Extraction and Purification of Alpha Amylase from *Bacillus amyloliquefaciens* and Identification of the Strain by 16s rRNA Gene Sequencing

A senior thesis submitted for partial fulfillment of Bachelor of Science in Biological Sciences

By

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Acknowledgment

I would like to take this opportunity to thank and show my sincerest gratitude to my advisor Professor AKM Moniruzzaman Mollah, Head of Science and Math Program, AUW, for his constant guidance, supervision and insightful responses to conduct this research successfully. Special thanks to lab officer Ms. Nabila Ishaque Ira for her assistance and support throughout the time. Thanks to lab officer Mr. Rahul Das, lab assistant Farzana Fayeza, and Montaha Chowdhury for their assistance in the lab. I would like to convey my deepest gratitude to Mr. Palash Kumar Sarker, Department of Microbial Biotechnology Laboratory, National Institute of Biotechnology (NIB) for his support to use the resources of NIB to conduct a part of the research at NIB.

I would like to thank Adity Shayontony Das, my fellow researcher, for her cooperation during the study. Lastly, I would like to extend my gratitude to AUW for funding this thesis, provide the lab premises and resources to conduct my research.

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Abstract

In the present study, alpha amylase was produced from *Bacillus amyloliquefaciens* in optimized condition by submerged fermentation technique. Biochemical tests were performed to identify the bacteria. The enzyme was extracted and purified by ammonium sulphate precipitation. Amylase activity and specific activity of the purified amylase was found to be 10 U/ml/min and 0.89 U/mg/min respectively. The enzyme was further purified by dialysis and size exclusion chromatography. The 16s rRNA gene was extracted from the bacteria and sent for sequencing to identify the strain. The result of the sequence analysis of the gene will be used to design primer and clone the amylase gene to over express the enzyme for industrial use in textile industry.

Key words: Alpha amylase, *B. amyloliquefaciens*, amylase activity, purification, 16s rRNA gene, cloning, textile industry

1) Introduction

1.1) Significance of amylase

Amylase makes up around one fourth of worlds enzyme market (Gopinath et al, 2017). This enzyme is one of the most important constituent of raw materials of various industries in Bangladesh including dairy, soft drinks, pharmaceuticals, food processing, leather, textile and paper industry (Hasan et al. 2017; Islam et al. 2016). It is one of the common enzymes used in various bioconversion processes in textile, food and agricultural industries (Rawat et al. 2012). The textile industries, the largest industrial sector of Bangladesh, require this enzyme for desizing and softening of fabric and are spending huge foreign currency every year to import this enzyme. Given the increased requirement and use of this enzyme in different industrial sectors of Bangladesh, the production and purchase of amylase is a huge part of the industrial budget (Khatiwada et al. 2016) This enzyme also comprises a good percentage of the world enzyme market, and thus have a large commercial value. Hence, characterization and production of the enzyme is of great importance to biotechnologist nowadays (Deb et al. 2013).

Use of enzyme to hydrolysis starch has become popular in industrial sectors than acid hydrolysis in most of the hydrolyzing processes (Gangadharan et al, 2006). The reasons for the increasing interest in microbial sources for desired industrially used enzymes include the short span of time needed to produce enzyme, cost effective procedures, ease of handling and manipulation of microbes to optimize and obtain desired features (Sundarram and Murthy, 2014).

1.2) Functions and features of amylase

Alpha amylases are extracellular endo enzymes that cleave the 1, 4- glycosidic bond between adjacent glucose molecules in the linear amylose chain generating glucose, maltose and maltotriose units (Gangadharan et al, 2006). The random cleavage of starch molecule by alpha amylase results in

the formation of linear and branched oligosaccharides of various chain lengths (Gopinath et al, 2017).

Alpha amylase family posses some common features. It belongs to the GH-13 family of glyosyl hydrolases (Mehta & Satyanarayana 2016; Maarel et al 2002). The enzymes work on 1,-4-glycosidic bonds and break this bond to produce monomers or oligosaccharides (Figure 1.1) (Held 2012; Maarel et al 2002).

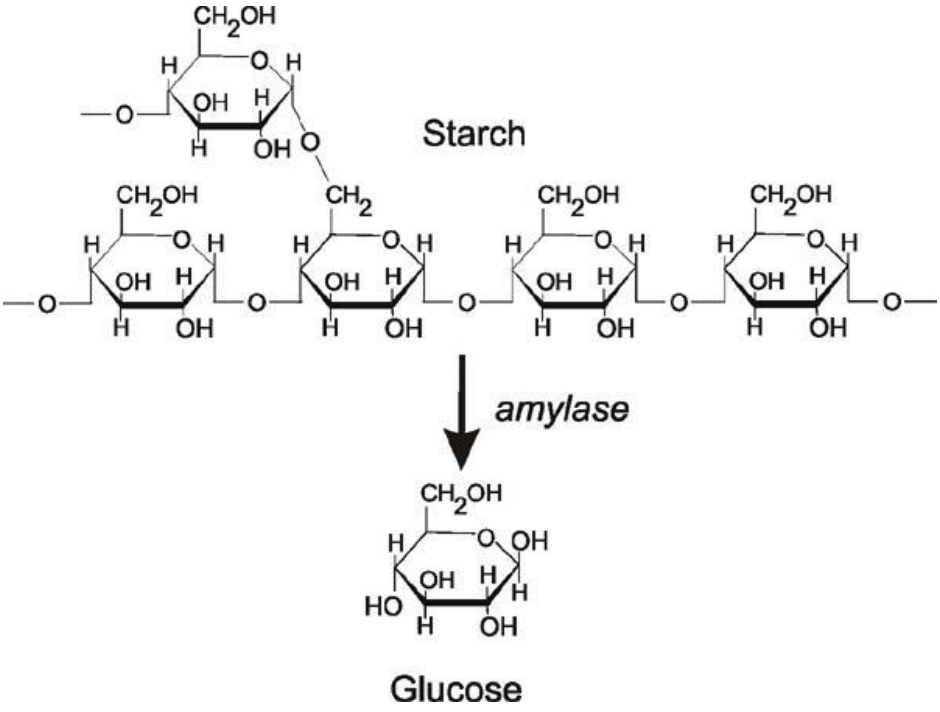

Figure 1.1: Catalytic activity of amylase to breakdown starch into glucose (Held 2012)

1.3) Structure of amylase

Amylase consists of the TIM barrel conserved protein folding where eight alpha helices and beta strands are present forming the catalytic site residues. The enzyme has four highly conserved regions in their primary structure which forms the catalytic site. Few amino acids of these conserved regions are essential for the stability of the conserved TIM barrel protein folding (Maarel et al 2002). The protein contains 3 domains: A, B, and C (Figure 1.2). The A domain is the largest and the B domain is connected to the A domain by disulphide bond. The C domain has a β sheet structure

linked to the A domain by a simple polypeptide chain. The C domain appears to be an independent domain with unknown function. The active site of the alpha amylase is located in a long cleft located between the carboxyl end of the A and B domains. A calcium ion is positioned between the A and B domains. This calcium maintains the stability of the three-dimensional structure of amylase and acts as an allosteric activator (De Souza & de Oliveira Magalhães 2010; Siddiqui et al. 2005)

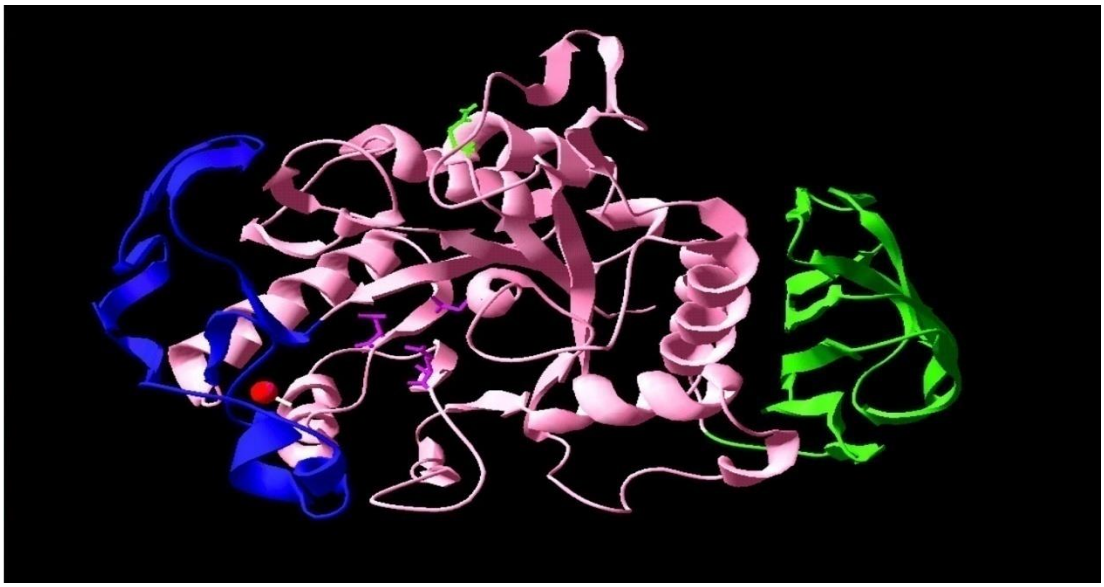

Figure 1.2: Structure of α-Amylase (Siddiqui et al. 2005)

The protein consists of approximately 496 amino acid forming the alpha helices and beta sheets. 170 water molecules and a chloride ion are also present in the protein structures; those are required for its structural stability. According to numerous researches, the molecular weight of the protein varies widely, from 10-210 kDa. Nonetheless, from different *Bacillus* a*myloliquefaciens* strains, the variation of molecular weight of amylase ranges from 40-60 kDa approximately (Mehta & Satyanarayana 2016).

1.4) Amylase producing bacteria

A large number of different bacteria were isolated to check the production of amylase and it has been found in many studies that Bacillus species are most efficient in amylase secretion (Gopinath et al. 2017). According to multiple sources, *Bacillus subtilis, Bacillus stearothermophilus, Bacillus licheniformis,* and *Bacillus amyloliquefaciens* are all well known producers of thermostable α-Amylase (Sundarram and Murthy 2014).

1.5) Bacillus amyloliquefaciens

Bacillus amyloliquefaciens is a plant growth promoting, non-pathogenic bacteria found in soil. They are also beneficial rhizobacteria. These bacteria suppress pathogenic organisms by producing antifungal and antibacterial substances, such as bacillomycin D, surfactin, and bacillaene. Two of the proteins produced by *Bacillus amyloliquefaciens* are proteases and amylases; these are widely used in industrial applications. This organism is well known for its thermostable amylase production. The thermostability is perhaps generated from the median 46.4% of GC content. 54 strains of the bacteria were identified, and genome sequences were recorded. *Bacillus amyloliquefaciens* are Gram positive, aerobic, terrestrial, and motile bacteria with bacilli shape (Genome 2010).

1.6) Amylase production in laboratory

The enzyme is produced in higher concentration when the bacteria are grown in submerged fermentation in a rotary shaker with controlled agitation (Haq et al. 2010). The rate of amylase production can vary depending upon many factors, such as, strain of the bacteria, original growing environment of the bacteria, pH, temperature, time of incubation, methods of cultivation, and carbon and nitrogen sources (Gopinath et al. 2017 & Haq et al. 2010). Also, amylases are metalloenzyme which requires Ca^{2+} for the structural stability and activity of the enzyme (Saha et al. 2014). The number of bound calcium ion per enzyme is between one and ten. Thus, the number of calcium ion binding with the enzyme has profound effect on its activity and thermostability (Demirkan et al.

2003). Therefore, optimization of the culture condition is one of the important factors needed to consider in bacterial culture in the laboratory to maximize the enzyme production and activity (Deb et al. 2013).

Two fermentation procedures are followed to cultivate the enzyme, submerged and solid state fermentation (Gopinath et al. 2017). Previously, production of industrially important enzymes have been carried out by submerged fermentation (SmF) since it is easier to handle; culture conditions such as pH and temperature can be kept in control. However, solid-state fermentation (SSF) is a good alternative since the products are concentrated in addition to the cost effective purification procedures (Gangadharan et al. 2006). Crude extract of amylase from fermentation product can serve its enzymatic function. However, for specific industrial use, purified enzyme is required. Therefore, it is important that the enzyme is purified following extraction. Purification of the enzyme can be accomplished by different protein purification methods such as chromatography (Gopinath et al. 2017).

1.7) Study Objective

Currently, there is no commercial production of industrially used enzymes in Bangladesh. Consequently, Bangladesh is dependent on import of the enzymes. However, it is high time to start working on the production of industrial enzymes locally which will proportionately increase the growth rate of industrial sectors (Hasan et al. 2017). In this research we focused on the maximum production of amylase from *Bacillus amyloliquefaciens* using optimized production conditions. The activity and efficiency of the enzyme was checked and compared to industrially compatible amylase. The DNA was extracted followed by sequence analysis of the 16s rRNA gene to indentify the unknown strain. The research will be continued following purification of the enzyme and the gene of interest will be cloned to amplify the production rate.

2) Materials and Methodology

2.1 Bacterial culture

Bacterial sample was collected from Dhaka University Microbiology Department and transported to laboratory in proper condition. Bacterial colony was streaked in nutrient agar plate and fresh subculture was prepared in nutrient agar medium before each experiment.

The bacterial colony morphology was studied from the freshly grown culture. Gram staining and series of conventional biochemical tests were carried out for the identification of the bacterial species (Appendices). All the biochemical tests had a control group. Log phage growth bacteria were maintained in 50% glycerol broth at −20°C.

2.2 Starch Hydrolysis

To test if the bacteria produce amylase, a preliminary test was carried out. A loopful of bacteria from freshly cultured nutrient agar (NA) plate was inoculated in 50 ml Luria Bertani (LB) growing media in sterilized condition. The inoculated broth was incubated at 37°C for 24 hours. 100µl of sample from the incubated LB broth was transferred to a starch agar plate using spread plate technique. The plate was incubated for 48 hours at 37°C. After 48 hours, the plate was flooded with Gram's iodine solution and formation of clear zone was observed.

2.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Protein was run in 12% discontinuous SDS gel. The gel was prepared manually in the lab. To make 12% of 10 ml separating gel, 3.2 ml distilled H_2O, 4 ml Acrylamide / Bisacrylamide, 2.6ml 1.5M Tris HCl (pH 8.8), 0.1 ml 10% (w/v) SDS, 0.1 ml 10% (w/v) Ammonium persulfate (APS), and 10 µl tetramethylethylenediamine (TEMED) was mixed and poured in a casting tray. The gel was allowed to polymerize for 20-30 minutes. To make the upper layer even, small amount of dH_2O was pipetted on top of the poured gel which was later dried using a filter paper after polymerization.

To make 5 ml stacking gel, 2.97 ml distilled H_2O, 0.67 ml Acrylamide / Bisacrylamide, 1.25ml 0.5M Tris HCl (pH 6.8), 50µl 10% (w/v) SDS, 50µl 10% (w/v) APS and 5µl TEMED was mixed and poured on top of the polymerized separating gel. APS was freshly prepared and added to the mixtures just before pouring each of the gel mixtures.

Two different methods were followed to prepare sample for SDS PAGE, boiled DNA method and using cell lysis buffer.

Using cell lysis buffer: 500 µl of 24 hours old cultured broth was mixed with 50 µl lysis buffer in a 1.5 ml eppendorf tube. The mixture was vortexed to mix properly followed by heating at 50°C for 5 minutes. 16.7µl of the mixture was then transferred to another eppendorf tube and 3.3 µl of SDS buffer was added to it. The mixture was vortexed followed by a brief spin. The sample was ready to be loaded in the gel.

Boiled DNA method: 500 µl of 24 hours old cultured broth was taken in an eppendorf tube. The sample was centrifuged at 13000 rpm for 8 minutes. 800 µl of Phosphate-buffered saline (PBS) was added to the centrifuged sample. The sample mixture was boiled for 10 minutes immediately followed by ice cooling for 20 minutes. The sample was centrifuged at 13000 rpm for 10 minutes after the heat-shock. 16.7 µl of the sample was then mixed with 3.3 µl of SDS buffer. The sample was vortexed followed by a brief spin. This mixture was then boiled for 5 minutes ready to be loaded in the gel.

The sample was loaded and the gel was run along with another sample from *Bacillus Subtilis* and a known protein ladder at 150V for 60 minutes. It was then stained with Comassie Blue for 35 minutes and then washed with destaining solution. The gel was submerged in destaining solution and kept in a shaker to destain overnight. It was then observed under UV light to detect protein band.

Bands which were in parallel line with the bands of the protein ladder were marked. The sizes of the bands were determined using the known sizes of the protein ladder.

2.4 Preparation of culture

A preliminary test was carried out with optimized culture condition and a SDS-PAGE was performed with the cultured broth.

A loopful of freshly grown culture from a 24 hour old plate was aseptically transferred to 50 ml of LB broth in a conical flask. The flask was incubated overnight at 37°C and in a water bath shaker.

The enzyme production was carried out in the basal medium containing 0.1% KH_2PO_4, 0.25% Na_2HPO_4, 0.1% NaCl, 0.2% $(NH_4)_2SO_4$, 0.005% $MgSO_4$.$7H_2O$, 0.005% $CaCl_2$, 0.2% tryptone and 1% soluble starch (Deb et al. 2013). 1 ml of 24 hours grown inoculums was cultured in 250-ml Erlenmeyer flasks containing 100 ml of medium with an initial pH 7.0. The cultures were incubated in a water bath shaker incubator at 37°C for 48 hours. The broth was used to run a SDS-PAGE using the previous protocol.

2.5 Enzyme production in shake flask culture

Using the same basal media composition, enzyme production was carried out in orbital shaker at 150 rpm agitation and 37°C for 24 hours. Total 200 ml culture was prepared in two replicates, each of 100 ml. After the incubation, the fermented broth was centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected and the pellet was discarded.

2.6 Enzyme assay

The presence of amylase in the collected supernatant was detected by using soluble starch, 1% (w/v), as the substrate in 0.05 M Sodium phosphate buffer of pH 6.5. The reaction mixtures for enzyme assay were prepared according to the following table:

Table 2.1: Sample preparation for absorbance at 540 nm

	Blank (ml)	SC (ml)	EC (ml)	Test (ml)
Buffer	2	0.2	1.8	0
Buffer Substrate	0	1.8	0	1.8
Enzyme	0	0	0.2	0.2
Reactive Media Volume	2 ml	2 ml	2 ml	2 ml
DNS	3 ml	3 ml	3 ml	3 ml
Total Volume (ml)	5 ml	5 ml	5 ml	5 ml

***Abbreviations
SC = Substrate Control (Substrate + Buffer)
EC = Enzyme Control (Enzyme + Buffer)
Test = Substrate + Enzyme + Buffer

The reaction mixtures were then incubated at 50°C for 10 min. The reaction was stopped by adding 3 ml dinitrosalicylic acid (DNS). The enzyme activity was determined by using the absorbance measured at 540 nm with spectrophotometer. One unit (U/ml) of alpha amylase activity is defined as the amount of α-amylase required to release 1 mg of reducing sugar from starch/min, under the assay conditions (Deb et al. 2013).

The equations used to calculate enzyme activity and specific activity were:

Enzyme activity (U /ml/ min) =

$$\frac{Maltose\ released\ (\mu g) \times TOtal\ Volume\ of\ Reactive\ Media\ (ml) \times Dilution\ Factor\ (DF)}{Molecular\ Weight\ of\ Maltose \times Enzyme\ Used\ (ml) \times Time\ of\ Incubation\ (min)} \quad \text{(Equation 1)}$$

Specific activity (U /ml/ min) = $\frac{Enzyme\ activity\ (U\ /ml/\ min)}{Extracellular\ Protein\ Concentration\ (\frac{mg}{ml})}$ (Equation 2)

2.7 Ammonium sulfate precipitation of enzyme

In a second trial, cultures were made using the previous protocol of enzyme production. The enzyme production was carried out in orbital shaker at 150rpm and 37°C for 24 hours. Total 500 ml culture was prepared in five replicates, each of 100 ml. After the incubation, the fermented broth was centrifuged at 10000 rpm for 10 minutes at 4°C. The supernatant was collected and the pellet was discarded. 50 ml of the supernatant was used for ammonium sulphate precipitation (75%). 26.08g of ammonium sulphate was slowly added and dissolved in the 50 ml of supernatant. After overnight incubation at 4°C the mixture was centrifuged at 10000 rpm for 15 minutes. The supernatant was discarded and the pellet was collected. The pellet was then resuspended in 2 ml of 0.05M sodium phosphate buffer (pH 6.5).

2.8 Protein Estimation

OD for the precipitated enzyme was taken at 600 nm to find the concentration of extracellular amylase which was estimated by the Lowry's method (1951). 2 ml of analytical reagent was added to 0.2 ml of the diluted sample obtained after precipitation. The amalgam was mixed well and incubated for 10 min at 50°C. Then the sample was cooled for a while. Immediately 0.2 ml of the folin-ciocalteau reagent was added to it and vortexed followed by incubation at room temperature for 30 minutes. OD of the reaction mixture was measured at 600 nm, against a blank prepared with 0.2 ml buffer. A standard curve was plotted using bovine serum albumin (BSA) as the known protein. The amount of the soluble protein was calculated in mg protein per ml of test samples from the standard curve. The OD for crude enzyme and the supernatant of the precipitated enzyme was also taken.

Analytical reagent: 4 ml of reagent B mixed with 200 ml of reagent A

Reagent A: 100 ml 2% sodium carbonate solution mixed with 100 ml of 0.1N NaOH

Reagent B: 10 ml of 1.56% copper sulphate solution mixed with 10 ml of 2.37% Rochelle salt solution

2.9 Dialysis and chromatography of protein

The precipitated enzyme was then dialyzed in sodium phosphate buffer to get concentrated protein which was purified by column chromatography. The column was prepared using resin beads Sephadex G-75.

Dialysis of protein

For dialysis, the dialysis tube was pre-treated in 1mM EDTA. It was boiled for 10 minutes and then allowed to cool. One end of the tube was closed by tying knot. 2 ml of the sample was pipetted into the tube and another knot was tied at the other end in a way to minimize formation of air bubbles. The dialysis tube was then placed inside a 500 ml beaker containing 200 ml of sodium phosphate buffer. A magnetic stirrer was put in the buffer and the beaker was kept inside another 1000 ml beaker containing ice to keep the buffer temperature at 4°C. Then the larger beaker was placed on a magnetic plate for 1.5 hours. After 1.5 hours the buffer was replaced with new buffer and kept for another 1.5 hours. Later the dialysis buffer was replaced with new buffer once more and the beaker was kept at 4°C for overnight incubation. Then the dialysis tube was removed from the buffer and the dialyzed protein was pipetted into an eppendorf tube for storage at -20°C.

For column chromatography, Sephadex G-75 resin beads were soaked in 20X phosphate buffer for 72 hours to swell up the beads. A burette was clamped and its nozzle was closed. The bottom of the burette was packed with a cotton ball to filter out the dust in the solution poured into the burette. The soaked resin beads were poured and allowed to settle to form a compact column. Total 30 ml of the burette was filled up. Then the packed column was washed with phosphate buffer thrice. When the total 30 ml of buffer ran through the column, 2 ml tryptophan of concentration 1

mg/ml and 2 ml BSA of concentration 1mg / ml was mixed together. 1 ml of this mixture was added to 1 ml of the dialyzed protein. This mixture of sample was loaded on drained bed surface. The outlet was closed and layered the sample on the top of the bed. The outlet was then opened to allow running buffer to pass through. Then fractions were collected. Total 60 fractions, 500 µl in each fraction, were collected. Then the OD was taken at 280 nm in a spectrophotometer to check for the presence of protein. Each cuvette for spectrophotometer reading required minimum sample volume of 1500 µl. Therefore, 1000 µl distilled water was added to each of the 500 µl sample to take reading. The spectrophotometer was set to take reading for DNA/protein at 280 nm. Phosphate buffer was used as the blank. Each time the spectrophotometer was set to zero before taking the OD. For each sample, three readings were taken and the average was used to check for the presence of protein from the absorbance.

2.10 DNA Extraction

Bacterial culture was grown in LB media from the 24 hour old culture.

1.5ml of the overnight grown culture of the bacteria was transferred into an eppendorf tube and centrifuged at 10000 rpm for 10 minutes. The supernatant was discarded and the previous step was repeated by taking another 1.5ml of bacterial culture to the pellet. The supernatant was discarded and the pellet was washed with 750µl of 1X PBS solution. This mixture was centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded and the pellet was dissolved with 250µl of extraction buffer by pipetting. This step was followed by the addition of 25µl of 10% SDS solution into the pellet. The eppendorf tube was incubated at 65˚C for 10 minutes. 10µl of Proteinase K was added into the tube and incubated the microcentrifuge tube at 56˚C for 30 minutes. After incubation 285µl of Phenol: chloroform: isoamyl alcohol (25:24:1) was added into the tube and mixed gently by vortex. The mixture was centrifuged at 13000 rpm for 15 minutes. The top layer was transferred into

a new eppendorf tube. 25µl of 3M sodium acetate pH 7 was added into the sample followed by the addition of 600µl of ice cold absolute ethanol and mixed. Then 70% ethanol was added to the mixture. The sample was then centrifuged at 10000 rpm for 5 minutes. The supernatant was discarded and the pellet was to air dry. The DNA was stored in TE buffer at -20 °C.

2.11 Polymerase Chain Reaction (PCR)

PCR reaction was performed using freshly prepared master mix. The components used in the master mix are given in table 2.2. $MgCl_2$ of concentration 20 mM was present in the DreamTaq Green Buffer from ThermoScientific, hence it was not added separately. the following cycling protocol was used to perform PCR, denaturation at 95 °C for 20s, annealing at 60 °C for 20s, and elongation at 72 °C for 30s for a total of 30 cycles. Seven separate samples, each of 50 µl, were prepared for PCR. DNA extracts of *B.amyloliquefaciens* and *B. subtilis* with PCR mix and primer, DNA extracts of *E.coli*, *B.amyloliquefaciens* and *B. subtilis* added to PCR mix without primer, and one sample of PCR mix along with primer only.

Table 2.2: Preparation of 50 uL PCR reaction mixture

Reagents	Stock Concentration	Final Concentration	Volume
Forward Primer	10 uM	1 uM	5 uL
Reverse Primer	10 uM	1 uM	5 uL
Buffer	10X	1X	5 uL
dNTPs	10 mM	200 uM	1 uL
Taq Polymerase	5U / uL	10U/uL	1 uL
DNA Template	-	-	5 uL
Water	-	-	28 uL
Total Volume			50 uL

2.12 Agarose Gel Electrophoresis

The PCR products were ran in 1% agarose gel to check if there was DNA in the extracted sample and determine the size of the DNA bands. 1g of agarose powder was dissolved in 100 ml of 1X TBE buffer and heated the mixture in microwave for 1.5 minutes until the agarose was dissolved. Then the solution was cooled to about 60 °C. Then 50 ul of ethidium bromide was added to the solution and poured in the casting tray. A comb of 10 wells were put in the gel and allowed it to cool. . 20 ul of sample was loaded in the gel along with extraction of *Bacillus subtilis* DNA, other known DNA and a DNA ladder. The gel was run at 150V for 60 minutes.

2.13 DNA Sequencing

The PCR sample was sent to National Institute of Biotechnology for sequencing. To identify the strain of the bacteria from the gene sequence, NCBI BLAST will be run.

3) Results

3.1 Morphology

The morphology of the bacteria were studied through close observation of the colony formed after 24 hours of incubation at 37°C in nutrient agar medium (Table 3.1 and Figure 3.1).

Table 3.1: Morphology of the colonies grown after 24 h of incubation

Size	Large
Shape	Irregular
Margin	Undulate
Elevation	Raised
Pigment / Color	White

Figure 3.1: Morphology of the bacterial colony

Gram Staining was done by the conventional method of staining. The cells of the bacteria were found to obtain purple color under the microscope indicating gram positive bacteria. The shape was found to be rod shaped.

3.2 Starch Hydrolysis

Starch hydrolysis of 24 hours old culture showed clear areas around the colony of bacteria from blue black color few minutes after iodine was added to the starch agar plate (Figure 3.2).

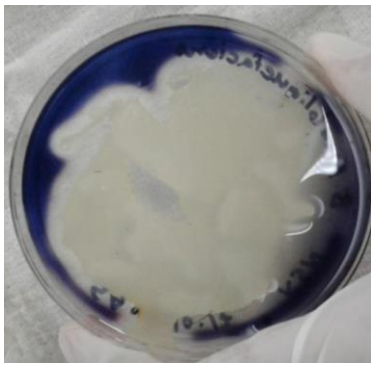

Figure 3.2: Starch hydrolysis showed formation of clear zone

3.3 Biochemical Tests

Table 3.2 summarizes the results of the biochemical tests for *Bacillus amyloliquefaciens*. The color of the broth changed immediately to red when methy red was added to the broth (Figure 3.3 a). In citrate test the incubated bacteria in agar did not change the color of the media to blue (Figure 3.3 b). The SIM test shows that *B. amyloliquefaciens* is a highly motile bacteria (figure 3.3 c)

Table 3.2: Biochemical Tests Results

Test	Result
Catalase	Positive
Oxidase	Positive
Methyl Red	Positive
VP Test	Negative
Sulfide +Indole	Positive
Citrate	Negative
Motility	Highly Motile

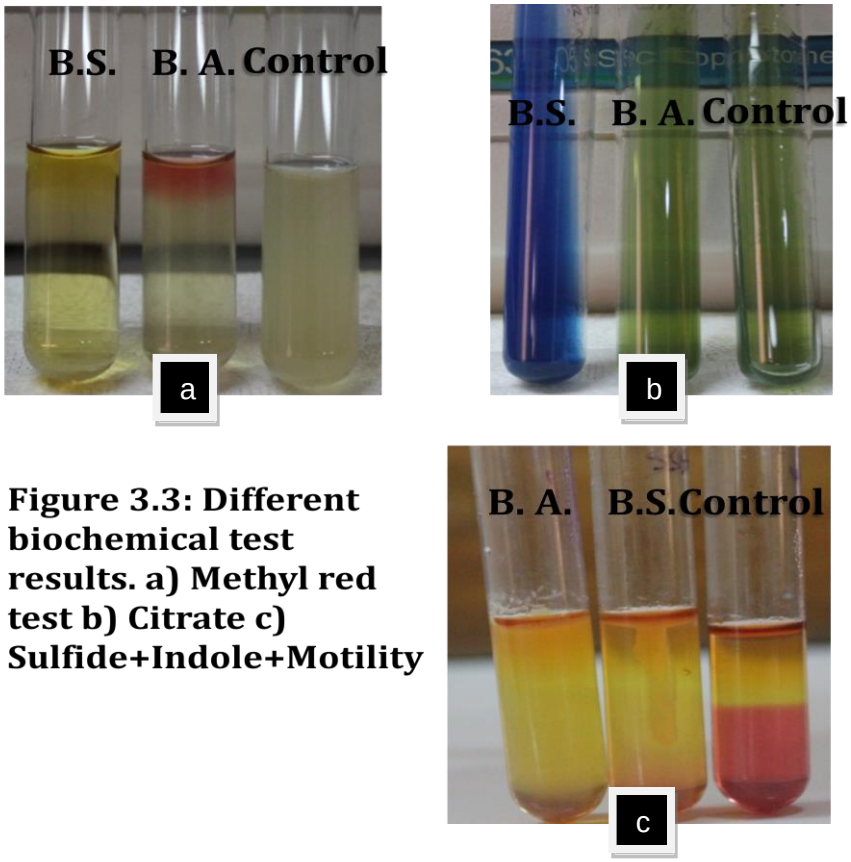

Figure 3.3: Different biochemical test results. a) Methyl red test b) Citrate c) Sulfide+Indole+Motility

3.4 SDS-PAGE

After the second SDS-PAGE, the gel was observed under white light to look for protein bands. Only one band was visible, which had approximate weight of 17kDa for both *B. amyloliquefaciens* and *B. subtilis* (Figure 3.6). The size was found from the known protein ladder used. No other bands were observed for any of the bacterial sample.

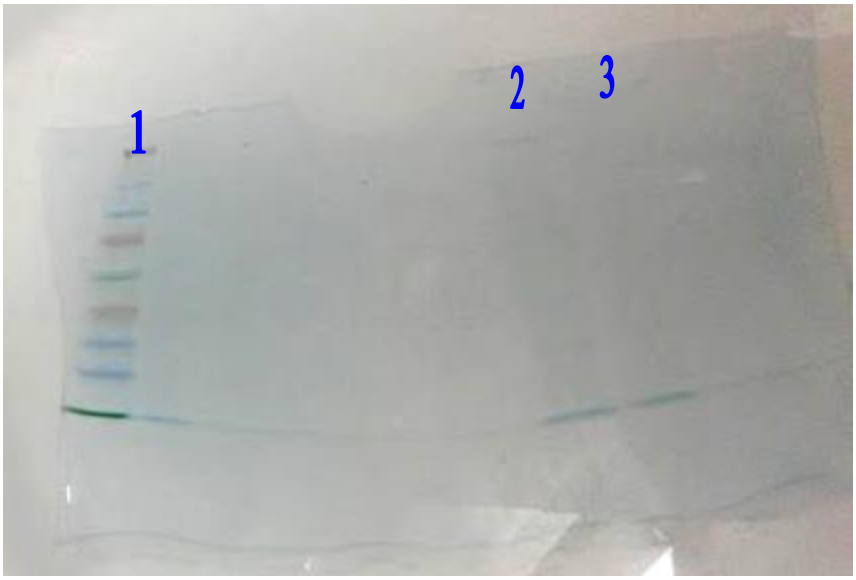

Figure 3.6: SDS-PAGE result after optimizing the basal media
Lane 1 = Protein ladder
Lane 2 = Protein from Bacillus Subtilis
Lane 3 = Protein from Bacillus Amyloliquefaciens

3.5 Enzyme Assay

After maintaining the bacteria in optimized culture condition to produce amylase, the extract of the broth was used to check the activity of amylase by taking spectrophotometer reading at 540nm. The value of the optical density shows that the activity of the enzyme is very low. However, the activity of the precipitated enzyme was higher and the OD value is nearly 1. The supernatant of the precipitated enzyme was found to have negative OD value which shows that enzyme was absent in the supernatant (table 3.3).

Table 3.3: OD value of enzyme with DNS at 540 nm for amylase activity

Test	Crude Enzyme 1	Crude Enzyme 2	Crude Enzyme 3	Crude Enzyme 4	Crude Enzyme 5	Precipitated Enzyme	Supernatant of Precipitated Enzyme
Blank	0	0	0	0	0	0	0
SC	0.022	0.022	0.023	0.023	0.023	0.024	-0.005
EC	0.015	0.068	0.075	0.004	0.392	0.324	-0.055
Test	0.041	0.106	0.078	0.030	0.464	1.357	-0.044
Final	0.004	0.016	- 0.02	0.003	0.049	1.009	-0.016

****Final = Test – SC – EC, SC = Substrate control, EC = Enzyme control

Enzyme activity was calculated from the standard curve of maltose (figure 3.7).

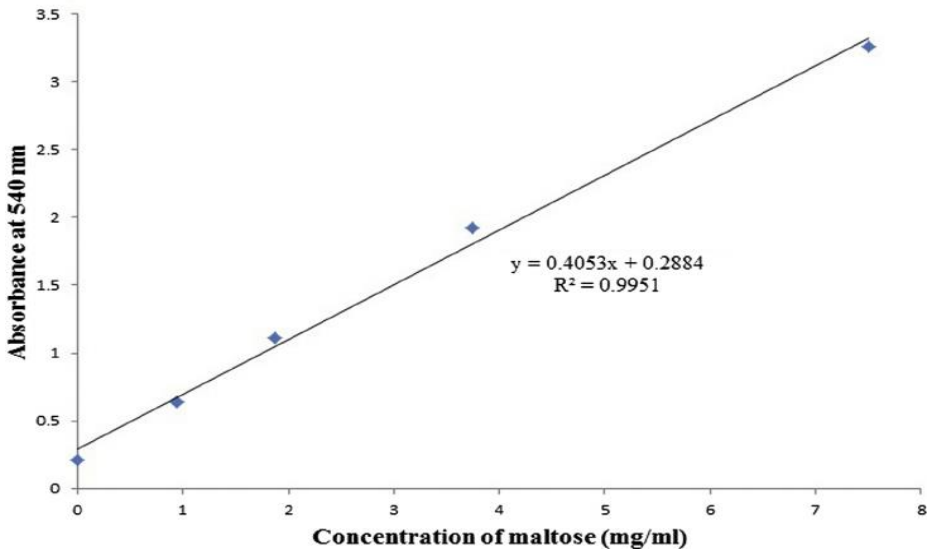

Figure 3.7: Standard curve for maltose (Dehgan et al 2015)

The equation y = 0.4053x + 0.2884 was used to calculated maltose concentration from the absorption value of the four replicates. An average of replicates was taken and the test 3 was excluded in the calculation of the average for the anomaly. The average absorption is 0.018 for the crude enzymes with DNS.

Maltose released from the crude enzyme activity = - 0.667 mg / ml = - 667 µg / ml. Since the value is negative, it is excluded for further calculation.

Maltose released from the precipitated enzyme activity = 1.778 mg /ml = 1778 µg / ml

Volume of the media was 2 ml. Therefore, total 3556 µg of maltose was released.

$$\text{Enzyme activity (U /ml/ min)} = \frac{\text{Maltose released (µg)} \times \text{Total Volume of Reactive Media (ml)} \times \text{Dilution Factor (DF)}}{\text{Molecular Weight of Maltose} \times \text{Enzyme Used (ml)} \times \text{Time of Incubation (min)}}$$

$$= \frac{3556(\mu g) \times 2\ (ml) \times 1\ (DF)}{342.3\left(\frac{g}{mol}\right) \times 0.2\ (ml) \times 10\ (min)}$$

$$= 10.4$$

3.6 Protein Estimation

The OD of both the precipitated protein and crude extract was taken at 600nm for protein estimation. Usually, the absorbance of protein is taken at 280nm. However, due to the addition of the reagents A and B, here the absorbance was taken at 600nm. Crude extract had more protein than the precipitated sample (table 3.4). Protein estimation was done using a standard curve for BSA (figure 3.8). The equation y = 0.066x + 0.0732 was used to calculate the concentration of the protein from the OD value. Accordingly, the concentration of protein in the crude extract was calculated to be 12.1 mg/mL whereas the concentration of protein in the precipitated sample was found to be 11.7 mg/mL.

Table 3.4: OD value at 600 nm for protein estimation

	Crude Protein	Precipitated Protein
Blank	0	0
Protein	0.873	0.845

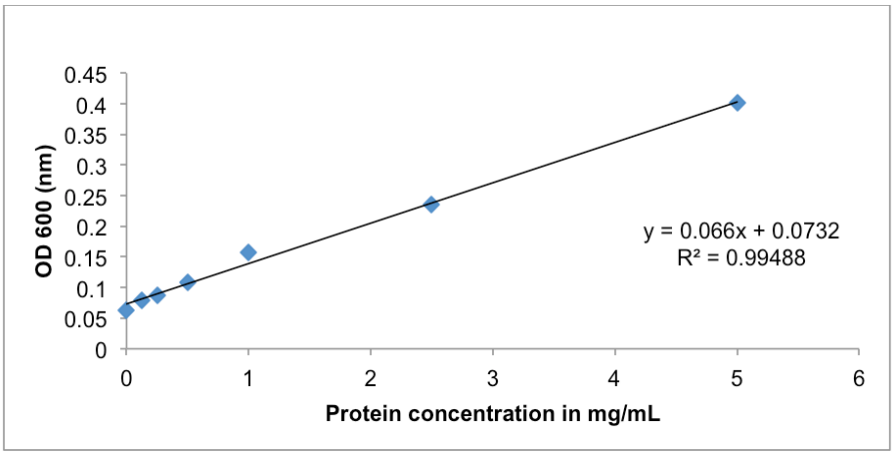

Figure 3.8: The standard curve for BSA (Giles et al. 2016)

$$\text{Specific activity (U /mg/ min)} = \frac{\text{Enzyme activity (U /ml/ min)}}{\text{Extracellular Protein Concentration } (\frac{\text{mg}}{\text{ml}})}$$

$$= \frac{10.4 \text{ (U /ml/ min)}}{11.7 \ (\frac{\text{mg}}{\text{ml}})}$$

$$= 0.89$$

The result for column chromatography was not very appreciable showing very irregular pattern of protein absorbance at 280 nm for the sixty fractions collected.

3.7 PCR, DNA Extraction and Gel Electrophoresis

After electrophoresis, the agarose gel showed DNA band when illuminated with UV light. The molecular weight of the 16s rRNA gene of *B.amyloliquefaciens* was found to be 1397 bp. This weight was calculated from the graph plotted (figure 3.10) using the known sizes of the 100 bp ladder and the distance travelled by each of the DNA bands in the gel during electrophoresis (figure 3.11).

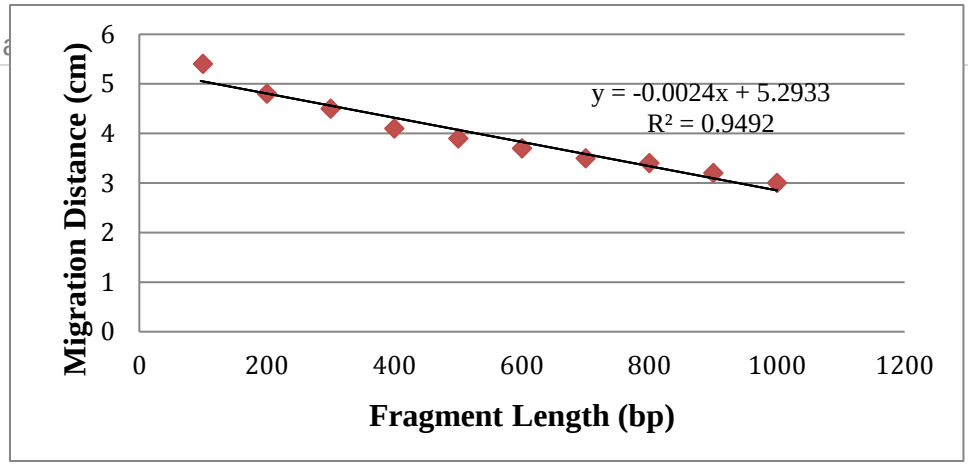

Figure 3.10: Graph plotted using the migration distance of each band and the known DNA ladder size

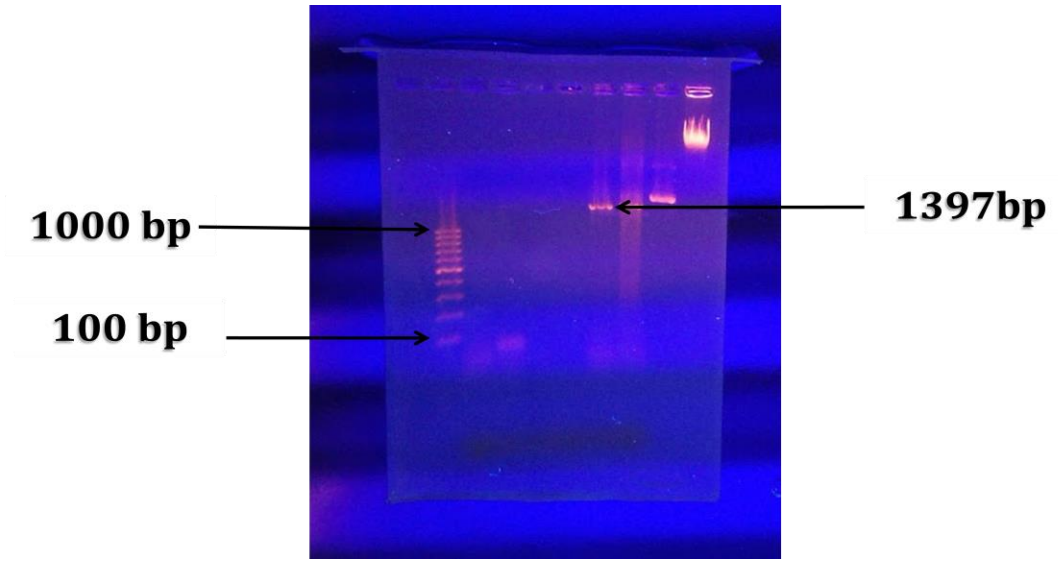

Figure 3.11: 16s rRNA gene were seen under UV light after gel electrophoresis
***Lane 1 = Blank, Lane 2 = DNA ladder (100 bp), Lane 3 = PCR mix + Extract of E.Coli DNA (no primer), Lane 4 = PCR mix + primer only, Lane 5 = PCR mix + DNA extract of *B.Amyloliquefaciens* (no primer), Lane 6 = PCR mix + DNA extract of *B. Subtilis* (no primer), Lane 7 = PCR product of *B.Amyloliquefaciens* DNA, Lane 8 = PCR product of *B.Subtilis* DNA, Lane 9 = pUC19, Lane 10 = Lambda DNA.

4) **Discussion**

Alpha amylase, for large scale industrial use, are preferred to be obtained from the Bacillus species because of its prodigious thermostability and because of the available expression systems (De Souza & de Oliveira Magalhães, 2010). In this study, the crude amylase obtained by centrifugation of the fermented bacterial culture showed very low activity. Previous studies with *Bacillus* species showed that enzyme activity can be as high as 940 U/ml for crude enzyme and 465 U/ml for precipitated enzyme (Annamalai et al. 2011) whereas in our experiment the activity for precipitated enzyme was found to be 10.4 U/ml.

The alpha amylase obtained from the *B. amyloliquefaciens* was purified by three steps. However, the yield of enzyme was very low. There are few possible causes which might have resulted in the low amylase production and activity. One of the primary reason could be the presence of protease in the solution which might have degraded the amylase (Gangadharan et al., 2006). The addition of protease phenyl methylsulphonyl fluoride (PMFS) to the enzyme mixtures could inhibit the function of the protease and improve the yield of amylase (Demirkan et al 2004). Additionally, some of the protein might have degraded during SDS-PAGE. Sample preparation is very crucial in SDS-PAGE to get a clear and high resolution protein band. Delay in the heating of sample after the addition of SDS buffer can result in protein degradation by peptide bond cleavage and, hence, the absence of the protein in the SDS-PAGE. Samples which are denatured by detergents are more sensitive to the proteolytic activities which increases the risk of degradation by protease given that proteases themselves are resistant to the effect of SDS (Grabski & Burgess, n.d.). Therefore, the samples should be prepared very carefully to overcome the aforementioned effects. Use of silver

staining which is 100 fold more sensitive, could be more useful than Coomassie blue stain in cases where the amount of protein is very less (Grabski & Burgess n.d.).

The activity of enzymes can be affected markedly by slight variation in the reaction conditions (Maarel et al 2002). The reaction condition for fermentation and enzyme activity used in this study was based on the conditions optimum for other strains of *B. amyloliquefaciens* since the strain of the bacteria was unknown. The temperature and pH in which the bacterial culture were maintained and enzyme activity was checked might not be optimum for this specific strain of *B. amyloliquefaciens*. Thus, characterization of the bacteria in various temperature, pH ranges could help to identify the optimum reaction conditions for its production and activity of alpha amylase. The experiment could be repeated later in the optimized condition to check for enzyme activity and production.

The total amount of protein in the sample obtained from ammonium sulphate precipitation was calculated to be 11.4 mg from the photometric absorbance at 600 nm and the standard curve for BSA. In each step of purification some of the protein is lost. The absorbance value of the eluted fractions collected from the resin column showed very irregular and insignificant reading. The amount of protein in the loaded sample might not be enough to provide an appreciable result. Perhaps, the concentration of protein was very low in the dialyzed protein or it was an artefact due to a problem in the preparative procedure of the packing of the column, the elution buffer or the resin bead preparation. The dialyzed sample was collected in Savar, Dhaka and then transferred to Chittagong in a ice box. The fluctuations in temperatures while transferring the dialyzed protein sample could have degraded the small amount of protein.

In previous studies, it was reported that enzyme concentration of 25-350 U/ml/min found to be effective in the desizing of fabric, which increases with an increase in the concentration of enzyme. However, for industrial purpose, optimum result is found with 200 to 225 units (Haq et al., 2010). Although the concentration of alpha amylase obtained in this study is very low, with a specific activity of 0.89 U/mg/min, it can be amplified by cloning the alpha amylase gene in suitable expression vectors. Studies claims that thermostable alpha amylase gene from *B. amyloliquefaciens* can be cloned and expressed in other bacteria such as *E.coli* to overexpress the protein by artificially inducing it with the help of a strong promoter (Demirkan et al., 2003). Cloning is a competent alternative in such cases where the expression of a gene is not satisfactory in the wild type strain (Aiba et al. 1983). Although, there will be limitations in the outcome of cloning as well.

Due to limitation of time, the initial goal of cloning the enzyme could not be conducted in this study. Nonetheless, the result of 16s DNA sequence can be used to run a NCBI BLAST to identify the strain and clone the enzyme for further research. The gene for amylase can be cloned in the cloning vector pBR322 and can be expressed in pET21d (Novagen) expression vector containing T7 promoter. The transformed plasmid can be transfected in to *E.coli* to over produce amylase in suitable reaction conditions.

The study can be extended further to find if random mutations of the gene have any effect in the enzyme activity. In a study, random mutation of *B.amyloliquefaciens* by ethyl methane sulphonate (EMS) showed amylase activity over 100 U/ml/min which was 1.4 fold higher than the activity of the parental strain (Haq et al., 2010). This strain of bacteria can be experimented with different kinds of mutant and compare with the parental strain. To obtain reliable result, the mutant bacteria should also be characterized because it is evident that the mutant enzyme might have different optimum reaction conditions (Demirkan et al. 2003)

5) **Conclusion**

The aim of this research was to produce and extract amylase from *B. amyloliquefaciens* for industrial use as a fabric desizing agent in textile industry. However, the enzyme activity and amount of protein was very less in the final product which is not industrially compatible. The research can be extended to over express amylase using the 16s rRNA gene sequence analysis result. The gene can be cloned in suitable vectors and by artificially inducing it to over express using a strong promoter. The study can be improved further by characterizing the bacteria and testing other parameters, such as mutation, to improve enzyme production.

6) References

Aiba, S., Kitai, K., and Imanaka, T. (1983). Cloning and Expression of Thermostable a-Amylase Gene from Bacillus stearothermophilus in Bacillus stearothermophilus and Bacillus subtilis. *Applied and environmental microbiology*. 46(5): 1059-1065.

Annamalai, N., Thavasi, R., Vijayalakshmi, S., and Balasubramanian, T. (2011). Extraction, Purification and Characterization of Thermostable, Alkaline Tolerant a-Amylase from Bacillus cereus. *Indian J Microbiol*.51(4): 424–429.

Deb, P., Talukdar, S. A., Mohsina, K., Sarker, P. K., & Sayem, S. A. (2013). Production and partial characterization of extracellular amylase enzyme from *Bacillus amyloliquefaciens* P-001. *SpringerPlus*. *2*. 154.

Dehghan H., Sarrafi Y., and Salehi P. (2015). Antioxidant and antidiabetic activities of 11 herbal plants from Hyrcania region, Iran. Journal of Food and Drug Analysis. 1-10.

De Souza, P. M., & de Oliveira Magalhães, P. (2010). Application of microbial α-amylase in industry – A review. *Brazilian Journal of Microbiology*, *41*(4), 850–861. http://doi.org/10.1590/S1517-83822010000400004

Gangadharan D., Sivaramakrishnan S., Nampoothiri K.M. & Pandey A. (2006) . Solid Culturing of *Bacillus amyloliquefaciens* for Alpha Amylase Production. *Food Technology. Biotechnology*. 44 (2) 269–274.

Genome [Internet]. (2010)- Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; [cited 2017 11 17]. Available from: https://www.ncbi.nlm.nih.gov/genome/?term=Bacillus+amyloliquefaciens+

Giles, S.K., Stroeher, U.H. and Brown, M.H. (2016). Measurement of Intracellular cAMP Levels

Using the Cyclic Nucleotide XP Enzymatic Immunoassay Kit in Bacteria. Bio Protocol 6(8): e1792.

Gopinath S. C. B., Anbu P., Arshad M. K. M., Lakshmipriya T., Voon C. H., Uda H. & Chinnin S. V. (2017). Biotechnological Processes in Microbial Amylase Production. *BioMed Research International*. (2017). 9 pages

Grabski A.C. and Burgess R.R.(n.d.). Preparation of protein samples for SDS-polyacrylamide gel electrophoresis: procedures and tips. Novagen, Inc. and 2 McArdle Laboratory for Cancer Research, University of Wisconsin-Madison, Madison, WI 53706. 10-12. http://wolfson.huji.ac.il/purification/PDF/PAGE_SDS/NOVAGEN_Prepare_Sample_PAGE_SDS.pdf

Hasan M. M., Marzan L. W., Hosna A., Hakim A. & Azad A.K. (2017).Optimization of some fermentation conditions for the production of extracellular amylases by using Chryseobacterium and Bacillus isolates from organic kitchen wastes. J*ournal of Genetic Engineering and Biotechnology*. 15(1). 59-68.

Held, P. (2012). Enzymatic Digestion of Polysaccharides. Retrieved from www.biotek.com.

Islam, M.R., Mondol, O.K., Rahman, M.S., Billah, M.M., Rahman, M.S., and Zohora, U.S. (2016). Screening of α-amylase producing bacteria from tannery wastes of Hazaribag, Bangladesh. *Journal of Biological Science*. 5(2): 1-10.

Khatiwada P., Ahmed J., Sohag M.H., Islam K., & Azad A.K. (2016). Isolation, Screening and Characterization of Cellulase Producing Bacterial Isolates from Municipal Solid Wastes and Rice Straw Wastes. *Journal of Bioprocessing & Biotechniques*. 6 (4). 5 pages.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem 193:265 (the original method)

Maarel, M. J. E. C. V. D., Veen, B. V. D., Uitdehaag, J. C. M., Leemhuis, H., & Dijkhuizen, L. (2002). Properties and applications of starch-converting enzymes of the -amylase family. *Journal of Biotechnology*. 94(2). 137-155.

Mehta, D., & Satyanarayana, T. (2016). Bacterial and Archaeal α-Amylases: Diversity and Amelioration of the Desirable Characteristics for Industrial Applications. *Frontiers in Microbiology*. 7. 1129.

Rawat, R. & Tewari, L. (2012). Purification and characterization of an acidothermophilic cellulase enzyme produced by Bacillus subtilis strain LFS3. Extremophiles. 16(4), 637-644.

Saha K., Maity S., Roy S., Pahan K., Pathak R., Majumder S. & Gupta S. (2014) Optimization of Amylase Production from B. amyloliquefaciens(MTCC 1270) Using Solid State Fermentation. *International Journal of Microbiology.* (2014) 7 pages.

Siddiqui, K.S., Feller, G., D'Amico, S., Gerday, C., Giaquinto, L., and Cavicchiolil, R. 2005. The Active Site Is the Least Stable Structure in the Unfolding Pathway of a Multidomain Cold-Adapted α-Amylase. *Journal of Bacteriology*. 187.

Sundarram, A. , & Murthy, T. P. K. (2014). α-Amylase Production and Applications: A Review. *Journal of Applied & Environmental Microbiology*, *2*(4), 166-175.

7) Appendices

Gram Staining

A smear of the bacteria was prepared on a clean glass slide. It was allowed to air dry followed by heat fixation by swiftly passing the slide over the flame of a spirit lamp. Then the fixed smear was stained with crystal violet for 1 minute and washed with distilled water. Then few drops of Gram's iodine was added and after 1 minute washed off with distilled water. Then it was flooded with 95% ethanol and washed with distilled water after 5 seconds. Safranin was added on the smear and washed off after 45 seconds. Then the smear was observed under the microscope.

Catalase

A clean glass slide was marked with B.A. in one end. A loopful of bacteria was taken and smeared on the glass in the middle. Few drops of hydrogen peroxide was added to the smear and was observed to see if there is any immediate formation of bubbles.

Oxidase

An oxidase test strip was taken and dipped one end in the colony of bacteria. Then dark purple color formation was observed

Methyl Red

6 ml MRVP broth was prepared in each test tubes and autoclaved. After autoclaving the broth was cooled and a loopful bacterial culture was inoculated. The test tubes were incubated at 37°C for 24 hours. After 24 hours, few drops of methyl red was added to the broth. Immediate formation of red colour was observed.

VP Test

6 ml MRVP broth was prepared in each test tube and autoclaved. After autoclaving the broth was cooled and a loopful bacterial culture was inoculated. The test tubes were incubated at 37°C for 48

hours. After 48 hours 12 drops of Barritt's reagent A was added followed by 6 drops of Barritt's reagent B. The tubes were left still for 10 minutes and formation of any red-pink colour of the broth was observed.

Sulfide Indole Motility Test

SIM media was prepared using the Sulfide Indole Motility Agar. A loop full bacterial colony was stabbed onto the cooled agar and incubated for 72hrs at 37^{o}C. Black color indicated a positive result for Sulfide test. Motility test results were confirmed by observing the turbidity of the tubes. If the entire tube is turbid, it indicates the bacteria are motile. The red color ring formation after the addition of few drops of Kovac's reagents was used to confirm the Indole test.

Citrate

6 ml media was prepared and autoclaved. Then the test tubes were left to cool in a slanted position in order to create agar slant. Using an inoculating loop, a single colony from a 24 hours fresh bacterial culture was taken and was streaked on the whole surface of the slant in a zigzag pattern. The test tubes were incubated at 37°C for 24 hours. The color of the media was observed to see if there is any color change of the agar.

Coomassie Blue Stain

1.25g Coomassie R-250, 225 ml methanol, 50 ml glacial acetic acid, and 225 ml distilled water was mixed to prepare 500 ml staining solution.

Distaining solution

100 ml distaining solution was prepared using 10 ml acetic acid, 50 ml methanol, and 40 ml distilled water.

Starch agar

To make 1% starch agar plate, 0.12g beef extract, 0.4g soluble starch, 0.48g agar was added to 40 ml of distilled water. The mixture was heated and then autoclaved before pouring in the plate.

Iodine Solution for Starch Hydrolysis (0.1N, 1%)

0.340g iodine and 0.660g potassium iodide was mixed with 100 ml distilled water.

Lysis Buffer

10g NaCl in 10 ml washing detergent was added and mixed. The volume was mad upto 100 ml with distilled water.

Sodium phosphate buffer

0.60g NaH_2PO_4 was dissolved in 100 ml of distilled water and 1.06g Na_2HPO_4 was dissolved in 150 ml distilled water. the two solutions were mixed to make the sodium phosphate buffer. The pH was adjusted by adding HCl.