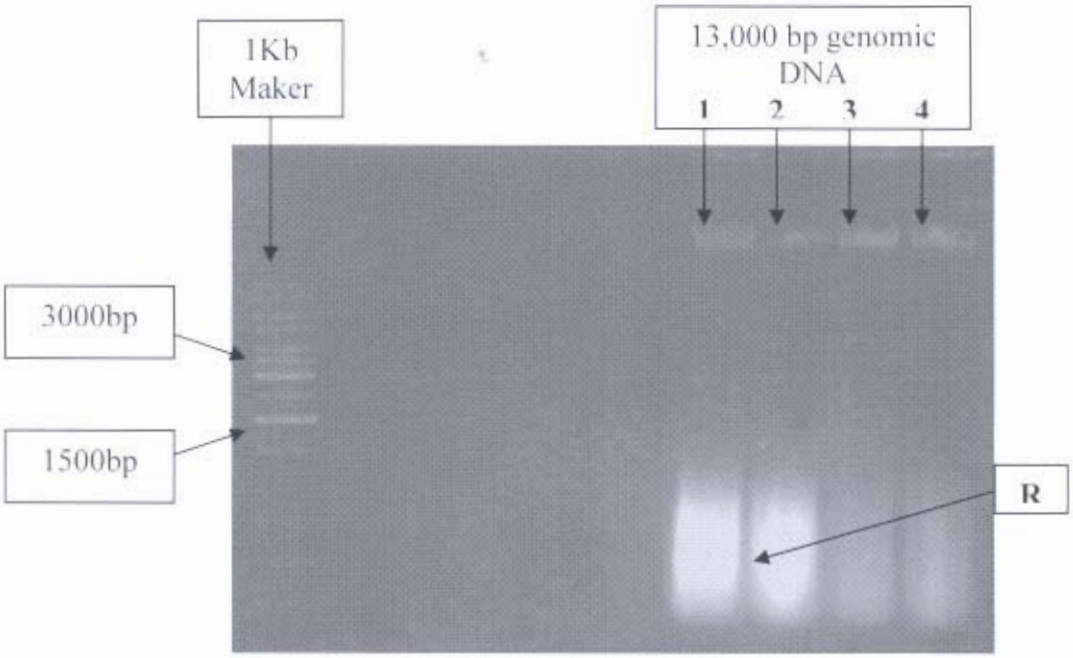

Figure 3: Agarose Gel Electrophoresis analysis of genomic DNA from *Bacillus amyloliquefaciens*. The DNA samples (1, 2, 3, 4) were run on a 1% agarose gel. The sizes of the DNA fragments were indicated using 1Kbp DNA Ladder Marker. The genomic DNA of this Bacillus was approximately 13,000bp. **R** represent for contamination of RNA

However, when the DNA was further purified using RNase A (10mg/ml), the bands appeared less brighter compared during the first stage of the DNA observation (**Figure 4**). The DNA might lose during this step. Therefore, the quantity and concentration of the DNA were reduced. Regardless of this condition, spectrophotometer analysis was carried out to make sure that the quality, quantity and concentration of the DNA is sufficient to undergo further analysis.

From all the samples, two tubes of samples that contained purified DNA were selected randomly for spectrophotometer analysis. Here, determination of the absorbance ratio and the DNA concentration were done. The absorbance ratio of A_{260}:A_{280} should be more than 1.8 (Brown, 1990). Respectively, the reading for each samples are as shown in (**Table 8**) below.

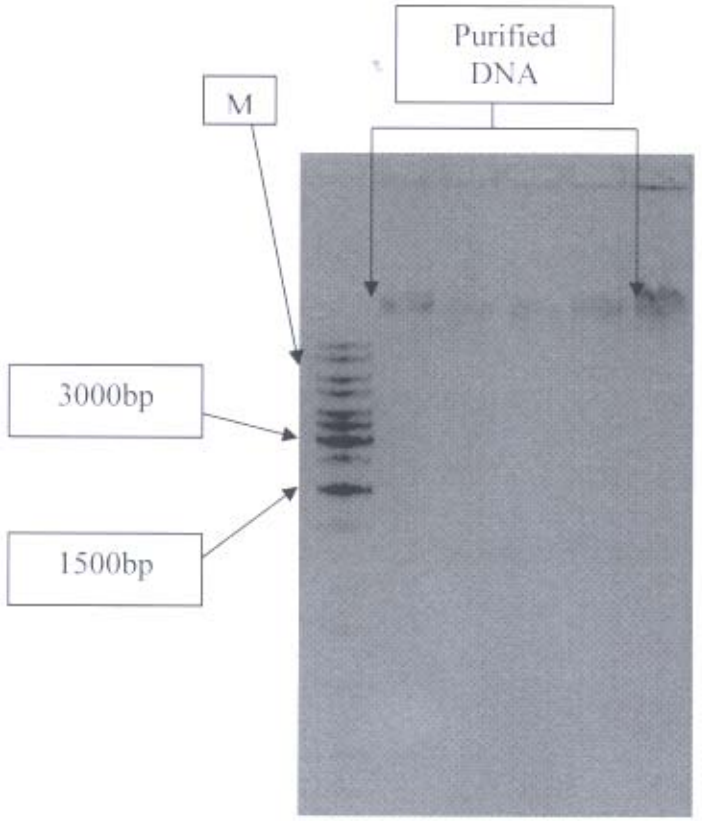

Figure 4: Purified DNA run on 1% Agarose Gel Electrophoresis and the size of the DNA fragments were indicated using M, 1Kbp Ladder Marker (Promega).

Table 8: The absorbance reading and the concentration of DNA for the selected samples measured by spectrophotometer.

Samples	**1**	**2**
A_{260}	0.382	0.441
A_{280}	0.210	0.234
A_{260}:A_{280}	1.790	1.975
DNA concentration	1.915	2.106