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In Vivo Modulation of Bacillus Subtilis Sub Specie for High Level Production of Thermo-Stable Amylase

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ABSTRACT

The aim of the investigation was to construct a plasmid-DNA inserted into an empty *Bacillus subtilis* host for growth stability and synthesis of thermo-stable amylase. The method of *in vivo* modification described as follows: (1). Construct of 5' – 3' forward primer (AAGGAGACGCGTATGTTTGCAAAACGATTCAAAACC) and 3' – 5' reverse primer (ATGTGATCTAGAATGGGGAAGAGAACCGCTTAAG), (2). Use the primers to amplify a target regions of a mesophilic *Bacillus subtilis* 16s sequence obtained from National Centre for Biotechnology Information (NCBI) data, (3). Infusion cloning of aprE signal peptide bond with amplified amylase gene inserted a pBE-S-DNA vector and finally (4). Production and characterization of amylase using new *Bacillus* construct. The newly constructed *Bacillus subtilis* sub specie was compared to a commercial *Bacillus subtilis* to ascertain improved expression in conditions. The results showed growth of *Bacillus subtilis* sub specie at 52°C while commercial *Bacillus subtilis* was stable at 37°C. Submerged fermentation was used for production of amylase using starch as substrate. Amylase activities of 5.2 U/ml was obtained at 60°C and 4.3 U/ml at pH 6.0 by *Bacillus subtilis* sub-specie while activities of 4.3 U/ml at 50°C and 3.4 U/ml at pH 7.0 by commercial strain respectively. The crude enzyme samples were partially purified by 70% ammonium sulphate precipitation and their molecular weights were determined using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). The weight of amylase sample by constructed *Bacillus subtilis* sub specie was observed to be in the range of 75 - 77KDa while sample of commercial *Bacillus subtilis* was in the range of 65 - 70kDa. In conclusion, in vivo modification technique proved to be useful in gene regulation and in this study developing a high active strain for synthesis of thermo-stable amylase. Further work on regulation of the amino acids positioning, developing cocktail of promoters and activation of microbial signaling are possible research targets that can influence properties of peptide bonds for optimal amylase functionality in industries.

Keywords: *Bacillus*, Signal Peptide bond, Submerged, Amylase, Thermostability,

INTRODUCTION

The major contribution of industrial amylase to global market has been through microbial application and it has been estimated to rise in the nearest future. Microbial amylase is mostly preferred due to its short growth period, higher productivity and thermo-stability [1]. They are widely used in industrial processes due to their low cost value, large productivity, stability, plasticity and vast availability [2]. Among the bacterial sources, *Bacillus subtilis*, *Bacillus staerothermophilus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus acidocaldarius*, *Bifidobacterium bifidum and Bifidobacterium acerans* are important species that have been used in production of industrial amylase amongst other enzymes [3]. The bacterial amylase is among the most widely studied amylase and highly homologous with respect to primary and tertiary structures [4]. Genetic improvement of bacterial extracellular amylase production is achieved by applying a range of strategies based on molecular cloning tools.

These include: 1) enhancement of expression level through amplification of gene copy number [5]; codon usage optimization [6], or strong promoters being used to boost gene transcription [7]; 2) enhancement of secretion by modulation of signal peptides [8]; fusion to heterologous signal peptides for efficient targeting to the translocase [9]; increasing the copy number of signal peptidase genes [10], or deregulation and/or co-expression of chaperon encoding genes to make efficient protein folding [11]; 3) improvement of productivity

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through re-designing the capacity of the secretion machinery by targeted deletion of genes encoding non-beneficial extracellular enzymes [12]; or genome reduction [13]; and 4) improvement of the productivity by preventing degradation of extracellular enzymes using protease deficient strains [14]. Overproduction of secreted amylase sometimes severely affects the secretory system of an organism [15]; and eventually results in a secretion stress response that may limit secretion [16; 17]. Moreover, it is almost impossible at the present stage to enhance the maximum synthesis and secretion capacity by site-directed gene disruption and expression in a specific strain. Alternatively, data from genome shuffling and genome size reduction studies; strongly suggest that a natural strain should exist with a maximum capacity for synthesis and secretion of extracellular amylase as a result of accumulation of mutations and shuffling of the genomes. Bacterial extracellular amylase production is a complex process, in which the efficiencies of transcription and translation of the amylase-encoding genes as well as translocation define the concentration in the growth medium and are under control of the bacterial host. The secretion of protein to the periplasm offers an attractive route to produce heterologous proteins that contain disulfide bonds [18]. In this approach, the N-terminus of the heterologous protein is fused to a signal peptide that mediates translocation of the protein from the cytoplasm to the periplasm [19]. The signal peptide is cleaved during the translocation process and its significance in protein secretion as a target recognition motif is a well-established concept but its role in directly modulating the properties of the pre-proteins and export process has not been well characterized [20]. The earliest studies on signal peptides were related mainly to their direct interaction with the membrane for protein insertion into and across the membrane barrier. Stability studies have only been reported with mutants of the mature protein which are known to refold at rates similar to the precursor form or with slow folding suppressor mutants of export defective preMBP [21]. Moreover, low concentrations of endogenous proteins in the periplasm make it easier to isolate the heterologous protein from host protein contaminants at laboratory scale [22]. Thus, the yields of heterologous proteins in the periplasm are often reported to be low. To improve protein accumulation in the periplasm, extensive studies have focused on the primary structures of signal peptides [23; 24]. Signal peptides are commonly composed of three distinct regions: a charged N-terminal region, a hydrophobic core region often referred to as the H-region, and a C-terminal region recognized by the signal peptidase [25]. A large body of literature using *E. coli* proteins or fusion proteins as cargo proteins suggests that increasing the hydrophobicity of the H-region promotes protein translocation. However, a few mutagenesis studies of heterologous protein production showed that increasing the signal peptide hydrophobicity did not improve the yields [26; 27). The aim and objective of the investigation is to construct a new plasmid DNA infused with aprE signal peptide bond inserted into an empty *Bacillus subtilis* host for improved growth stability and secretion of thermo-stable amylase.

MATERIALS AND METHODS

1. Identification of Bacillus subtilis Genome

A complete *Bacillus subtilis* 16s genome was obtained from the National Center for Biotechnology Information (**NCBI**) database. The sequence was use for the purpose of this investigation.

2. Primer construct

The complete sequence obtained from NCBI was analyzed and used for construct of specific forward and reverse primers having signal peptide targeted amylase gene insert. Primer design and quality are critical for the success of the In-Fusion reaction. The following properties were considered: 1). 5' end of the primer contained 15 bases that are homologous to 15 bases at one end of the insert. 2). The 3' end of the primer also contained sequence that is specific to the target gene. 3). Other properties considered were GC-content between 40–60%, melting temperature (Tm) between 58–65°C with ≤ 4°C.

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3. Construct of plasmid library

Construction of Expression Plasmid Library for in-fusion reaction procedures was followed according to the user manual of the In-Fusion™ HD PCR Cloning Kit: 1). Construct of expression plasmid by inserting a target gene into the multicloning site downstream from the secretory signal peptide of pBE-2 DNA. 2). Use the restriction enzymes Mlu I and Eco52 I to completely digest the expression plasmid constructed. 3). Isolate and recover the expression plasmid fragment using agarose gel electrophoresis. 4).Linearize the expression plasmid by PCR amplification. 5). Prepare the In-Fusion™ reaction solution by mixing the SP-DNA mixture and the expression plasmid digested by the restriction enzyme and mix the reaction solution by pipetting. 6). Allow the mixture to incubate at 50 for 15minutes and then incubate on ice. 7). Thaw the *E. coli* HST08 premium competent cells on ice immediately prior to their use and after thawing the cells, mix them gently to homogenize and transfer 100 µl of competent cells to a 14 ml rounded-bottom test tube. 8). Add 2µl of the In-Fusion™ reaction solution diluent from Step 5 to the tube in Step 7 and incubate on ice for 30minutes. 9). Incubate at 42°C for 45seconds and incubate on ice for 1 to 2minutes. 10). Add SOC culture medium pre-warmed to 37°C to obtain a final volume of 1ml and shake at 160 to 225 rpm for 1 hour at 37°C. 11). Spread a suitable volume on an LB plate containing ampicillin (100 µg/ml) and incubate overnight at 37. After suspending the colonies from the plate in LB and harvesting them, purify the produce plasmid library.

4. Genomic DNA, plasmid isolation and purification

Genomic deoxyribonucleic acid (DNA) was extracted using the Sigma-Aldrich GenElute™ bacterial genomic DNA Kit (**Cat No: NA2120**) while following standard protocol and purification was carried out for transformation purpose using commercial available purified kits. The Thermo Scientific GeneJET Miniprep Kit (**Cat. No: #K0503**) was used for plasmid isolation while Thermo Scientific GeneJET Gel extraction and DNA cleanup Microkit (**Cat. No: #K0831**) was used for purification.

5. Construct of Bacillus sub specie

High efficient *Bacillus subtilis* sub specie was constructed by inserting an infusion of aprE signal peptide and amplified target amylase gene using In-Fusion HD Cloning Kit from Takara Laboratories Inc. Cat. Nos. (011614).]. Further isolation of plasmid DNA from the transformed isolates was done using Thermo Scientific GeneJET Plasmid Miniprep Kit, Cat. No. #k0503. The plasmid DNA isolated from the constructed *Bacillus subtilis* sub specie was used to confirm successful insert.

6. Growth stability

A pure 24hour old colony of *Bacillus subtilis* sub specie and *Bacillus subtilis* respectively were grown on separate Luria Bertani Broth (Tryptone 1.0g, Yeast extract 0.5g, NaCl 1.0g, Starch 1.0g) at 37°C, pH 7.2 ± 0.2 for 24 hour. Every hour, 1 ml of each culture specimen was collected and used to obtain optimal growth condition. Spores were concentrated and subsequently centrifuged for 10 min at 30,000 × *g*. The wet density of the whole spore was measured according to the method in reference [28].

7. Amylase production

Submerged fermentation technique is a unique production method for enzyme extraction. 100ml of the following ingredients(%): (Glucose 1.0g, $Ca(PO4)_2$ 0.5g, $(NH4)_2SO_4$ 0.05g, $MgSO_4.7H_2O$ 0.001g, $MnSO_4.7H_2O$ 0.001g, $FeSO_4$ 0.001g, KCl 0.02g, Yeast extract 0.05g) was prepared in a sterile 250ml conical flask. Microbial suspension of both constructed *Bacillus subtilis* sub specie and commercial *Bacillus subtilis* were prepared respectively and from each sample 1.0ml was measured and further inoculated into separate sterile 250 ml conical flask containing the ingredients. A submerged fermentation protocol according to [29]; was used for the production of amylase using starch as carbon source.

8. Enzyme purification

The crude amylase extracts were purified and concentrated using ammonium sulfate. The procedure according to [30]; descried as

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follow: 1). Use of fresh, desiccated ammonium sulfate to ensure uniform and rapid dissolution. 2). Ammonium sulfate was placed overnight at 120°C drying oven in a large beaker (ammonium sulfate decomposes at 220°C). 3). Dried clean sample was carefully grinded into a fine powder. Make sure you wear a dust mask and use ground powder immediately. 4). Add the powder slowly but steadily with thorough mixing. (For 70% saturation, 43.6 grams of the powder was mixed with 100 mls of sample). 5). Allow precipitation to continue for 30 minutes at 4°C with stirring. 6). Recover precipitate by centrifugation. 7). Remove supernatant and re-spin briefly to clear remaining ammonium sulfate. 8). Re-suspend pellets in a volume of buffer equal to the volume of the extract.

9. Enzyme Assay

The method of [31]; was used to estimate amylase activity. 250 µl of enzyme sample was incubated with 250 µl of 1% soluble starch solution in 20 mM phosphate buffer pH 7.0 at 37°C for 30 min. The reducing sugar from each sample was measured by adding 250 µl of 3, 5-dinitro salicylic acid reagent to stop the reaction. The tubes were boiled at 100°C for 5 min, cooled and absorbance was measured at 540 nm with the UV spectrophotometer (Spinco Biotech. PVT LTD). The formula below was used to calculate the amylase activity:

$$\text{Amylase Activity} = \frac{\text{Quantity of Reducing Sugar x Dilution Factor}}{\text{Incubation Time (min) x Total Vol. of Reaction}}$$

10. Optimal temperature (°C)

The reaction mixtures for assay were incubated at various temperatures of 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, 90°C and 100°C. To 1ml of the incubated samples, 0.5 ml of the enzyme from the different temperature was mixed with 0.5 ml of iodine and the resulting colour (**blue black**) was determined for its absorbance at 540 nm.

11. Optimal pH

The reaction mixtures prepared were adjusted to different pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 using 6N HCl and 5N NaOH solutions intermittently. 0.5 ml of each sample was mixed with 0.5 ml of iodine and incubated for 40 minutes at 30°C. The resulting colour (**blue black**) was determined for its absorbance at 540 nm. The concentration of reducing sugar release was then calculated for glucose.

12. Protein estimation

The protein concentration of the crude amylase samples was determined according to [32]; using bovine serum albumin (BSA) as standard.

13. Amylase Molecular Weight

The molecular weight of partially purified (70% ammonium sulphate) amylase samples was determined using sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE). Each sample was loaded onto a 12.5% SDS-PAGE gel. The gel was left to run at an initial 50 voltage, and then increased to 70 voltage and finally 100 voltage. After the completion of gel run, it was flooded with commasie blue staining reagent for 1h, thereafter a prepared solution mixture of (400 ml ethanol, 100 ml glacial acetic acid and 500 ml distilled water) was applied to remove the commasie blue stain. Molecular weight of each sample was identified using a protein marker.

RESULTS AND DISCUSSION

Plasmid Construct

Studies on bacteria plasmid stability have been problematic and antibiotics have been used to ensure plasmid maintenance. This has been a major limitation during *in vivo* studies. The ability to provide genes for complementation or reporter constructs on plasmids is an important tool in studying bacterial viability. In this study *Bacillus subtilis* sub specie plasmid was constructed with resistance to antibiotic such as kanamycin which is usually taken up by eucaryotic cells. Similar studies carried out has shown that construct of promoter-less *cat* genes have successfully been used as reporter genes in cloning bacterial promoters that are active inside host cells [33].

Also [34]; had reported that large-scale experiments requiring protein expression from thousands of genes require an efficient method for cloning the genes into protein expression

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vectors. Traditional cloning methods based on restriction enzyme digestion and ligation was not practical due to the individual requirements and specifications for each construct. In contrast, recombinational cloning, or the transfer of DNA from one vector to another based on sequence homology, allows high-throughput cloning of genes into protein expression vectors under largely universal conditions without the use of restriction digests or ligation reactions. Recombinational cloning uses site-specific recombination, where both the insert and vector contain the required nucleotide sequences recognized by the cloning enzymes necessary for the recombinational event to occur. These nucleotide-specific sequences are referred to as the recombination sequences. Another advantage of recombinational cloning is the construction of an entry clone, an intermediate clone that functions as a holding or storage clone that allows the flexibility of transferring a single insert into multiple expression vectors. The PCR primers used to amplify a gene are designed to contain a 3' gene-specific portion along with a common 5' tail sequence for adding flanking recombination sites. For convenience, cost, and lower error rates, it was ideal to employ 3 PCR steps to build a recombination-competent fragment, which was possible with the In-Fusion® cloning scheme where only 15 base pair (bp) are needed for successful homologous recombination. All primers had a similar melting temperature and anchored in start or stop positions of the open reading frame. The gene-specific 5′ primer started with ATG, and the 3′ primer started at the end of the position of the common stop codon. These primers designed using bases from the relevant end (5′ or 3′) of the target sequence and adding additional bases until the desired melting temperature (often 60°C) was reached. The In-Fusion® system of combining a linearized vector with one or more PCR products with overlapping ends to generate clones is based on the simple principle of homologous recombination [35]. The molecular mechanism of this ligation-independent cloning system started with the incubation of the In-Fusion® enzymes, PCR product(s), and linearized vector. The 3'- to -5' exonuclease activity removed nucleotides from the ends of the double-stranded DNA samples [36]. The exposed complementary single stranded ends of 15 bp homologous regions of the PCR fragments and linearized vector anneal, in which were incited with the addition of arpE signal peptide binding protein. A DNA polymerase was present to insert bases into the gaps of DNA, and a DNA ligase functions to seal in the nicks, resulting in a seamless double stranded linear construct of a vector and the sequence of interest. Similar to the Gateway® system, positive selection for the correct entry clone was mediated by a Kanamycin resistance marker.

Bacilli Growth Stability

Fig. 2a is the genomic DNA (> 1,500bp) and amplified by polymerized chain reaction and used in the construction of plasmid DNA. Fig. 2b shows the molecular weight of the plasmid DNA band (> 7,000bp). The specimens were obtained using the 1.5% agarose gel and images recovered with the aid of UV trans-illuminator. Microbial growth curve describes the differential activities occurring within the cell through log phase, lag phase, stationary to death phase. But these activities are controlled by genes and activation timing of such genes between a wild type strain and transformed strain is usually different. Relevance to virulence, several research activities have differentiated effect of optimal temperature between wild type and engineered strain especially constructed plasmid DNA for optimal performance. In this experiment *Bacillus subtilis* sub specie showed optimum growth at 52°C and the most suitable temperature for the growth of mesophilic bacterium has been found to be 45°C. The growth curve pattern was studied by growing the bacterium in pivoskaya broth and comparing with a commercial strain *Bacillus subtilis*. The optimal growth (O.D) was determined after 5th hour for *Bacillus subtilis* sub specie while optimal growth of commercial strain was observed after 6th hour of growth as illustrated in Fig. 3. Since the first report on transformation of *Bacillus subtilis*, the competency of *B. subtilis* has intensively been

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studied revealing highly interconnected regulatory networks [37]. These interconnected regulatory networks are responsible for fine-tuning of the single-cell and social behavior of *B. subtilis* in a culture medium, especially during their entrance to stationary phase. When *B. subtilis* cells enter stationary phase due to nutrient deprivation and high cell density, they start to differentiate into various subpopulations. Some of them become motile, while the others form biofilm [38]; secrete degradative enzymes and antibiotics [39]; or finally sporulate [40]. Another small subpopulation differentiates into competent cells able to take up extracellular DNA [41].

Effect of optimal temperatures (°C) and pH

The effect of plasmid construct showed enhanced growth performance of the *Bacillus subtilis* sub specie in growth curve appearance, optimal time and temperature (°C) as compared to the commercial *Bacillus subtilis* strain. Although many microorganisms produce this enzyme, bacterial most commonly used for different industrial application are: *Bacillus licheniformis, Bacillus amyloliquifaciens and Bacillus subtilis* amongst others. Amylase stand out as a class of enzymes, which are of useful applications in the food, brewing, textile, detergent and pharmaceutical industries because they are mainly employed for starch liquefaction to reduce their viscosity, production of maltose, oligosaccharide mixtures, high fructose syrup and maltotetraose syrup. The use of the submerged culture is advantageous because of the ease of sterilization and process control is easier to engineer in these systems. The production of extracellular amylase in bacilli is regulated by a number of genes. The most significant of these appear to be amyR, papM, and tmr. The amyR gene is a specific regulator of amylase that can be linked to its structural gene (amyE) by transformation. The papM gene regulates the production of amylase and protease as well as a number of other pleiotropic properties [42]. The tmr gene encodes resistance to tunicamycin. This mutant also produces high levels of extracellular amylase. *Bacillus subtilis* Marburg 6160 carries amyRI and *B. subtilis* var. amylosacchariticus carries amyR3 [43]. It is possible to introduce genes that are also involved in the production of amylase from closely related bacilli into *B. subtilis* by deoxyribonucleic acid (DNA)-mediated transformation. Frequently, this procedure results in the improvement of enzyme production. The present report not only proved this assumption but also established that the effect of multiple genes is not additive, but of a synergistic nature. Production of enzyme was done by submerged fermentation technique using the newly constructed strain and activities obtained were compared to that of amylase by commercial *Bacillus subtilis*. From the results, sample of measured amylase activities against optimal temperature is shown in Fig. 1 while against optimal pH is shown in Fig. 2 respectively. From Fig. 1, the *Bacillus subtilis* sub specie had measure an optimal activity of 5.2U/ml at 60°C while value of 4.3U/ml at 50°C was recorded as activity for amylase by commercial strain in Figure 2. Velocity of an enzyme reaction increases with increase in temperature up to a maximum and then declines. The optimum temperature for most of the enzymes has been recorded between 40°C - 45°C. However, a few enzymes (e.g. venom phosphokinases, muscle adenylate kinase) are active even at 100°C. In general, when the enzymes are exposed to a temperature above 50°C, denaturation leading to derangement in the native (tertiary) structure of the protein and active site are seen.

Earlier investigation reported that there are two temperatures that need to be in optimum range during production and they are temperature for the growth of the microbial source and optimum temperature at which maximum production of amylase takes place. The optimum temperatures for both were studied in strains of *B. licheniformis* and *B. subtilis* 29. The optimum temperatures for growth and amylase production were found to be 45°C to 46 °C and 50 °C, respectively. In *B. subtilis* 29 the optimum temperature for growth (42°C) was higher by 5°C than that of amylase formation (37°C). Thermophilic archaeal amylase is active and grows at high temperatures. An optimum range of 95-100°C was observed to yield maximum enzyme

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activity in a study involving *Pyrococcus furiosus* [44]. *Penicillium fellutanum* showed an optimum activity of 98±4.6 U/ml at a temperature of 30°C [45].

Increase in the hydrogen ion concentration (pH) considerably influences the enzyme activity and a bell-shaped curve is normally obtained. Each enzyme has an optimum pH at which the velocity becomes optimal. The activities (U/ml) for optimal pH recorded from pikovskaya media by constructed Bac*illus subtilis* sub specie and commercial *Bacillus subtilis* are shown in Fig. 3 and 4 respectively. From the Fig. 3, optimal activity of 4.3U/ml at pH 6.0 was recorded for amylase produced by *Bacillus subtilis* sub specie while amylase from commercial strain recorded activity of 3.4U/ml at pH 7.0 as shown in Fig. 4 respectively. Most of the enzymes of higher organisms show optimum activity around neutral pH (6-8). There are, however, many exceptions like pepsin (pH 1-2), acid phosphatase (pH 4-5) and alkaline phosphatase (pH 10-11) for optimum pH.

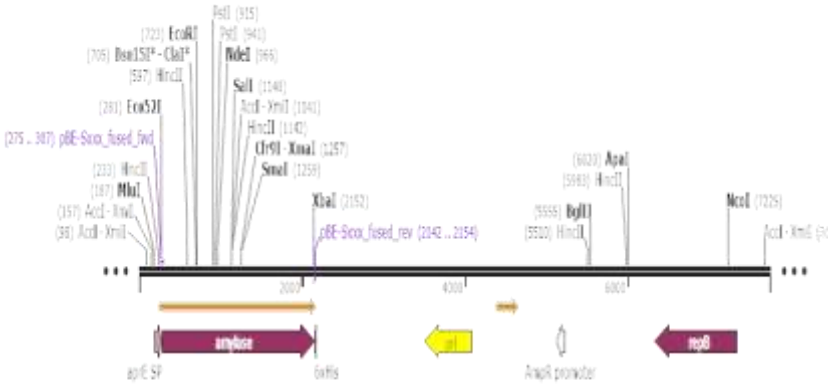
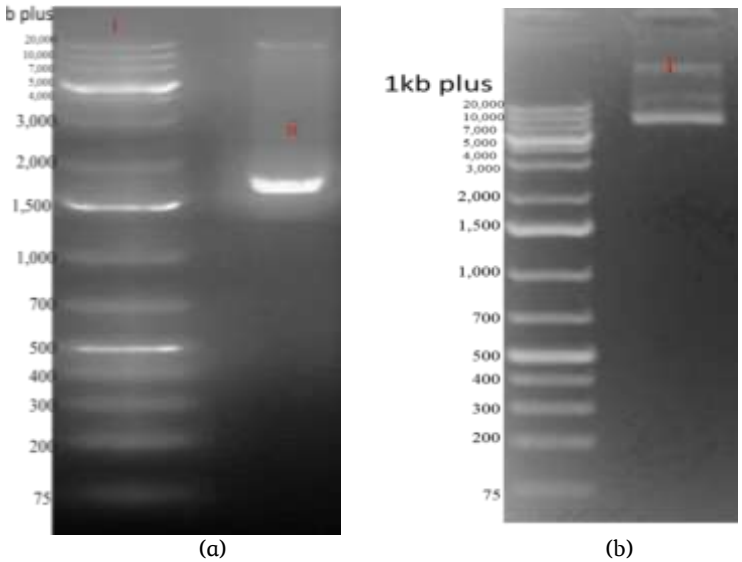


Fig. 1: A summary of *Bacillus subtilis* sub specie plasmid construct for.



(a) (b)

Fig. 2: The molecular weight of genomic and plasmid samples identified in these images.

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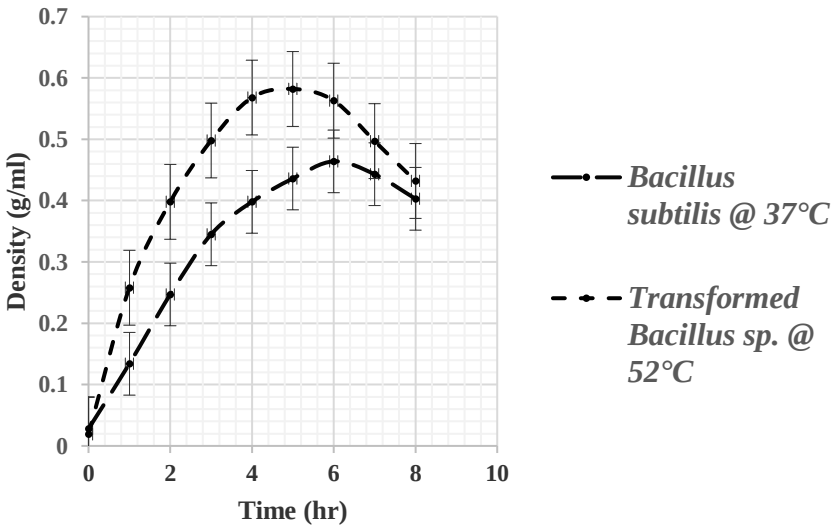

Fig. 3: Comparative growth curves of *Bacillus* species

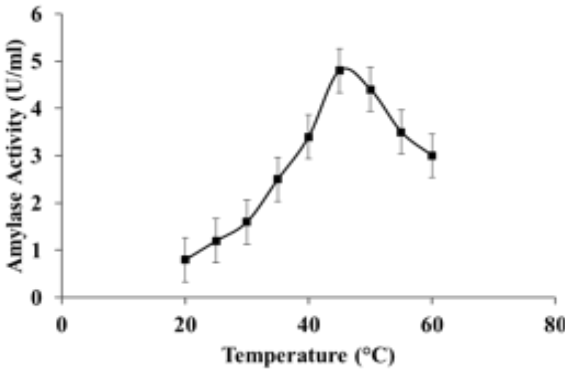

Fig. 1: Effect of Temperature (°C) on amylase activity by *Bacillus subtilis* sub specie

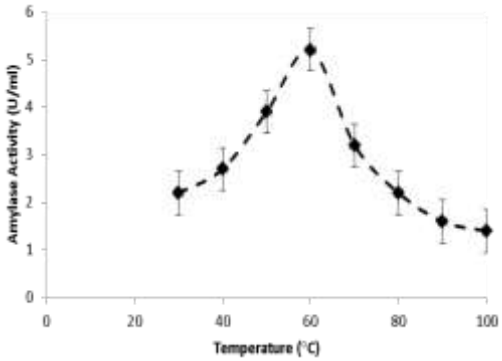

Fig. 2: Effect of temperature (°C) on amylase by commercial *Bacillus subtilis*

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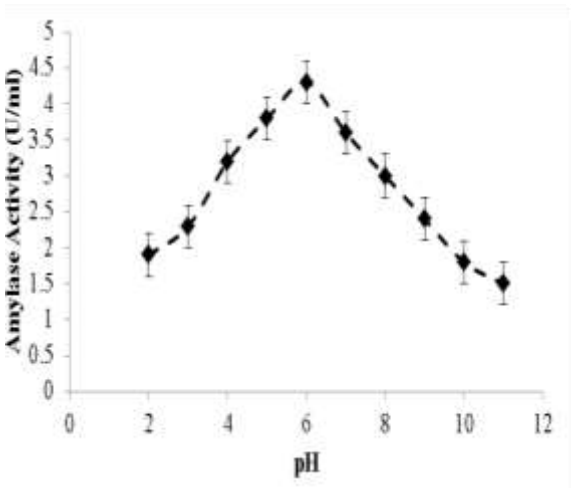

Fig. 3: Effect of pH on amylase activity by *Bacillus subtilis* sub specie

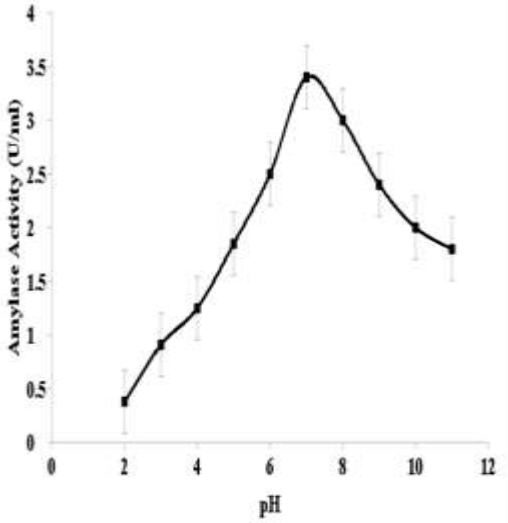

Fig. 4: Effect of pH on amylase activity by *Bacillus subtilis*

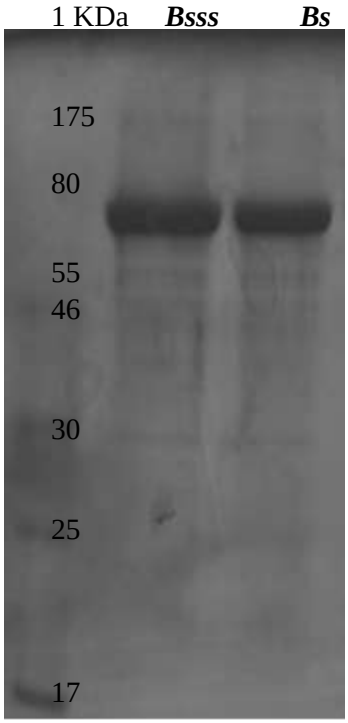

Fig. 5: Protein expression

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An earlier investigation recorded that optimum pH was a critical factor for the stability of enzyme produced. Amylase is pH sensitive and hence care must be taken to control the pH of the production process. *Pyrococcus furiosus* produces amylase which showed activity at an optimum pH of 6.5–7.5. The maximum enzyme activity was 534 U/g when wheat bran was used as the substrate with optimum pH conditions of 6.0. *Bacillus amyloliquefaciens* produced the enzyme with an optimum pH of 7.0 [46]. *Halomonas meridiana* was studied for optimization of amylase production. The study revealed that the amylase exhibited maximal activity at pH 7.0, being relatively stable in alkaline conditions [47]. The optimum pH for production of the enzyme by *Bacillus* sp. isolated from dhal industry waste was found to be 6.5 [48].

Determination of molecular weight of amylase samples using 12.5% SDS-PAGE analysis was carried out. From the Fig.m 5, amylase bands are amylase from *Bacillus subtilis* sub specie (*Bsss*) and commercial *Bacillus subtilis* respectively. The weight of amylase sample by constructed *Bacillus subtilis* sub specie (Bsss) was observed to be in the range of 75 - 77KDa while sample of commercial *Bacillus subtilis* (Bs) was in the range of 65 - 70kDa. Also from the image it was observed that yield of amylase from constructed strain was more than the commercial strain.

CONCLUSION

The successful completion of this investigation was achieved by meeting up with expected aims and goals. A novel strain of *Bacillus subtilis* sub-specie was successfully developed with a constructed plasmid DNA for overexpression of amylase enzyme. The new strain was able to grow at extreme conditions allowing possessing properties of more than strain. The amylase produced gave a high activity and was stable at high temperature (°C) and low Ph. This research has the potential to contribute to an academic understanding of differential metabolic reactions of living organisms, also an industrial understanding in production and development of novel biological agents.

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