International Journal of Biotechnology and Microbiology
www.biotechnologyjournals.com
Online ISSN: 2664-7680, Print ISSN: 2664-7672
Received: 16-03-2021; Accepted: 11-04-2021; Published: 20-05-2021
Volume 3, Issue 1, 2021, Page No. 86-93

# Determination of alpha amylase shelf life concentration at increasing temperatures

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**Abstract**

An investigation was carried out on the synthesis and characterization of alpha amylase (EC.3.2.1.1). The main aim was achieved using a statistical tool to determine concentration of shelf life at increasing temperatures (ºC). The methodology involved a *Bacillus subtilis* sub sp. construct used for the synthesis of a high efficient alpha amylase (EC.3.2.1.1) and compared to the activities of a wild type *Bacillus subtilis*. The results showed that the novel Am *Bacillus subtilis* sub sp. expressed optimal growth density of 0.588mg/ml at the 4th hour as compared to the native strain with 0.464mg/ml at the 6th hour. Assay of sample by the construct recorded 4.2U/ml and 5.2U/ml at pH 5.0 and 60ºC respectively as compared to activities of 1.86U/ml and 1.64U/ml recorded at pH 7.0 and 40ºC respectively for native *Bacillus subtilis*. The shelf life concentration of alpha amylase [EC.3.2.1.1] from construct was estimated after 24 hr and 48 hr respectively at different temperatures (ºC) using Microsoft Excel 2010 and the results showed that the secretome from novel strain recorded values of 100% at 50ºC, 115.9% at 60ºC and 88.38% at 70ºC after 24hrs and 94.62%, 98.81%, and 88.38% after 48hr respectively. The observations confirmed efficiency of stable alpha amylase [EC.3.2.1.1] by a construct. This further implies that advanced research on alpha amylase (EC.3.2.1.1) is required for high throughput industrial activities and research diversity.

**Keywords:** gene, construct, fermentation, enzyme, bioassay, stability

## Introduction

Thermophilic *Bacillus* species have been identified with high temperature (ºC) tolerance properties and used to synthesize high performance alpha amylase (EC.3.2.1.1). It has been reported that alpha amylase (EC.3.2.1.1) from natural thermophilic *Bacillus licheniformis, Bacillus coagulans, Bacillus amyloliquefaciens* amongst others are of special interest because they are not usually denatured by high temperatures and are even active at elevated temperatures and lower pH (Eijsink *et al*. 2004, Turner, 2009, Vielle and Zeikus, 2001) [7, 37, 38]. The genus *Bacillus subtilis* produces a large variety of extracellular enzymes and alpha amylase (EC.3.2.1.1) has had a high level of significant industrial application in degrading starch used in food, pharmaceutical, brewing, sugar production, designing in textile industries (Shivange *et al*. 2009) [29], and in detergent manufacturing processes (Romero and Arnold, 2009) [28]. Each application of alpha amylase (EC.3.2.1.1) requires unique properties with respect to specificity, and stability at increasing temperature (Wintrode and Arnold, 2001) [42]. Current research activities have focussed on improving the thermostable activities of alpha amylase produced by *Bacillus subtilis* and had led to development of statistical manipulation, enzyme optimization software, and strain development techniques such as *Bacillus* expression system used for development of optimized alpha amylase and novel strains of *Bacillus* species. The implementation of these techniques in industries is to improve enzyme activities, specific activities, and shelf life stability towards achieving desirable by products (Morimoto *et al*. 2008, Kumari and Kayastha, 2011) [21, 18]. Another recent research into alpha amylase (EC.3.2.1) stability is determination of residual activity which has further increased the studies of measuring ratios of alpha amylase (EC.3.2.1) stability before and after its activity within a reaction. The design of novel studies into enzyme performance has further increased application diversity into biotechnological processes in many sectors. As a result of this, the research aim was to determine the shelf life concentration of alpha amylase (EC.3.2.1) synthesized by a novel *Bacillus subtilis* sub sp. at different temperatures (ºC) using statistical application.

## Materials and Methods

### Construct of *Bacillus subtilis* sub sp.

A *Bacillus subtilis* complete genome sequence was obtained from the National Centre for Biotechnology Information (NCBI) online. With the aid of SnapGene tool, the genome was analysed and used for construct of specific short forward and reverse primers.

Primer design and quality are critical for the success of an In-Fusion reaction such that 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 was specific to the target gene. 3). GC-content was between 40 - 60%, 4). melting temperature (Tm) was between 58 - 65°C with ≤ 4°C. 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. 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. The plasmid DNA isolated from the constructed *Bacillus subtilis* sub specie was used to confirm successful amplification which was done inserting an infusion of aprE signal peptide and target amylase gene using In-Fusion HD Cloning Kit from Takara Laboratories Inc. Cat. Nos. (011614).

A genome amplification as described here was used to develop a novel *Bacillus subtilis* sub specie in our earlier investigation.

**Microbial Growth**

A pure *Bacillus subtilis* colony of approximately 1.67 X $10^5$ cell size in volume was inoculated into 10ml test tube containing 5ml Luria Bertani broth. This reaction was incubated for 6 hrs at 37ºC. At the end of the incubation, 1 ml of the specimen from the suspension was further inoculated into a 250 ml conical flask containing 100 ml Luria Bertani Broth and then incubated for 18 hrs at 37ºC. To determine growth density of the cell, 1 ml of the suspension was poured into a cuvette and concentration of the suspension was measured against absorbance at 600nm using a uv spectrophotometer (Spinco Biotech. PVT LTD). This procedure was repeated with the newly constructed *Bacillus subtilis* sub sp. which was obtained following a *Bacillus* expression system protocol as described above. Further analysis was carried out to confirm a successful construct. This was done by growing the novel strain on Luria Bertani fortified with 50ul kanamycin. The ability of the novel strain to grow on the antibiotic is a characteristic of the high performance activity differentiating it from the wild type strain.

**Amylase Synthesis**

The following ingredients were composed for submerged fermentation technique used for the synthesis of alpha amylase (EC.3.2.1.1): 10.0g tryptone; 5.0g Yeast extract; 10.0g NaCl, 1.0L $DdH_2O$ and pH was adjusted to 7.0. The mixture was homogenized using magnetic stirrer with hot plate at a low temperature (ºC), and furthermore sterilized at 110ºC for 10minutes using an autoclave. The temperature of the medium was allowed to drop to room temperature before use. Into two separate 250ml conical flasks labelled A and B, 100ml from the medium above was measured and dispensed into each flasks. 0.5g of potato starch was added respectively into the separate flasks and with the aid of a magnetic stirrer, homogenous mixtures were obtained. 1ml of the wild type *Bacillus subtilis* suspension and 1ml of the constructed *Bacillus subtilis* sub sp. suspension was inoculated separately into flask A and B respectively. The flasks were then incubated at 37ºC, speed of 115 rpm for 24 hr. At the end of incubation period, crude extracts of alpha amylase (EC.3.2.1.1) were extracted using a cold centrifuge and kept in 4ºC for further analysis

**Assay of alpha amylase (EC.3.2.1.1)**

Determination of reducing sugars was carried out by dinitrosalicylic acid (DNS) method following the method described according to (Keharom *et al*. 2016) [16]. The stock solution of standard maltose (2,000 mg/L) was prepared by dissolving 0.2 g of D-(+)-maltose monohydrate in 100 mL deionized water (DI) water. 20 mM sodium phosphate buffer solution with 6.7 mM sodium chloride, was prepared in 250 mL deionized water using sodium hydrogen phosphate 0.7148 g, sodium dihydrogen phosphate 0.6898 g and sodium chloride 0.0980 g, and then adjusted to pH 7.0 with 1M sodium hydroxide. 1% (w/v) starch solution was prepared by dissolving 0.25 g of potato starch in 25 mL of 20 mM sodium phosphate buffer solution (pH 7.0). To facilitate the solubility of starch solution, it was heated directly on a hot plate using constant stirring, bring to boil and maintain the solution at that temperature for 5 min. DNS reagent was prepared by dissolving 1 g of 3,5-dinitrosalicylic acid in 20 mL deionized water. It was then mixed with sodium potassium tartrate tetrahydrate solution (30 g of potassium tartrate tetrahydrate in 2N sodium hydroxide), and heated directly on a hot plate with constant stirring at 50°C and further diluted to 100 mL with deionized water. The stock solution of standard enzyme (10 activity units/mL) was prepared by dissolving 1.949 mg of the enzyme in 10 mL of the sodium phosphate buffer solution under cool condition.

The method of (Morimoto *et al*. 2007) [21]; was used to validate alphase amylase activity as described above. 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 100ºC for 5 min, cooled and absorbance was measured at 540 nm with the UV spectrophotometer (Spinco Biotech. PVT LTD).

**Protein estimation**

The protein concentration of the crude amylase samples was determined according to (Mergulhao *et al*. 2005, Lowry *et al*. 1951) [20, 19]; using bovine serum albumin (BSA) as standard.

**Effect of temperature (ºC) and pH**

The reaction mixtures for assay were incubated at various temperatures of 30 - 100ºC. 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. Also, for effect of pH, the reaction mixtures prepared were adjusted to different pH values of 2 - 11 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 30ºC. The resulting colour (blue black) was determined for its

absorbance at 540 nm. The concentration of reducing sugar released was then calculated for glucose.

**Statistical Application**

The shelf life of alpha amylase samples was determined after 24hr and 48hr using Microsoft Excel (2010) version. To determine the shelf life, concentration (%) of thermos-stability at various temperature (°C) calculated and the formula below was used:

Eqn 1 = 'AGX'/'AG$Y' x 100

Where:

AGX: Relative observations being the determined alpha amylase activities

$AG$Y: Absolute observations being determined alpha amylase residual activity.

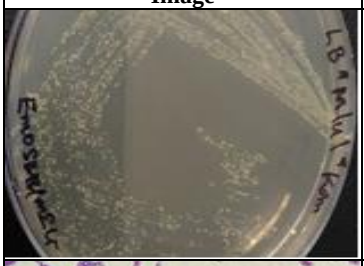
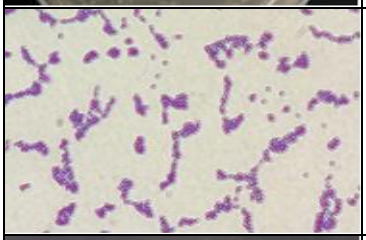
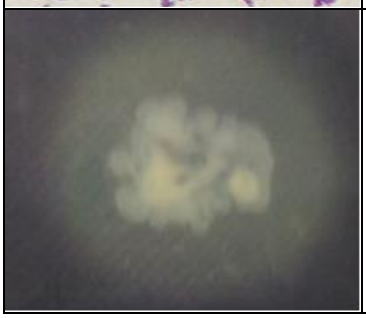
**Results**

>160930-R02_E01_Mlu_1_Mlu_1_Reverse.ab1 and 160930-R02_C01_Mlu_1_Xba_1_Forward.ab1 Length: 812

```
Identities:337/472(71%),  Gaps:23/472(4%), Strand: Plus/Plus
Query  32   GCGgccccgcccgccccccgccggccgaccgattctgttatccccccctttaaccc--tg  89
            |||||||  |||||  |||||||  || || | |  |||||||||||||| | |||  |
Sbjct  35   GCGGCCCT-CCCGCGCCCCGCCC-CCTACTGTTATTGTTATCCCCCCCTTCACCCCCCTC  92

Query  90   cctccccctcacaccc-ctcctcacccctcccctttttccctcctctttttttctctt  148
            || ||||||| |||||  |||||||| ||||  |||| | | |||||||  ||| | || ||
Sbjct  93   CCACCCCCTC-CACCCACTCCTCACACCTTTCCCT-CATCTCCCTCCATTTTCTACTTTT  150

Query  149  ctttttttattgcaactttaacggaacacatattctt-tcttaacccttttttttctttt-  206
            ||||||| |||  ||||||||   ||| ||  ||||| || |  | ||| || || ||| |
Sbjct  151  CTTTTTTGATTCGAACTTTAATT-AACCCAATTTCTTCTCCTTCCTCTTCTTCTCCTTTA  209

Query  207  tttctattgttt-ttgtctc-at-tcct-ttcctttccctactcattcttcctcctcctt  262
            |||||  |  ||  || |||| || |||  |||||  ||| | ||   | ||| ||  |||
Sbjct  210  TTTCTCTATTTCCTTTTCTCCATCTCCACTTCCTACCCCCAATCCCCCATCCCCCCACTT  269

Query  263  cctccttctttt-ttcATATTAATCTCTTTTACTGCCTTATTGATGCctctcattgtttc  321
            ||||  ||||  |  |||  | |||||||||  ||||||  |||||  || | |||  || || |
Sbjct  270  CCTCTTTCTAATATTCTAAATAATCTCTTATACTGCTTTATTTATTCTTCTTTTTTTTCC  329

Query  322  tctcattaattctctttcactctatcc-tcccccaattctctc-tctctcccGATACACC  379
            | |||||||||   | | | ||| ||  | | ||||||  |||  |||||  | ||| || |
Sbjct  330  TTTCATTAATTTATTCTAAATCTCTCGATTCTCCAATTTTCTAATCTCTTCTGAT-CAGC  388

Query  380  CCTTTCTTTGATCAATCAAGTCGGGCCCACGCGGCGGCCTGATCCATCCCGTGTATTAGA  439
            | ||||||||||||  || | | |  ||||   ||||||| ||||||  || ||   ||||
Sbjct  389  C-TTTCTTTGATCAGACATGCCTGATCCACTAAGCGGCCTAATCCATGCCTTG--ATAGA  445

Query  440  --TGGTTCCTTCACAGATCGAGTTTTGGTTCAGTCAACGCGGTGGGCGCATC  489
               ||||||   || || || | |  |||||  ||||||||  ||||  |||||
Sbjct  446  TTTGGTTCGGGCAAAGGTCTAATCATGGTTTTGTCAACGCTATGGGTGCATC  497
```

**Fig 1:** A complete aligned forward and backward sequence of the constructed Bacillus subtilis sp. using the Nucleotide FASTA (Aligned Sequence) programme within the National Centre for Biotechnology Information (NCBI) software. The information about the sequence was also provided indicating Sequence ID: Query_10235, Length: 916, Range 1: 35 to 497, Score:174, bits (192), and Expect:2e-47

**Table 1:** Microbial activities showing a complete analytical routine carried out using the constructed *Bacillus subtilis* sub sp.

| Image | Description |
|---|---|
|  | (a) Isolation and purification of constructed *Bacillus subtilis* sub sp. on Luria Bertani agar. The growth medium was fortified with 50µl kanamycin to ascertain growth ability of a modified strain and confirm differential characteristic between wild type *Bacillus subtilis* and novel *Bacillus subtilis* sub sp. |
|  | (b) Gram staining reaction of the constructed *Bacillus subtilis* sub sp. The image was obtained using a fluorescence microscope at X100 magnification. The image observed had differential properties: i. bluish rod shaped positive colouration of *Bacillus subtilis* sub sp. and ii. oval shaped rods indicating the effect of antibiotic on cellular structure but having no adverse effect on cellular mechanism. |
|  | (c) Zone of hydrolysis was observed and recorded as positive starch degrading ability of the constructed *Bacillus subtilis* sub sp. A medium of 1% starch + 100ml Luria Bertani agar fortified with 50µl kanamycin was used for the hydrolysis test. |

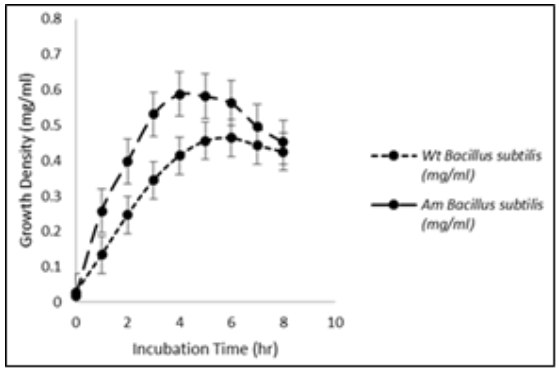



**Fig 2:** Microbial Growth Curves. A comparative growth curve representative growth density of wild type *wt Bacillus subtilis* and constructed *Am Bacillus subtilis* sub sp.

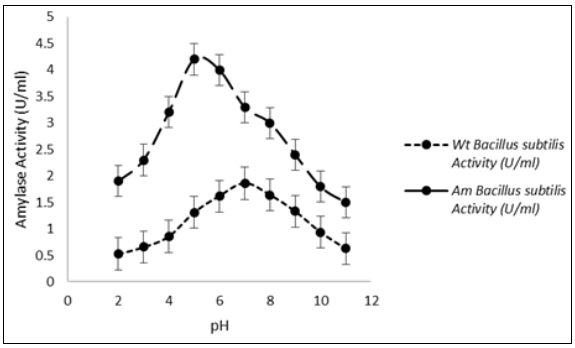



**Fig 4:** Effect of pH range on different samples of alpha amylase activity synthesized by wild type *wt Bacillus subtilis* and constructed *Am Bacillus subtilis* sub sp.

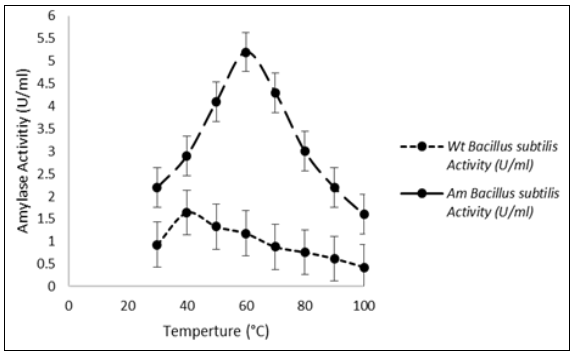



**Fig 3:** Effect of temperature (°C) range on different samples of alpha amylase activity synthesized by wild type *wt Bacillus subtilis* and constructed *AmBacillus subtilis* sub sp.

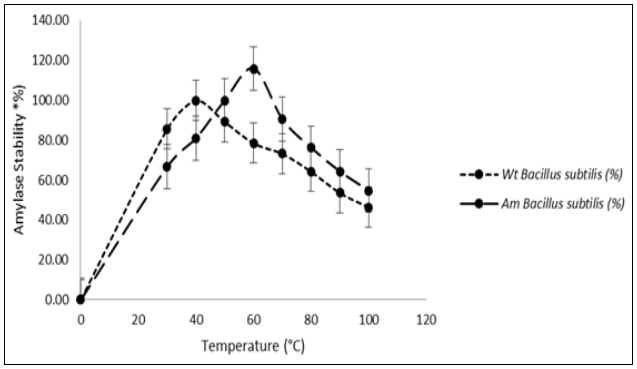



**Fig 5:** Alpha amylase shelf life curves after 24hr. The concentration of thermo-stability of alpha amylase samples produced by both wild type *wt Bacillus subtilis* and constructed *Am Bacillus subtilis* sp. are represented in the above figure

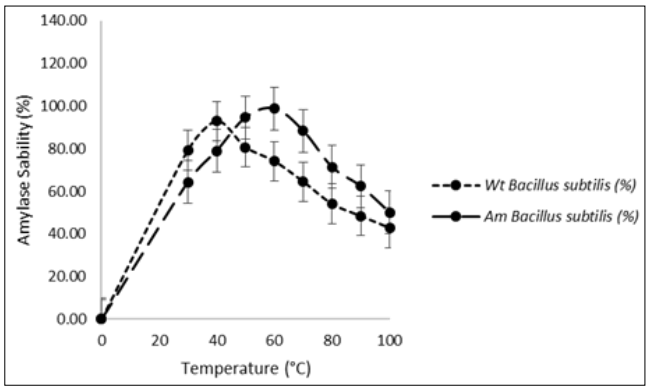



**Fig 6:** Alpha amylase shelf life curves after 48hr. The concentration of thermo-stability of alpha amylase samples produced by both wild type wt *Bacillus subtilis* and constructed *Am Bacillus subtilis* sp. are represented in the above figure

## Discussion

Current research investigations have shown that the selection of an appropriate host and suitable production conditions is necessary for an efficient downstream processing. It has been established that extracellular proteins from thermophiles offer several advantages compared to secretomes from mesophilic strains, such as longer useful shelf life, less contamination problems and increased chemical resistance (Dey *et al*. 2016, Drejer *et al*. 2018, Haq *et al*. 2010, Dai *et al*. 2020) [5, 6, 13, 2]. In this investigation, an area of research interest was identified, resulting from the activities of the high performance alpha amylase synthesized by a constructed Am *Bacillus subtilis* sub sp.

## Alpha amylase genome construct

The development of a newly constructed *Bacillus subtilis* sub sp. was carried using the *Bacillus* expression system kit obtained from Takara. The procedure led to the production of competent cells which were infused with designed primers, signal peptide bond and amyl promoters, all supplied along with the kit except for the designed primers which were modulated using the SnapGene tool. The infusion was amplified using 3 step PCR amplification protocol and further expressed in an empty *Bacillus subtilis* host. The confirmation of a successful infusion was tested on a kanamycin Luria Bertani agar. Isolation of Plasmid DNA was carried out to identify the sequence of the newly alpha amylase genome as shown in Figure 1. Using the National Centre for Biotecnology Information (NCBI) software to analyse the genome, the analysis showed the successful aligned nucleotide, and sequence length. In this investigation, the newly *Bacillus subtilis* sub sp. was used for the production of high performance alpha amylase [EC.3.2.1.1]. Related article according to (Yao *et al*. 2019, Hamoen *et al*. 2003, Ravn *et al*. 2003) [43, 12, 27] confirmed that the performance of protein precursor transmembrane transport is related to the amino acid sequences of the signal peptide and the mature protein, as well as their interaction with related intracellular elements. Compared to *B. licheniformis* and *B. amyloliquefaciens*, constructed *Bacillus subtilis* have shown great potential as a host for secretion of alpha amylase [EC.3.2.1.1] (Wei *et al*. 2015, Niu *et al*. 2009) [40, 22].

## Microbial Analysis

The protocol of *Bacillus* express system was carried out to achieve a new strain and its activities were studied monitored following standard conditions as illustrated in Table 1. From Table 1, a pure colony of the newly constructed Am *Bacillus subtilis* sub sp. was used to express high performance on as described in the images 1a, 1b, and 1c.

From the table, the strain was re-isolated and purified using Luria Bertani Agar fortified with 50µl kanamycin. The ability of the strain to grow on an antibiotic is now a known characteristic attributed to an optimized genome which is an added advantage over the wild type *Bacillus subtilis*. Both biochemical and physiological tests were employed to identify and characterise the construct. As shown in 1b, following the techniques prescribed by the Bergey's Manual of Systematic Bacteriology (BMSB) (Kersters and Vancanneyt, 2005) [17] a singular colony was used for further identification on gram's reaction. The result showed a rod shaped cellular structure, sometimes in cluster, singular or paired as recorded with aid of a fluorescence microscope at X100 magnification as shown in 1b. Screening ability of the new construct was further determined to express high performance. A pure colony was grown on 1% starch Luria Bertany agar fortified with 50µl kanamycin for the screening of amylase. The medium was incubated at 45 °C for 24 h. A positive reaction was observed by the size of the clearing zone as shown in 1c. It has been determined that the higher the diameter of clearing zone, the higher the activity produced by the enzyme, in this case alpha amylase [EC.3.2.1.1], measured by centimetres (Thebti *et al*. 2016) [36].

## Microbial Growth Rate

With much relevance to the performance of a strain, several research activities have differentiated effect of temperature (°C), and time (hr) on strain growth density (g/ml) which can be illustrated with the growth curve differentiating activities occurring within the cell. These conditions help to define the growth curves which are obtained by measuring the optical density (OD) of cell populations over time and thus apply differently to various microorganisms that are controlled by genes and activation timing. In this experiment Am *Bacillus subtilis* sub specie recorded optimum growth density of 0.588 (g/ml) at 4th hour while wild type recorded 0.464 (g/ml) at 6th the hour, by growing the bacteria in Luria Bertani broth at 45°C for 48hour separately as shown in Figure 2. The increasing growth rate activity of a competent cell has intensively been studied revealing improved regulatory activities within a culture medium, especially during their entrance to stationary phase (Hazebrouck *et al*. 2007) [14]. The growth rate of the constructed Am *Bacillus subtilis* sub sp. confirmed with studies that have shown that when cellular type enters stationary phase depending of physiological factors, they start to differentiate into various subpopulations.

Some of them become motile, while the others form biofilm (Westers *et al*. 2004) [41]; secrete degradative enzymes and

antibiotics (Haki and Rakshit, 2003) [11]; or finally sporulate (Ramachandra et. al. 2004) [26]. Another small subpopulation differentiates into competent cells able to take up extracellular DNA (Gonzalez-Pastor *et al*. 2003) [10]. This finding further agrees with the reports of (Slimane *et al*. 2015, Henshaw ans Wakil, 2019, Singh *et al*. 2014) [34, 15, 32] stating that growth of natural and reengineered thermophilic *Bacillus spp.* is faster than wild type and increases in density along with time.

**Effect of optimal temperature (ºC)**

Temperature have been shown to affect an enzyme either by a direct influence on the reaction or in thermal denaturation of the enzyme at elevated temperatures (Demirkan *et al*. 2011) [4]. In this investigation, the effect of temperature (ºC) range of 30 – 100ºC on samples of alpha amylase samples synthesized by amplified (Am) *Bacillus subtilis* sub sp and wild type (wt) *Bacillus subtilis* was determined and the results are illustrated in Figure 3. From the figure above, activities of alpha amylase synthesized by Am *Bacilus subtilis* sub sp and wt *Bacillus subtilis* recorded 5.2U/ml and 1.64U/ml at 60ºC and 40ºC respectively. The relevance of temperature effect is a highly sensitive parameter for α-amylase productivity and this is varied from one strain to another (Sivakumar *et al*. 2011) [33]. Our result can be compared to studies of (Far *et al*. 2020) [9] while investigating the thermostability of alpha amylase from other *Bacillus* species, and report by (Simair *et al*. 2017) [30] on maximum amylase production from optimized *Bacillus* WA21 was observed above 45°C. Bacilli alpha amylase activities against temperature (ºC) effect determined by various research investigations have shown that optimal temperature range are 50 - 90ºC, and this has made the enzyme highly favourable at high temperatures to various industries utilizing starch.

**Effect of optimal pH**

The normal pH ranges from 6.0 to 7.0 have been reported for wild type Bacillus subtilis growth and enzyme activity and when pH is altered, the activity is affected by denaturation. The effect of ionic concentration often defines an enzyme functionality on substrate active site complementary to the shape, size, and rate of reaction. In this investigation, the effect of pH ranges 2 – 11 on the activities of alpha amylase samples was determined. The results obtained showed various activities of the alpha amylases produced by wild type wt *Bacillus subtilis* and the newly constructed Am *Bacillus subtilis* sub sp. as described in Figure 4.

From the figure, activities of 4.2U/ml and 1.86U/ml were recorded at pH 5.0 and pH 7.0 for the newly constructed strain and wild type respectively. A good industrial catalyst should be stable under the toughest operating conditions and for long durations (Pal and Khanum, 2010) [23]. These findings are similar to results by (Singh *et al*. 2012, Suribabu *et al*. 2014, Dash *et al*. 2015, Asgher *et al*. 2007) [31, 35, 3, 1] recorded for a both wild type and thermophilic *Bacillus* spp.

**Estimation of Shelf Life Concentration**

Recent research investigations have identified alpha amylase [EC.3.2.1.1] to be stable at increasing temperatures and adaptive to harsh industrial process but also can be denatured buy the same conditions. In this investigation we went ahead to justify the performance of the alpha amylase produced by the constructed Am *Bacillus subtilis* sub sp. by a statistical approach. Using Microsoft Excel 2010 version, shelf life concentration was calculated after 24hr and 48hr, and the results are illustrated in Figure 5 and 6 respectively. From Figure 5, shelf life concentration recorded 100%, 115%, and 90.84% stability at 50ºC, 60ºC and 70ºC respectively after 24hr, From Figure 6, the shelf life concentration recorded 694.62%, 98.81% and 88.38% at 50ºC, 60ºC, and 70ºC after 48hr. Several methods have been utilized to achieve a stable alpha amylase [EC.3.2.1.1], such as i. extraction from native extremophile microorganisms, ii. extraction from genetically manipulated microorganisms; iii. protein engineering, iv. enzyme immobilization and some statistical approach have also been used to isolate and determine performance of alpha amylase [EC.3.2.1.1.1] (Prajapati *et al*. 2013) [25]. Similar studies involve the use Response Surface Methodology (RSM) which is described as collection of experimental strategies, mathematical methods and statistical inference for constructing and exploring an approximate functional relationship between a response variable and a set of design variables have been documented (Elibol, 2004, Zhao *et al*. 2011, Pilai *et al*. 2011, Vishwanatha *et al*. 2020) [8, 44, 24, 39]. The relevance of this type of statistical approach can be used to estimate shelf life concentration to further explain the combined effects of all the factors in a fermentation process.

**Conclusion**

Although the importance and relevance of a hyper active strain and an efficient enzyme such as Bacilli and alpha amylase [EC.3.2.1.1] have been well documented as keys factors promoting biotechnological significance towards industrialization, there is need to diversify research investigations into understanding interactions between and within a reaction as in the case of enzyme and substrate concentrations.

The demand for hyper active alpha amylase is increasingly required for biotransformation and biodegradation but the need to understand its shelf life concentration is most relevant to every reaction. In this research investigation, we used a statistical approach to predict and confirm alpha amylase activity at different temperature, and thermostability by evaluating shelf life concentration using a statistical approach

**Acknowledgment**

We wish to sincerely appreciate the Microbial Engineering Group, International Centre for Genetic Engineering and Biotechnology (ICGEB), Delhi, New Delhi, for providing a Takara Infusion Kit *Bacillus* Expression System and the Department of Microbiology, Olabisi Onabanjo University for supporting the research investigation.

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