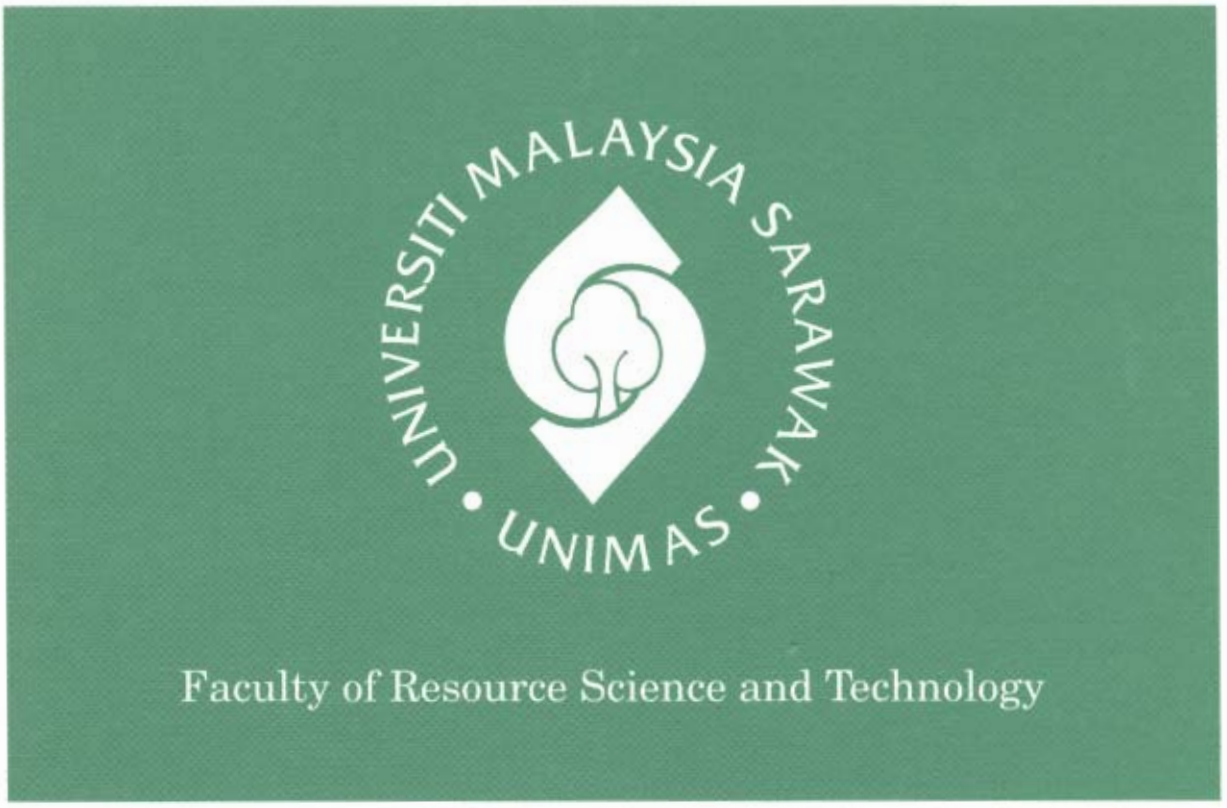

ISOLATION AND CHARACTERIZATION OF AMYLASE GENE FROM *BACILLUS AMYLOLIQUEFACIENS* UMAS 1002

Aida Sabrina Bt. Zainal

Bachelor of Science with Honours
(Resource Biotechnology)
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the requirements for the degree of Bachelor of Science with Honours
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ABSTRACT

Isolation and characterization *of Bacillus amyloliquefaciens* UMAS 1002 were conducted in order to determine the existence of amylase gene from this bacterium. The strain possessed the ability to hydrolyse starch as indicated by the presence of amylolytic activity that resulted in clear haloes within each colony when visualized in minimal media plate agar containing soluble starch. These colonies were selected for molecular analysis, which included genomic DNA extraction, restriction endonuclease digestion, Polymerase Chain Reaction (PCR) and cloning. Characterization of amylase gene from this bacterium using random cloning and PCR was unsuccessful.

Keywords: *Bacillus amyloliquefaciens* UMAS 1002, amylase, amylolytic

ABSTRAK

Pemencilan dan ciri-ciri molekular bagi gen yang mengkodkan enzim amylase daripada Bacillus amyloliquefaciens UMAS 1002. Bakteria ini menunjukkan aktiviti amilolitik kerana mempunyai keupayaan untuk menghidrolisiskan kanji dengan menunjukkan zon terang di sekeliling koloni di atas media minima kanji. Koloni-koloni ini dipencilkan dan analisis molekular dilakukan,. di mana melibatkan pengekstrakan DNA genomik, penghadaman oleh enzim pemotongan, penggandaan dan pemanjangan rantai (PCR), dan penklonan. Pengklonan secara rawak dan PCR gen amylase daripada bacteria ini tidak berjaya dilakukan.

Kata kunci: Bacillus amyloliquefaciens UMAS 1002, amylase, amilolitik

INTRODUCTION

Sarawak is known for the sago palm in Malaysia, where Mukah is the largest producer with over 50% from 90% production of sago starch. There are two main species of sago palm in Sarawak basically, the thorny (*Metroxylon rumphii*) and the thornless (*Metroxylon sagu*) palm (Jeffery, 1986). Sago is an important industrial starch in the local food industries and in manufacturer of monosodium glutamate. Besides that, the other applications of this palm are in a variety of industries such as in textiles, brewing, sewage treatment and paper production (Ferrari *et al*, 1993). Sago pith waste or sago hampas is the final residual product in the sago industries. According to Vikineswary and Shim (1996), sago pith waste contains a high level of starch (65.7%), crude protein (1.0%) and crude fiber (14.8%). The degradation of starch components in sago pith waste is catalyst by microorganisms such as bacteria and fungi.

According to Apun *et al.* (2000) a strain of *Bacillus sp.* has been successfully screened and isolated, which is called *Bacillus amyloliquefaciens* UMAS 1002. *B. amyloliquefaciens* is a strain of *Bacillus sp.*, which can naturally secrete high levels of amylase and protease. It is a Gram-positive bacterium, rod shaped, motile, aerobic and can be found in soils naturally. These bacteria are identified for its ability to degrade protein extracellularly. Due to its amylotic and cellulolytic enzymatic properties ability, this bacterium is capable of degrading both cellulase and starch from the sago pith waste.

Amylase is a group of hydrolases that has been known to split complex carbohydrates, such as amylose, amylopectin and glycogen into small fragment. There are several types of amylases. They are α-amylase (α-1,4-D-glucan glycanohydrolase) used to hydrolyse the α-1,4-glycosidic bonds, β-amylase (α-1,4-D-glucan maltohydrolase), glucoamylses (α-1,4-D-glucan

glucohydrolase) and pullulanases (α-dextrin 6-glucanohydrolase). α-amylase is an endo-acting amylase that hyrolyses internal α-1,4-linkages in amylose and amylopectin producing linear and branced saccharides with various lengths.

This project was part of the research work being carried out to understand the activities of amylase and cellulase from *Bacillus amyloliquefaciens* UMAS 1002. This project described the attempt in isolation and characterization of the amylase gene from the bacterium. Work done in this project included the isolation of genomic DNA, restriction digestion, ligation and transformation of fragments genomic DNA into expression vector. Polymerase Chain Reaction (PCR) work was also carried out to isolate the gene.

MATERIALS AND METHODS

Confirmation Test

Confirmation test were done by using biochemical test, which was the starch hydsrolysis test. *B. amyloliquefaciens* UMAS 1002 from the stock were grown in Luria Bertani (LB) plate agar. The bacterium was then subcultured in a minimal media plate agar consisted of yeast extract (0.2% w/v), KH_2PO_4 (0.1% w/v), $MgSO_4$ (0.5% w/v), and soluble starch (0.5 % w/v) (Apun *et al*, 2000) and incubated overnight at 32°C. To visualize hydrolysis zones, the plate was flooded with an aqueous solution of Lugol's iodine solution, which to screen for amylolytic properties in plate containing soluble starch.

Molecular Analysis

Genomic DNA extraction

15 ml fresh overnight cultured in McCartney bottle was centrifuged at 13,000 rpm (KUBOTA 7820) for 15 min to collect the cell pellets. The cell pellets were resuspended with 5 ml of TE Buffer (50 mM Tris [pH 8.0], 50 mM EDTA). The cells were frozen overnight in -20°C freezer. An amount of 0.5 ml 250 mM Tris [pH 8.0] and 10 mg/ml of Lysozyme were added to frozen suspension and thawed at room temperature on ice. Then, 1 ml Lysis Buffer (0.5% w/v SDS, 50 mM Tris [pH 7.5], 0.4 M EDTA, 1 mg/ml Proteinase K) were added and placed in 50°C water bath with gentle shaking for 1 hour. Next, 6 ml of phenol: chloroform: isoamyl alcohol (25:24:1) were added and centrifuged at 13,000 rpm for 15 min. The top layer (DNA aqueous layer) was transferred into a new bottle by avoiding the interface. Then, 0.1 volume of 3 M Natrium acetate (NaOAC) were added and mixed gently. Next, 2 volumes of cold 95% ethanol were added and mixed by inverting. The suspension was centrifuged at 13,000 rpm for 15 min and spooled out the DNA pellet. The pellet was air dried and dissolved in 2 ml TE Buffer (50 mM Tris [pH 7.5], 1 mM EDTA). The DNA samples were concentrated by incubated the samples at 37°C for 10 min. Then, 0.1 volumes of Sodium acetate and 2 volumes of ice-cold absolute ethanol were added, mixed gently and incubated at –20°C for 1 hour. The samples were centrifuged at 13,000 rpm for 15 min. The supernatant was discarded and the DNA pellet was washed with 70% ethanol and centrifuged at 13,000 rpm for 15 min. The supernatant was discarded again and let the DNA pellet to dry before adding 100 µl of TE Buffer (50 mM Tris [pH 7.5], 1 mM EDTA). Finally, 5 µl of genomic DNA were analyzed in 1% agarose gel electrophoresis.

Calcium chloride ($CaCl_2$) bacterial competent cells preparation

5 ml of LB cultured with *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* $I^qZ\Delta$ M15 Tn5 (Tet)]} were grown overnight at 37°C with shaking at 200 rpm. All 5 ml of the cultures were added into 50 ml fresh LB Broth and incubated at 37°C for 2 hours with shaking at 200 rpm to give an OD_{600} reading reached about 0.5. The cells were cooled on ice for 10-20 min and centrifuged at 3500 rpm at 4°C for 5 min. The supernatant was discarded and the cells were washed by gently resuspending them in 25 ml iced-cold 100 mM $CaCl_2$. The cells were kept on ice 10 min before centrifuging once again. The supernatant was discarded and the pellet was resuspends in 2.5 ml of cold, sterile 100 mM $CaCl_2$. Next, the cell suspensions were incubated on ice for 1 hour until use. For preparing the stocks, 20% (v/v) pure glycerol were added to the cell suspensions and mixed well. Aliquots (200 µl) were 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* (DE3) pLys] was also prepared using the same method.

Bacterial plasmid transformation

The objective of this transformation was to obtain high quantities of pET-22b(+) vector from stock plasmid. The stock plasmid (provided by Dr. Awang Ahmad Sallehin b. Awang Husaini) contained an insert of cellulase gene. An amount of 5 µl of plasmid was added to an Eppendorf tube contained 200 µl of freshly prepared *E.coli* XL1-Blue (competent cells) and mixed gently. The mixture was then, incubated on ice for 30 min in order to allow DNA to be absorbed onto the cells. The cells then were heat shock by incubation at 42°C for 45 seconds in a water bath

and followed by incubation on ice for 5-10 min. Next, 900 µl of fresh Luria Bertani (LB) Broth were added to the cells and incubated at 37°C for 1 hour with shaking at 250 rpm, which the cells were incubated as the transformation mixtures. This mixtures then were aliquoted and spread on LB-Ampicillin plates using a sterile bent glass rod. The liquids were allowed to diffuse into the agar for 20 min and then the plates were inverted a few times. Afterward, the plates were incubated overnight at 37°C. Subsequently, a single colony was isolated from each plate and transferred into 5 ml LB-Ampicillin Broth by using sterile inoculating loop. These cultures then were incubated overnight at 37°C with shaking at 250 rpm.

Alkaline lysis plasmid miniprep

1.5 ml of cell cultured overnight containing pET-22b(+) 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 1.5 ml of cells from the same culture was added to the tube. The cells were centrifuged again as before and supernatant was then discarded and leaving the pellet in the tube. Next, 400 µl of iced-cold Solution I (50 mM Glucose, 25 mM Tris-HCl [pH 8.0], 10 mM EDTA [pH 8.0]) were added and mixed gently to dissolve the pellet. The suspension was incubated on ice for 10 min. 600 µl of fresh prepared Solution II (200 mN NaOH, 1% (w/v) SDS) were added and mixed by inverting. The suspension was incubated on ice for 5 exactly min. Then, 600 µl of iced-cold Solution III (3 M Potassium acetate) were added and mixed by inverting. Again, the suspension was incubated on ice for 10 min and centrifuged at 13,000 rpm for 10 min at 4°C. Then, the upper layer of supernatant was transferred to fresh tube and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) were added. The mixture was mixed

gently and centrifuged at 12,000 rpm for 10 min at 4°C. Again, the upper layer of supernatant was transferred to fresh tube. After that, 0.1 volume of iced-cold 3M Sodium acetate (pH 5.2) and 2/3 volume of isopropanol for precipitation were added. Centrifuged at 12,000 rpm for 10 min at 4°C and then the supernatant was discarded. Next, the pellet was washed with 800 µl of iced-cold 70% (v/v) ethanol and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was discarded and air dried the pellet. Finally, the pellet was resuspended in 30 µl of TE Buffer pH 8.0 containing RNase. Finally, 5 µl of plasmid were analyzed in 1% agarose gel electrophoresis.

Cloning of fragments from genomic DNA of *B. amyloliquefaciens* UMAS 1002

An analytical scale restriction enzyme digest was performed in a volume of 20.0 µl by using *Hin*d III restriction enzyme. By using Eppendorf tube, 6.8 µl of sterile deionized water, 2.0 µl of RE 10X Buffer, 0.2 µl of Acetylated BSA 10 µg/µl, 10.0 µl of genomic DNA and mixed the mixture by gentle pipetting. Next, 1.0 µl of *Hin*d III was added to the mixture. Total reaction volume was mixed gently by pipetting before incubating at 37°C for 1-3 hours. Prior to use in further protocol, the enzyme was inactivated by adding 4 µl of 6X Loading Dye to the mixture after incubating. Next, 5 µl of this mixture was analyzed in 1% agarose gel electrophoresis. Beside genomic DNA, pET-22b(+), which identify as the expression vector was also digested using *Hin*d III. Vector fragment was ligated to DNA fragment and transformed BL 21 by using Rapid DNA Ligation and Transformation Kit (Fermentas). The ligated product was spread over LB-Ampicillin plates with Isopropyl-β-D-thiogalactopyranoside (IPTG) (25 mg/ml water). Next,

the plates were incubated overnight at 37°C to observe white colony. The existence of white colony indicated that the bacteria carrying recombinant plasmid.

Polymerase Chain Reaction (PCR)

PCR was carried out using the Peltier Thermal Cycler (PTC –200 DNA Engine Version 4.0) with *B. amyloliquefaciens* DNA as the template. 2 µl of genomic DNA was amplified with 2.5 µl of 25 pmol/µl of Amy1 Forward Primer (5'-GAT GTG GTT TTG AAT CAT AA- 3') and 2.5 µl of 25 pmol/µl Amy2 Reverse Primer (5'-CCA TCG TTT ACA TGA AAC TC- 3'). The final volume is 50.0 µl with consisted of 31.5 µl of steriled dH_20, 5.0 µl of 10X PCR Buffer, 1.0 µl of 10mM PCR Nucleotide Mix (dNTPs), 0.5 µl of 5u/µl *Taq* Polymerase and 4.0 µl of genomic DNA. These PCR reagents were obtained from Promega. PCR profile were; denaturation at 94°C for 1 min, annealing at 40°C for 2 min, extension at 72°C for 3 min, 7 min for final extension and allowed to cool at 4°C. Amplification was performed 25 cycles. Finally, 5 µl of PCR product was analyzed in 1% agarose gel electrophoresis.

RESULT AND DISCUSSION

Confirmation Test

Confirmation test on *Bacillus amyloliquefaciens* UMAS 1002 was done by using starch hydrolysis biochemical test. The physical appearances of this bacterium colonies were observed on LB plate agar were white in colour, circular shapes and creamy colonies (Figure 1). When

plated on minimal media containing soluble starch and flooded with Lugol's iodine, halo or clear zones within the colonies were visualized (Figure 2).

The existence of amylolytic activity was detected when bacterium possessed the ability to hydrolyse soluble starch. The levels of the amylase gene activity depend on the carbon source used, which from the soluble starch to stimulate the amylase formation (Santos, 2000). It was confirmed that the bacterial stock solution from the previous study (provided by Dr. Awang Ahmad Sallehin b. Awang Husaini) was *Bacillus amyloliquefaciens* UMAS 1002.

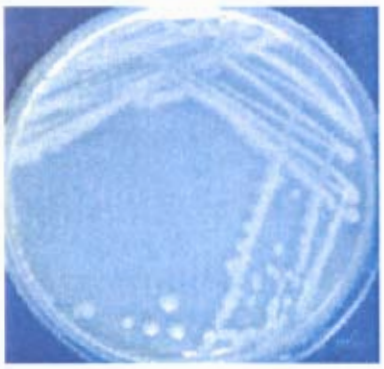

Figure 1: Colony of *Bacillus amyloliquefaciens* UMAS 1002 on LB plate agar.

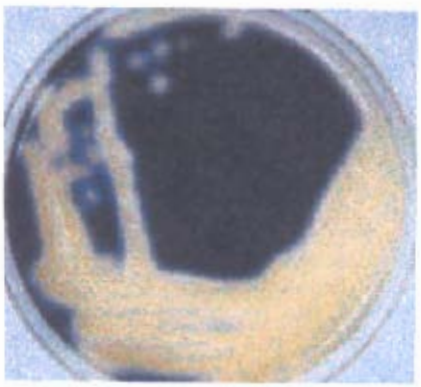

Figure 2: Starch soluble media plate after flooding the plate with Lugol's Iodine. Clear zone indicated the amylolytic activity of *B. amyloliquefaciens* UMAS 1002.

Molecular Analysis

B. amyloliquefaciens UMAS 1002 has been extracted by using general genomic DNA extraction with lysozyme usage. It was not easy to extract this bacterium due to it extracellular properties. Several modifications have been done on certain method from the original protocol in order to obtain precised genomic DNA of this bacterium. As a Gram-positive bacteria, the bacteria cells have to be treated with lysozyme in order to degrade the cell envelope which as a multi-layered (Sonenshein *et al*, 1993). The multi-layered cell envelope is consisted of protoplast membrane, cell wall and proteinacious outer layer called S-layer. By treatment with lysozme, these multi-layered cell envelope could be degraded. Following purification, 5 µl of the genomic DNA were analyzed by 1% agarose gel electrophoresis to visualize the DNA (Figure 3). It showed that the genomic DNA of *B. amyloliquefaciens* UMAS 1002 was approximately 13,000 bp. The position and size of the DNA fragments are indicated using 1 Kbp DNA Ladder Marker.

The genomic DNA and pET-22b(+) vector plasmid were digested with *Hind*III. The digestion revealed that the digested genomic DNA was approximately 1500 bp (Figure 4) while the digested pET-22b(+) vector was approximately 5000 bp (Figure 5).

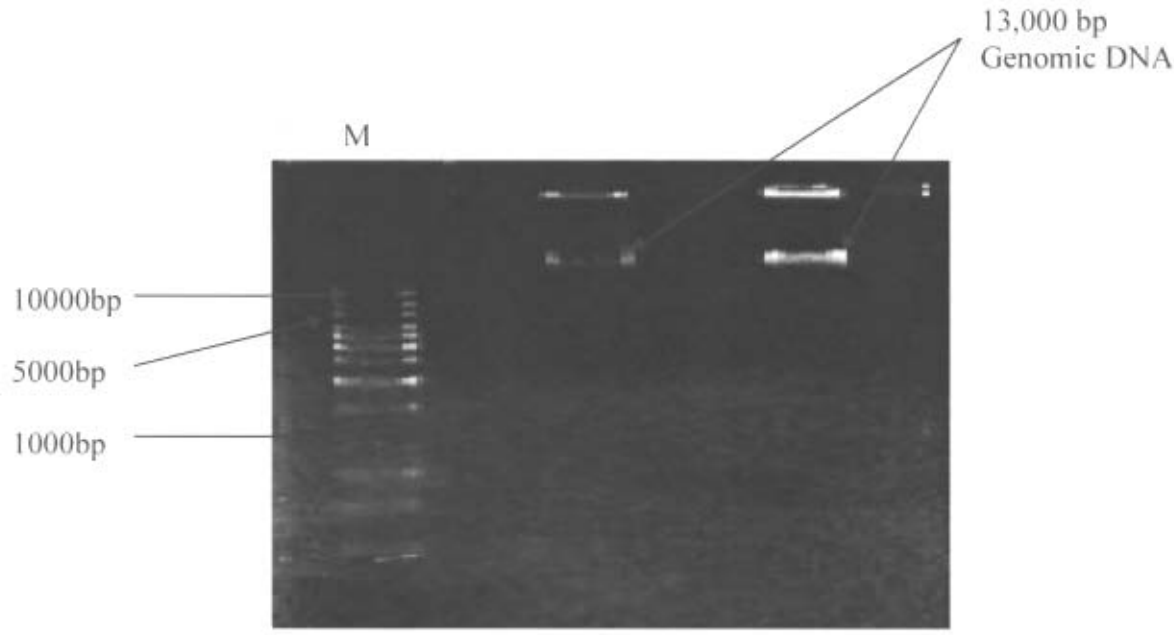

Figure 3: Agarose Gel Electrophoresis analysis of genomic DNA from *B. amyloliquefaciens* UMAS 1002. M indicated the 1 Kbp DNA Ladder Marker

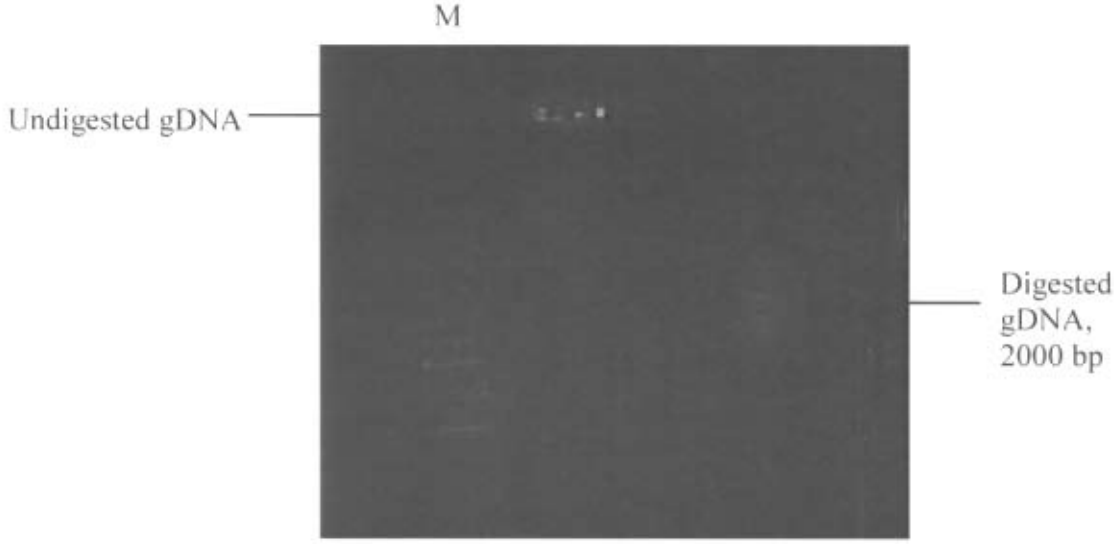

Figure 4: Agarose Gel Electrophoresis analysis of restriction digestion of genomic DNA of *B. amyloliquefaciens* UMAS 1002. M indicated the 1 Kbp DNA Ladder Marker.

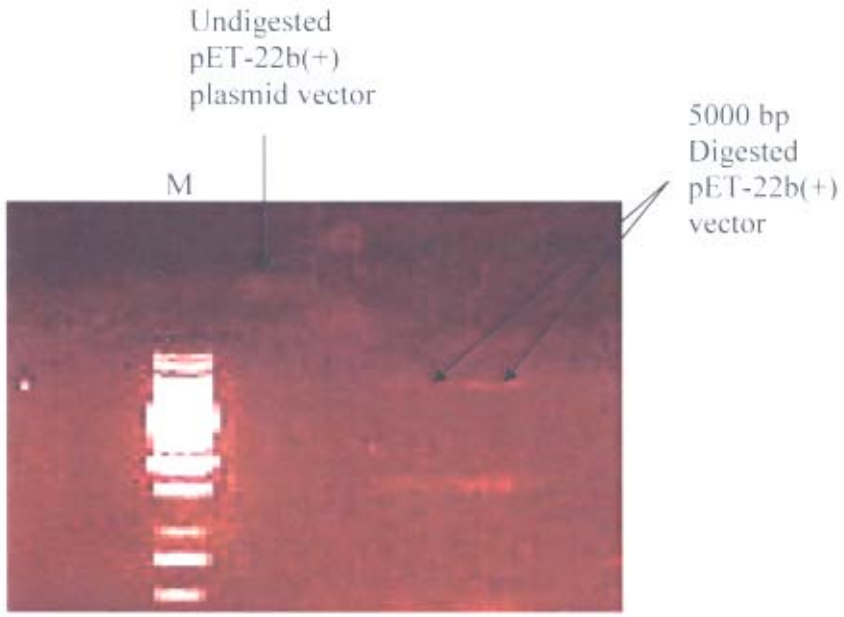

Figure 5: Agarose Gel Electrophoresis analysis of restriction digestion of pET-22b(+) vector plasmid. M indicated the 1 Kbp DNA Ladder Marker.

The digested genomic DNA and vector were ligated for cloning and expression of amylase gene by using BL-21 as competent cells. The transformation was done on LB plates with 1% soluble starch, IPTG and ampicillin, then overnight incubation at 37°C to screen white colony. IPTG was used as an inducer of β-galactoside activity by binding to and inhibit the *lac* repressor. An antibiotic, ampicillin was added to the media for selecting recombinant plasmid growth. Only cells transformed by a plasmid that provides resistance to ampicillin will be able to grow and multiply in the presence of this antibiotic. Bacteria carrying recombinant plasmid therefore should give rise to white colonies. From this study, attempts to clone the digested genomic DNA into pET-22b(+) vector was successful, as indicted by the white colonies observed on the antibiotics plate. However, the other aim of this ligation experiment, which was

to obtain clone containing active amylase gene, was unsuccessful. This was indicated by absence of any halo on the plates of white colonies after the plates were flooded with the Lugol's iodine solution.

The unsuccessful transformation of amylase gene might be due to some reasons. The most likely explanation was that all the inserts in the positive (white) colonies do not have the amylase gene in-frame with the promoter region and start codon on the pET-22b(+) vector. Theoretically, there should be at least one amylase gene in *B. amyloliquefaciens* UMAS 1002 as biochemical test has shown amylase activity on soluble starch. Therefore, the absence of halo on the digested colonies indicated the gene was inserted in the wrong frame in pET-22b(+) vector (Figure 6). Another explanation was that during digestion of genomic DNA, the amylase gene was probably cut at the N-terminal of the gene. This could probably produce an inactive amylase (Dr Mohd Hasnain B. Md. Hussain, personal communication). Although this procedure was unsuccessful for the expression of amylase gene, in some cases it was shown that random digestion of genomic DNA from bacteria followed by ligation and transformation into expression vector has been successful (Dr Awang Ahmad Sallehin b. Awang Husaini, personal communication).

There are some other reasons for the unsuccessful transformation. Firstly, it might because of the DNA isolated was impure with contaminants such as phenol, chloroform, detergent, EDTA and salts. These contaminants would partially or completely inhibit the digestion of genomic DNA.

Secondly, the problem might come up during the restriction digestion. An enzymatic reaction was affected by incubation temperature, buffer ionic strength, pH and Mg^{2+}

concentration. BSA was added in the reaction mix to influence the enzyme activity because it stabilizes the enzyme, binds some impurities and prevents enzyme adsorption on the test tube surface.

Other possible reasons might arising from the *E. coli* strain BL-21 competent cells that been used. The competent cells might not be competent enough to receive the DNA insert and vector. This occurs mainly because the improper handling of samples during the preparations of the competent cells, which decreased the efficiency of the cells to uptake the ligated plasmid vectors.

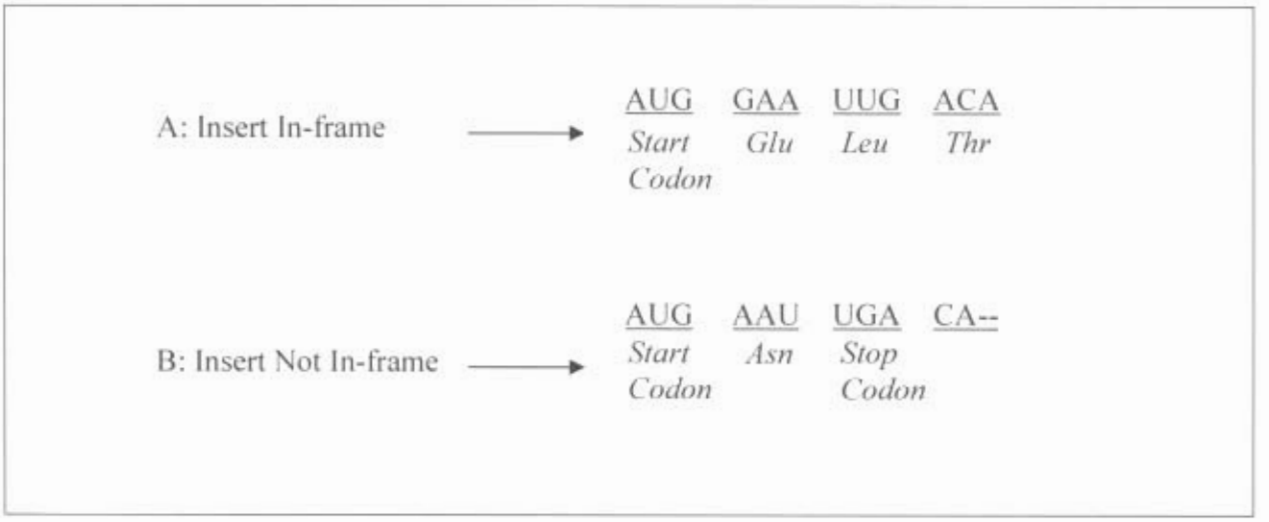

Figure 6: Correct insert will produce in-frame sequence for protein translation (A). Insert that are not in-frame will not produce protein during translation (B).

PCR was also carried out to amplify the amylase gene. The primers used in the PCR (Amy1 and Amy2 primers) were based on amylase gene from other bacterium species (Dr Mohd Hasnain B. Md. Hussain, personal communication). No amplification was obtained from this experiment hence it was not possible to do the sequencing.

The unsuccessful in obtaining any PCR product might be due to some possible reasons. First of all, it is possible that the amylase sequence in the primer is totally different from the amylase sequence in *B. amyloliquefaciens* UMAS 1002. During the process of finding suitable fragment for the amylase primer, amino acid sequence that are homolog in several species are chosen for the primers. However, there are instances where at amino acid level the amylase sequence are similar but at nucleotide level, there are differences. Furthermore, this was the first attempt using this newly designed primer. Besides the Polymerase Chain Reaction (PCR) profile used as described in the Materials and Methods, other profiles were also used in attempts to amplify the amylase gene. This attempts also failed to get any amplification. So, much more effort needs to be done in order to get the PCR product.

Other possible reasons come up from the PCR reagents itself such as dNTPs, $MgCl_2$ and *Taq Polymerase*. The dNTPs are very sensitive to heat. It might be damaged when melting it by hand gasping. *Taq Polymerase* also must be in stable condition. In room temperature, it still may work but could shorten its life span. Also, the concentration of $MgCl_2$ might be effecting the amplification of the DNA. Too much $MgCl_2$ would cause high levels of non-specific amplification while too little would inhibit the reaction.

CONCLUSION

The present has shown that *Bacillus amyloliquefaciens* UMAS 1002 contains amylolytic activity, as shown by the culture in minimal media containing soluble starch. This study has also shown that random cloning of digested genomic DNA was not suitable for obtaining a desired gene. The PCR was shown that Amy1 and Amy2 primers was not suitable for amplification of

amylase gene from *B. amyloliquefaciens* UMAS 1002. Therefore, new primers should be constructed for the PCR.

Further study should be conducted in order to achieve the characterization of amylase gene from this bacterium. Assisted by the data given, better result for the molecular characteristics should be obtained in the future.

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