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# Optimization of Growth Conditions to Identify the Superior *Bacillus* Strain Which Produce High Yield of Thermostable Alpha Amylase

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## Abstract

Thermostable $\alpha$-amylases hold a very important place in commercial industrial applications in Sri Lanka. Therefore, the main aim of this study was to identify superior *Bacillus* strain and optimize growth conditions that could yield high $\alpha$-amylase production. Three *Bacillus* strains, *B. amyloliquefaciens* ATCC 23350, *B. licheniformis* ATCC 14580 and *B. megaterium* ATCC 14581 were used for the study. Shake flask culture experiments were conducted to identify the effect of various fermentation conditions such as growth temperature, incubation period, carbon source, nitrogen source, initial pH and carbon concentration on extracellular $\alpha$-amylase production. DNSA assay was carried out to determine the enzyme activity. The highest temperature for enzyme activity was reported by *B. licheniformis* at 85˚C, followed by *B. amyloliquefaciens* at 75˚C and *B. megaterium* at 45˚C. Both *B. amyloliquefaciens* and *B. licheniformis* were able to give their optimum enzyme production at 37˚C, while *B. megaterium* at 30˚C in 150 rpm with initial pH of 7. *B. licheniformis* and *B. amyloliquefaciens* gave their optimum yield of the enzyme after 48 h of incubation while *B. megaterium* gave after 24 h of incubation. Among the carbon sources tested cassava starch was able to give the highest enzyme production. For *B. amyloliquefaciens*, the highest yield of the enzyme was obtained with 2% of starch, tryptone as a nitrogen source and initial pH of 7. Maximum enzyme production for *B. licheniformis* was obtained with 1.5% of starch, $KNO_3$ as a nitrogen source and initial pH of 6. For *B. megaterium* 1% of starch, tryptone and pH 7.5 induced the optimum $\alpha$-amylase production. According to the results obtained, *B. amyloliquefaciens* is the highest thermostable alpha amylase producer. However, according to the industrial requirement, *B. licheniformis* can also be used as an enzyme producer due to its stability in higher temperatures.

**Keywords**

Thermostable, *α*-Amylase, *Bacillus* Strain, Fermentation, Incubation Period

## 1. Introduction

Enzymes act as catalysts for biological processes that regulate specific biochemical reactions. The main constituents of enzymes are proteins. Almost all pathways in a biological cell required enzymes to bring the reaction to its equilibrium position more rapidly and maintain the reaction at a significant rate. Enzymes are selective for their substrates and therefore have the ability to determine which metabolic pathways take place in the cell [1].

Amylases are among the most valuable enzymes and hold great significance in biotechnology [2]. There are three different amylase groups known as *α*-amylase, *β*-amylase and glucoamylase. *α*-Amylases (E.C.3.2.1.1) have the ability to catalyze the hydrolysis of internal *α*-1,4-glycosidic linkages in starch into reducing sugars, such as glucose, maltose and maltotriose units [3]. Alpha amylases are members of a class of enzymes called glucosidases. The starch degrading enzymes are among the most important enzymes widely used industrially [4].

Using enzymatic catalysts industrially is more advantageous than using synthetic catalysts. They act faster than synthetic catalysts and highly specific producing desired product and therefore able to carry out efficient reactions at normal conditions and eagerly used in industrial processes to save energy and resources [5]. Also, another advantage of the use of enzymatic catalysts is the ability to act in extreme conditions. One of the best examples is thermostable enzymes.

Thermostability is a desired characteristic of most of the industrial enzymes. Some of the advantages of using thermostable enzymes are low risk of contamination by mesophillic bacteria, higher reaction rates due to a decrease in viscosity, cut down of cooling cost, and substrates are more soluble and increase of mixing and pumping due to lower viscosity [6].

The sudden demand for biological catalysts is attributed to the advance of biotechnology, which has led to a rapid development in the enzyme technology. Current experiments are carried out to improve thermostable amylolytic enzymes for industrial processes of starch degradation and have high importance to produce beneficial products such as glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins [3].

Amylases are used commercially for starch liquefaction, paper industry, desizing of textile fabrics, in removing wallpaper, brewing industry, production of sugar syrups from starch which consists of glucose, maltose and higher oligosaccharides, pharmaceutical and in preparing cold water solvent laundry starches [4] [7]. Furthermore, the application of amylase is extended into many areas of Science such as clinical, medicinal and analytical chemistry [8].

Amylases are produced by various living organisms ranging from animals to plants to microorganisms. Microbial origin $\alpha$-amylase is mainly used in industrially because they are more stable, cost-effective and can be easily genetically manipulated to obtain desired products. Also, another major advantage of using microorganisms for the production of amylases is the economical bulk production capacity. Some of them also have the ability to produce thermostable amylases [9].

The enzymes can be produced from microbes through either optimized fermentation of the microorganisms or cloning of genes from fast-growing microbes by recombinant DNA technology [10].

The composition and concentration of media, play a major role in the growth and secretion of extracellular amylases in bacteria. As an example, amount of carbon source, nitrogen source, metal ions, and initial pH of the growth medium are known to influence amylase production and the growth of the organism [11]. The growth medium for amylase production should obviously be one that provides a good yield of extracellular amylase. Therefore, growth medium can be optimized for high production of $\alpha$-amylase according to the specific organism.

Mainly Submerged Fermentation (SmF) and Solid-State Fermentation (SSF) are used to produce $\alpha$-amylase on an industrial scale [4]. In SmF microorganisms grow in a liquid culture medium such as molasses and broth culture. The products are secreted into the fermentation broth. This method is commonly used when secondary metabolites such as enzymes are harvested [3]. This method is considered as the best method to cultivate bacteria, since they require high moisture content, while solid state fermentation can be applied for microbes with low moisture requirements such as fungi. Advantages of SmF are ease of sterilization and purification. Also control of environmental factors such as temperature, pH, aeration, oxygen transfer and moisture can be conducted easily [4].

Amylase can be produced by different species of microorganisms. However, for commercial applications amylase is mainly obtained from the genus *Bacillus* as the members of the genus *Bacillus* can produce extracellular enzymes with a large range of variety. In previous researches the ability to produce extra cellular amylase of the three *Bacillus* strains of *Bacillus licheniformis* [12], *Bacillus megaterium* [13] and *Bacillus amyloliquefaciens* [14] have been found. Therefore, selected *Bacillus* strains for this study were *B. amyloliquefaciens* strain ATCC 23350, *B. licheniformis* strain ATCC 14580 and *B. megaterium* strain ATCC 14581 which were procured by MicroBioLogics, Inc.

Commercial production of thermostable $\alpha$-amylase is not conducted in Sri Lanka as of now due to the high cost of production. Hence, amylase enzyme is largely imported to the country for industrial applications which will be result of a huge loss of foreign exchange. One of the main aims of this study was to identify an alternative starch source and optimize growth conditions that could yield high $\alpha$-amylase production.

Cassava (*Manihot esculenta*), which selected as the best carbon source for the enzyme production, is grown mainly as a food or animal feed, with a little information of any industrial application in Sri Lanka. Cassava roots have high starch content and resistant to pest attacks, drought and able to produce acceptable yields under unfavorable weather conditions. These tendencies of cassava make it a good raw material to use in enzyme production industry in Sri Lanka [15]. Therefore, outcomes of this research project will be beneficial to commence production of thermostable $\alpha$-amylase as an industry in Sri Lanka.

## 2. Materials and Methods

### 2.1. Materials

Molecular biology grade chemicals, purchased from Sigma Aldrich (St. Louis, Missouri, United States) were used for this study unless otherwise stated.

### 2.2. Microorganisms and Maintenance of Culture

#### 2.2.1. Microorganisms

*Bacillus amyloliquefaciens* strain ATCC 23350, *B. licheniformis* strain ATCC 14580 and *B. megaterium* strain ATCC 14581 were obtained from American Type Culture Collection (ATCC). They were maintained at nutrient agar medium.

#### 2.2.2. Maintenance of Culture

**1) Preparation of glycerol stock cultures**

Cultures from the three *Bacillus* strains were grown in sterile nutrient broth (NB) at 37˚C and 150 rpm for overnight. For long time preservation purpose, stock cultures were prepared by mixing 800 µL of exponential phase cultures in 200 µL of 100% glycerol and stored at −80˚C.

**2) Preparation of subcultures**

Subcultures were prepared for laboratory use. Each bacterial strain from their glycerol stocks was streaked on the nutrient agar plates. Growth plates were incubated at 37˚C for an overnight. Subcultures were prepared every month and maintained at 4˚C.

### 2.3. Preparation of Inoculum

The inoculum was prepared by transferring the bacterial growth from a distinct single colony of freshly grown nutrient agar plate into a 15 mL of sterile NB medium by using an inoculation loop under aseptic conditions. It was incubated overnight at 37˚C and 150 rpm in a shaking incubator.

After overnight incubation, optical density (OD) value at 600 nm was measured and, 0.5 mL of the inoculum was introduced into 50 mL of the respective culture media in 250 mL conical flasks and incubated at 37˚C and 150 rpm in a shaking incubator unless otherwise stated. Every experiment was conducted in triplicates.

## 2.4. Determination of $\alpha$-Amylase Activity

### 2.4.1. Extraction of Amylase from the Fermentation Medium

Aliquots of 1.5 mL were taken out from each of the triplicates. Then, they were harvested by centrifugation at 10,000 rpm for 10 minutes at 4˚C in a Hermle Labortechnik centrifuge and the cells free supernatant was used as the crude enzyme extract [12].

### 2.4.2. Procedure of DNSA Assay

Amylase activity was measured by the method, known as 3,5-dinitro salicylic acid (DNSA) assay developed by [16] and modified by [17]. Amylase assay was carried out by incubating, a mixture of 0.5 mL of crude enzyme extract and 0.5 mL of 1% starch solution (starch in 0.05 M potassium phosphate buffer at pH 7) in screw cap test tubes at 55˚C in a water bath for 30 minutes [1] [18]. At the end of the incubation period, the reaction was terminated by adding 1 mL of DNSA reagent. Then the reaction mixture was kept in a boiling water bath for exactly 10 minutes [1]. After temperature of test tubes came to ambient temperature, the final volume was adjusted to 10 mL by adding distilled water. Every assay was conducted in screw cap test tubes to prevent the loss of solution from the evaporation. For the blank, instead of supernatant from the culture medium, uninoculated medium was added. Then, using Agilent Cary 100 Ultraviolet (UV) visible spectrophotometer, color intensity difference between the blank and the test sample was measured at 540 nm [1].

### 2.4.3. Development of Glucose Standard Curve

The standard curve for colorimetric assay method was developed by using D-glucose solution. First 0.01 M stock solution was prepared. Then, 11 different dilutions (0 mg/mL - 1.80 mg/mL) were prepared using distilled water. For the blank, instead of the glucose solution, only distilled water was added. Then 1 mL of DNSA reagent was added to each tube and kept in a boiling water bath for 10 minutes. Final volume was adjusted to 10 mL and spectrophotometric readings were taken at 540 nm.

One enzyme unit (U) is defined as the number of μmoles of glucose liberated by 1 mL of enzyme supernatant per minute under the assay conditions. [$U \cdot mL^{-1}$ = glucose produced (mg/mL) × 1000/incubation period (minutes) × enzyme volume (mL) × molecular weight (MW) of glucose (180.16 $g \cdot mol^{-1}$)].

## 2.5. Effect of Growth Temperature for Extracellular $\alpha$-Amylase Production

To determine the effect of temperature on amylase production, three *Bacillus* strains were grown at 25˚C, 30˚C, 37˚C, 45˚C, 55˚C and 65˚C. Experiments were carried out in 250 mL conical flasks containing 50 mL of NB medium and inoculated with 0.5 mL of inoculum culture which was prepared as mentioned in section 2.3. Then cultures were incubated at respective temperatures at 150 rpm in a shaking incubator. Growth and amylase production was measured after 24

hours (h), 48 h and 72 h. Negative control was conducted by adding 0.5 mL of sterile NB medium instead of the inoculum culture [19]. The growth of the microorganisms in culture media was measured by taking OD measurements at 600 nm using Agilent Cary 100 UV visible spectrophotometer. Dilution was carried out to keep the data in the linear absorbance range. Samples were diluted by 4 times using uninoculated sterilized NB medium. Readings were taken against a blank containing sterilized NB medium [19]. DNSA method was carried out to determine the amylase production as mentioned in section 2.4.

### 2.6. Effect of Incubation Period on Extracellular $\alpha$-Amylase Production

To determine the effect of incubation period on amylase production, cultures of three *Bacillus* stains were prepared in NB medium with three replicates and incubated at 37˚C and 150 rpm. Amylase assay as mentioned in section 2.4 was conducted after 24 h, 48 h, 72 h, 96 h and 120 h. Negative control was maintained by adding 0.5 mL of sterile NB medium instead of the inoculum culture [12].

### 2.7. Effect of Temperature on Enzyme Activity

The stability of the enzymes always depends mainly upon the temperature. To determine the effect of temperature on enzyme activity, shake flask experiments were conducted in 250 mL flasks containing 50 mL of NB medium and incubated at 37˚C for 48 h at 150 rpm. Then samples were collected, harvested and cell free supernatant was obtained. After that a mixture of 0.5 mL of crude enzyme extract and 0.5 mL of 1% soluble starch were incubated for 30 minutes at different temperatures namely 37˚C, 45˚C, 55˚C, 65˚C, 75˚C, 85˚C and 95˚C. Negative control was conducted incubating sterilized NB medium at respective temperatures, instead of the culture supernatant. Then, 1 mL of DNSA reagent was added to terminate the enzyme reaction and steps of assay procedure were carried out as mentioned in section 2.4.2.

### 2.8. Optimization of Culture Conditions for Amylase Production

#### 2.8.1. Preparation of the Basal Medium

Basal medium was prepared as it composed of ($g \cdot L^{-1}$): starch, 10; $KNO_3$, 0.5; $K_2HPO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.1; $FeCl_3$ traces and initial pH was adjusted to 7.0 using 2N NaOH or 2N HCl and autoclaved for 20 minutes at 121˚C at 15 lbs/inch$^2$ pressure [20].

The fermentation process was carried out in 250 mL of conical flasks containing 50 mL of the medium. After the media was allowed to cool to the ambient temperature, inoculated with 0.5 mL of inoculum culture which was prepared as mentioned in section 2.3 and incubated at 37˚C in a shaking incubator at 150 rpm. Every experiment was conducted in triplicates. After 48 h, aliquots of 1.5 mL were taken out and amylase assay was conducted as mentioned in section 2.4.

#### 2.8.2. Effect of Carbon Source on Extracellular $\alpha$-Amylase Production

To determine effect of carbon source on amylase production of three *Bacillus* strains, three different carbon sources; corn (*Zea* mays), sweet potato (*Ipomoea batatas*), and cassava (*Manihot esculenta*) were selected and bought from local market, Colombo, Sri Lanka. They were washed well with tap water to remove dust and impurities. The washed substrates were chopped and then dried at 60˚C for about 12 h to obtain a consistent weight. The dried starch materials were grinded in a laboratory grinder and a particle size larger than 1 mm were sieved out [20].

Starch of the basal medium was replaced by corn, cassava, sweet potato to identify the best carbon source for the amylase production. Basal medium without an added carbon source and basal medium with 1% of soluble starch as the carbon source were used as negative and positive controls, respectively [21].

According to the results (section 3.5) cassava was selected as the best starch source for the amylase production. For forthcoming studies starch source was replaced by 1% cassava starch, using it as the sole carbon source.

#### 2.8.3. Effect of Starch Concentration on Extracellular $\alpha$-Amylase Production

The three *Bacillus* sp. were inoculated to sterilized basal medium prepared as mentioned in section 2.8.1., but instead of the 1% cassava, media containing different cassava starch quantities to give a final concentration [Weight (w)/Volume (v)] of 0.5%, 1.0%, 1.5%, 2% and 2.5% were prepared. Experiments were carried out as mentioned previously. Briefly, to 50 mL of sterilized fermentation media 0.5 mL of inoculation was added and incubated at 37˚C at 150 rpm. After 48 h, aliquots of 1.5 mL were taken out and amylase assay was conducted as mentioned in section 2.4. Negative control was maintained without adding cassava starch.

#### 2.8.4. Effect of pH on Extracellular $\alpha$-Amylase Production

Sterilized basal medium was prepared as mentioned above in section 2.8.1. pH values were adjusted to 5 to 9 with the regular interval of 0.5 using 2N NaOH or 2N HCl. The inoculum was prepared as mentioned in section 2.3. Then inoculated media were incubated at 37˚C for 48 h in 150 rpm. After the time period, aliquots of 1.5 mL were taken out and amylase assay was conducted as mentioned in section 2.4.

#### 2.8.5. Effect of Nitrogen Source on Extracellular $\alpha$-Amylase Production

The *Bacillus* strains were inoculated into the basal media, prepared as mentioned above in section 2.8.1., but nitrogen source ($KNO_3$) was replaced by 0.5% (w/v) different organic and inorganic nitrogen sources namely, tryptone, peptone, yeast extract and ammonium sulphate [$(NH_4)_2SO_4$] [12]. Negative control was conducted by without adding any nitrogen source to the medium. After *Bacillus* strains were inoculated into the medium, cultures were incubated at 37˚C for 48 h at 150 rpm. Standard assay procedure was conducted as mentioned in section 2.4.

### 2.9. Statistical Analysis

All data are expressed as mean ± standard error (SE) of the mean. Statistical analysis was carried out using Minitab 17 statistical software. One way analysis of variance (ANOVA) was used to statistically analyze the data obtained from the research. For equal variation between groups Tukey-Kramer Post Hoc test was carried out. Differences were considered significant when $P < 0.05$.

## 3. Results and Discussion

### 3.1. Development of the Standard Curve

A standard curve relating glucose concentration to the color reaction was obtained as mentioned in section 2.4.3. The determination of amylase concentration in an unknown sample was done by plotting the absorbance of the reaction product at 540 nm against a standard amylase preparation (**Figure 1**). Units of amylase activity were expressed as the number of μmoles of glucose liberated by 1 mL of enzyme supernatant per minute under the assay conditions.

### 3.2. Effect of Growth Temperature for Extracellular *α*-Amylase Production

Temperature is an important factor that has the ability to control both growth of the organism and production of extracellular enzymes, such as *α*-amylase. The optimum temperature for the microbial growth may depend on the type of organism [22]. Production of *α*-amylase by microorganisms occurs in a broad range of temperatures. *B. amyloliquefaciens*, *B. licheniformis*, *B. megaterium*, *B. subtilis*, and *B. stearothermophilus* are among the most commonly used bacteria for amylase production. *Bacillus* sp. reported to producing *α*-amylase at temperatures ranging from 37˚C - 60˚C [11]. In this study to identify optimum temperature for enzyme production, the fermentation was carried out at different temperatures ranging from 25˚C to 65˚C.

According to the results obtained, both *B. licheniformis* and *B. amyloliquefaciens* have shown their optimum enzyme production 0.133 ± 0.006 $U{\cdot}mL^{-1}$, 0.417 ± 0.004 $U{\cdot}mL^{-1}$ respectively at 37˚C after 48 h while *B. megaterium* has shown to have optimum enzyme production, 0.057 ± 0.004 $U{\cdot}mL^{-1}$ at 30˚C after 24 h of incubation (**Table 1**).

According to values obtained at $OD_{600nm}$, optimum growth temperatures were 30˚C, 37˚C, and 45˚C for *B. megaterium*, *B. licheniformis* and *B. amyloliquefaciens*, respectively (**Table 2**). Even through, optimum cell density and optimum enzyme production for both *B. licheniformis* (37˚C) and *B. megaterium* (30˚C) were reported at the same temperature, for *B. amyloliquefaciens*, optimum cell density (45˚C) and optimum enzyme production (37˚C) were reported at different temperatures. Hence, it can be assumed that, there wasn't any relationship between temperature of the optimum enzyme production and optimum growth temperature.

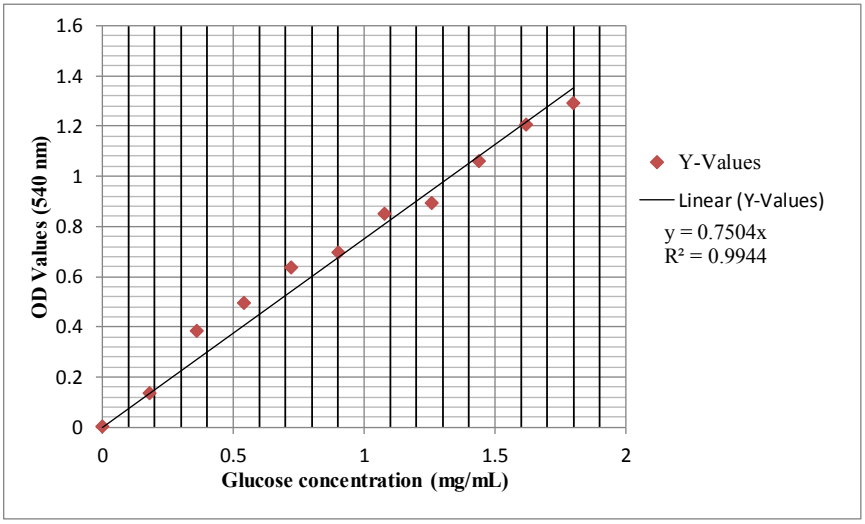


**Figure 1.** Calibration curve showing absorbance at 540 nm against reducing sugar concentration.

**Table 1.** Effect of temperature on extracellular *α*-amylase production of *Bacillus* species in shake-flask cultivations. Each value is an average of three parallel replicates.

| Temperature | Incubation period | Enzyme activity (U/mL) of *B. licheniformis* (Mean ± SE of mean) | Enzyme activity (U/mL) of *B. amyloliquefaciens* (Mean ± SE of mean) | Enzyme activity (U/mL) of *B. megaterium* (Mean ± SE of mean) | Enzyme activity (U/mL) of control (Mean ± SE of mean) |
|---|---|---|---|---|---|
| 25˚C | 24 h | 0.029 ± 0.003 | 0.017 ± 0.003 | 0.012 ± 0.002 | 0.005 ± 0.001 |
| | 48 h | 0.040 ± 0.003 | 0.024 ± 0.004 | 0.025 ± 0.002 | 0.004 ± 0.001 |
| | 72 h | 0.034 ± 0.003 | 0.016 ± 0.001 | 0.019 ± 0.003 | 0.004 ± 0.000 |
| 30˚C | 24 h | 0.032 ± 0.001 | 0.041 ± 0.000 | **0.057 ± 0.004** | 0.009 ± 0.001 |
| | 48 h | 0.057 ± 0.008 | 0.041 ± 0.002 | 0.033 ± 0.003 | 0.013 ± 0.014 |
| | 72 h | 0.030 ± 0.008 | 0.033 ± 0.001 | 0.030 ± 0.004 | 0.004 ± 0.001 |
| 37˚C | 24 h | 0.033 ± 0.004 | 0.356 ± 0.014 | 0.023 ± 0.002 | 0.003 ± 0.000 |
| | 48 h | **0.133 ± 0.006** | **0.417 ± 0.004** | 0.028 ± 0.001 | 0.003 ± 0.000 |
| | 72 h | 0.074 ± 0.001 | 0.377 ± 0.000 | 0.025 ± 0.002 | 0.002 ± 0.000 |
| 45˚C | 24 h | 0.027 ± 0.002 | 0.031 ± 0.004 | 0.003 ± 0.001 | 0.001 ± 0.000 |
| | 48 h | 0.010 ± 0.001 | 0.027 ± 0.000 | 0.005 ± 0.002 | 0.001 ± 0.000 |
| | 72 h | 0.020 ± 0.001 | 0.028 ± 0.006 | 0.007 ± 0.001 | 0.001 ± 0.000 |
| 55˚C | 24 h | 0.037 ± 0.003 | 0.015 ± 0.001 | 0.002 ± 0.001 | 0.000 ± 0.000 |
| | 48 h | 0.013 ± 0.001 | 0.009 ± 0.002 | 0.002 ± 0.000 | 0.000 ± 0.000 |
| | 72 h | 0.022 ± 0.000 | 0.025 ± 0.003 | 0.06 ± 0.000 | 0.000 ± 0.000 |
| 65˚C | 24 h | 0.007 ± 0.000 | 0.001 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| | 48 h | 0.009 ± 0.001 | 0.003 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| | 72 h | 0.006 ± 0.000 | 0.001 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |

**Table 2.** Effect of temperature on growth of *Bacillus* species in shake flask experiments. Each value is an average of three parallel replicates.

| Temperature | Incubation period | Growth ($OD_{600}$) of *B. licheniformis* (Mean ± SE of mean) | Growth ($OD_{600}$) of *B. amyloliquefaciens* (Mean ± SE of mean) | Growth ($OD_{600}$) of *B. megaterium* (Mean ± SE of mean) | Growth ($OD_{600}$) of control (Mean ± SE of mean) |
|---|---|---|---|---|---|
| 25˚C | 24 h | 0.339 ± 0.004 | 0.245 ± 0.006 | 0.355 ± 0.006 | 0.002 ± 0.000 |
| | 48 h | 0.282 ± 0.013 | 0.175 ± 0.007 | 0.416 ± 0.015 | 0.002 ± 0.000 |
| | 72 h | 0.259 ± 0.016 | 0.230 ± 0.004 | 0.447 ± 0.006 | 0.002 ± 0.000 |
| 30˚C | 24 h | 0.422 ± 0.005 | 0.248 ± 0.003 | 0.383 ± 0.008 | 0.003 ± 0.000 |
| | 48 h | 0.339 ± 0.010 | 0.190 ± 0.008 | 0.477 ± 0.036 | 0.002 ± 0.000 |
| | 72 h | 0.400 ± 0.010 | 0.215 ± 0.014 | **0.512 ± 0.045** | 0.009 ± 0.001 |
| 37˚C | 24 h | **0.814 ± 0.097** | 0.286 ± 0.019 | 0.331 ± 0.016 | 0.009 ± 0.001 |
| | 48 h | 0.480 ± 0.003 | 0.114 ± 0.011 | 0.444 ± 0.003 | 0.017 ± 0.001 |
| | 72 h | 0.504 ± 0.030 | 0.200 ± 0.016 | 0.507 ± 0.008 | 0.010 ± 0.001 |
| 45˚C | 24 h | 0.746 ± 0.010 | 0.454 ± 0.017 | 0.009 ± 0.000 | 0.002 ± 0.000 |
| | 48 h | 0.380 ± 0.008 | **0.636 ± 0.008** | 0.009 ± 0.000 | 0.003 ± 0.000 |
| | 72 h | 0.303 ± 0.020 | 0.579 ± 0.001 | 0.060 ± 0.011 | 0.003 ± 0.000 |
| 55˚C | 24 h | 0.297 ± 0.040 | 0.001± 0.001 | 0.000 ± 0.000 | 0.002 ± 0.000 |
| | 48 h | 0.075 ± 0.002 | 0.054 ± 0.006 | 0.009 ± 0.000 | 0.003 ± 0.000 |
| | 72 h | 0.111 ± 0.029 | 0.043 ± 0.012 | 0.036 ± 0.004 | 0.002 ± 0.000 |
| 65˚C | 24 h | 0.006 ± 0.001 | 0.001 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| | 48 h | 0.020 ± 0.002 | 0.005 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |
| | 72 h | 0.006 ± 0.001 | 0.000 ± 0.000 | 0.000 ± 0.000 | 0.000 ± 0.000 |

Tukey post hoc analysis was conducted under ANOVA test for results obtained after 48 h, since maximum enzyme productions were obtained after 48 h. Both *B. licheniformis* and *B. amyloliquefaciens* were able to show a significantly high enzyme production at 37˚C than the other temperatures. However, *B. megaterium* was not shown any significant difference in enzyme production at 25˚C, 30˚C, and 37˚C. Therefore, it was decided to grow all the three strains at 37˚C for further studies.

*B. megaterium* was the most thermo labile organism among three strains, since it was not able to show any significant enzyme production or growth, above 37˚C when compared with the negative control. After 37˚C, enzyme production by all three organisms gradually decreased. This might be due to their mesophilic nature. Since they are mesophiles, they do not have special mechanisms to tolerate high temperatures. Similar results have been reported by [23] for *B. amyloliquefaciens* and [24] for *B. megaterium*. According to those studies, the optimum temperature for the enzyme production for both strains was reported at 37˚C. However, [1] and [11] have revealed that optimum enzyme production for *B. amyloliquefaciens* P-001 and *B. licheniformis* were 42˚C and 30˚C, respec-

tively.

### 3.3. Effect of Incubation Period on Extracellular *α*-Amylase Production

Results were shown that optimum yield of the enzyme was given after 48 h of incubation by all three strains (Table 3). Maximum enzyme production, 0.465 ± 0.004 U·mL$^{-1}$ was obtained in *B. amyloliquefaciens.* It was followed by 0.132 ± 0.006 U·mL$^{-1}$ of *B. licheniformis* and 0.028 ± 0.001 U·mL$^{-1}$ of *B. megaterium*. According to the Tukey comparison test, enzyme production of *B. licheniformis* after 48 h of incubation has shown a significant difference than the production of amylase after any other incubation period. There were no significant difference in enzyme production after 48 h and 72 h in *B. amyloliquefaciens* and for *B. megaterium* there weren't any significant difference in enzyme production after 24 h, 48 h and 72 h.

Our findings are in accordance with the results obtained by [11] in case of *B. amyloliquefaciens* P-001. Similar results were obtained by [25] in case of *B. subtilis*, [26] in case of *Bacillus* sp. DLB 9 and [27] in case of *B. subtilis*. Nevertheless, there are also findings contrasting to this. As an example, [28] reported that *B. amyloliquefaciens* gave the highest enzyme yield after 24 h, which was not in accordance with our results.

It was revealed that production of the *α*-amylase begins with the start of the expontential phase. Accumulation of by products may be the reason for decrease in amylase activity after it reaches for maximum activity. Sudden decrease in the enzyme production might be due to the accumulation of protease enzyme in culture medium [27]. As it is reported by [28] high level of protease activities is concomitant with the sporulation process at the end of the exponential growth phase.

According to [19], amylase could not be even detected in the growth medium before 12 h incubation. According to [29], efficient induction of the enzyme occurred, only after organisms reached to the stationary phase and the available carbon source was reduced.

Table 3. Effect of incubation period on extracellular *α*-amylase production of *Bacillus* species in shake-flask cultivations. Each value is an average of three parallel replicates.

| Incubation period | Enzyme activity (U/mL) of *B. licheniformis* (Mean ± SE of mean) | Enzyme activity (U/mL) of *B. amyloliquefaciens* (Mean ± SE of mean) | Enzyme activity (U/mL) of *B. megaterium* (Mean ± SE of mean) | Enzyme activity (U/mL) of control (Mean ± SE of mean) |
|---|---|---|---|---|
| 24 h | 0.033 ± 0.004 | 0.365 ± 0.014 | 0.023 ± 0.002 | 0.002 ± 0.000 |
| 48 h | **0.132 ± 0.006** | **0.465 ± 0.004** | **0.028 ± 0.001** | 0.003 ± 0.001 |
| 72 h | 0.074 ± 0.001 | 0.425 ± 0.004 | 0.025 ± 0.001 | 0.005 ± 0.000 |
| 96 h | 0.050 ± 0.002 | 0.387 ± 0.007 | 0.008 ± 0.001 | 0.001 ± 0.000 |
| 120 h | 0.040 ± 0.004 | 0.365 ± 0.008 | 0.006 ± 0.002 | 0.002 ± 0.000 |

### 3.4. Effect of Temperature on Enzyme Activity

Effect of temperature on enzyme activity was assayed at different temperatures ranging from 37˚C to 95˚C. The highest enzyme activity 0.127 ± 0.003 U·mL$^{-1}$ for *B. licheniformis* was obtained at 85˚C and lowest at 37˚C (**Figure 2**). Similar results have reported by [30] for *B. licheniformis* CUMC 305. They obtained maximum activity for *B. licheniformis* at 90˚C. Comparison analysis conducted from Tukey post hoc test also showed a significant difference of optimum enzyme activity than any other enzyme activity obtained.

For *B. amyloliquefaciens* highest enzyme activity, 0.098 ± 0.002 U·mL$^{-1}$ was obtained at 75˚C. There was a gradual increase in enzyme activity until optimum enzyme activity was achieved and then the activity gradually decreased. According to the Tukey comparison analysis conducted there was not actually a significant difference between data obtained for 65˚C and 75˚C. *B. amyloliquefaciens* P-001showed optimum enzyme activity at 60˚C [11]. According to [31], *Bacillus* sp. AB68 has the ability to be active in a broad range of temperatures with 50˚C being the optimum value.

For *B. megaterium* optimum enzyme activity, 0.067 ± 0.003 U·mL$^{-1}$ was observed in 45˚C. However, the enzyme has shown to have activity in a broad range of temperatures. Tukey comparison analysis also proved that there is no significant difference in results obtained for 37˚C to 85˚C. According to [18], optimum enzyme activity of *B. megaterium* was obtained at 60˚C. Best activity obtained at in a range of temperatures 50˚C - 75˚C was reported by [32]. Optimum activity obtained at 35˚C was mentioned by [13].

Results of study concludes that all three organisms produce enzymes that moderately stable in a broad range of temperatures, 37˚C to 95˚C. In accordance with reports of many authors, the majority of the bacterial amylases have an optimum temperature in range of 30˚C to 100˚C [33].

According to the results, highest thermostabaility is reported from *B. licheniformis* followed by *B. amyloliquefaciens*. Specific characteristics in their structure might be the reasons for this remarkable thermostability. According to [34], revealed that *B. licheniformis* 584 (ATCC 27811) $\alpha$-amylase is a monomeric enzyme with molecular mass of 55,200 Da (483 amino acid residues) and its amino acid sequence is 80% similar with that of *B. amyloliquefaciens* strain El8. Thermophilic enzymes usually consist of higher number of charged amino acids residues such as Arginine or higher ratio of Arginine/(Arginine + Lysine) [35]. Numbers of reported Arginine residues are 22 and 20 for *B. licheniformis* and *B. amyloliquefaciens*, respectively, while the Arginine/(Arginine + Lysine) ratio is 0.440 and 0.400, respectively [36].

Histidines are involved in ionic interactions and significantly higher amounts can be observed in *B. licheniformis* $\alpha$-amylase type XII-A structure than any other bacterial amylase [36]. Salt bridges are also a major factor that prevents denature of the enzymes due to the heat. There are 39 salt bridges and extensive ionic interactions in $\alpha$-amylase structure of *B. licheniformis* reported by [37].

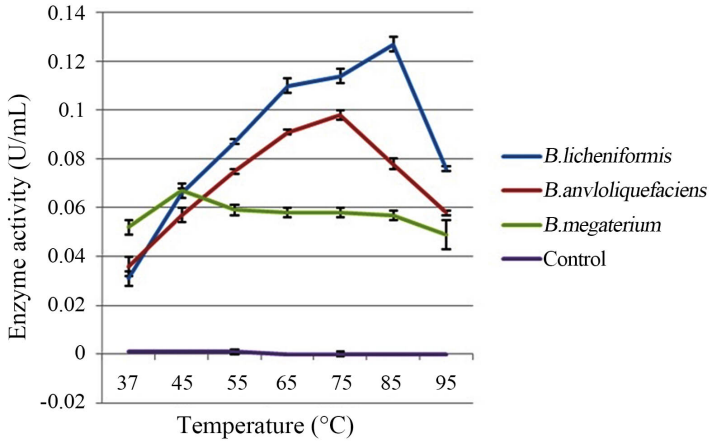


**Figure 2.** Effect of temperature on enzyme activity of *B. licheniformis, B. amyloliquefaciens* and *B. megaterium*. Each value is an average of three parallel replicates. Error bars indicate the SE of mean values.

Reduced surface area and increased packing interactions in the interior of the protein also responsible for thermostability. Efficiency of packing can be evaluated by the fraction of atoms in a protein with zero accessible surface area [36]. For *B. licheniformis* it is reported to be −0.55, which is significantly higher than the average and similar to aldehyde ferredoxin oxidoreductase, which is a hyperthermophilic enzyme from *Pyrococcus furiosus* [38]. Also *B. licheniformis*h as a very tightly packed hydrophobic core and this is considered as the one of the most important thermo stabilizing factors [36].

Alpha amylases are calcium metalloenzymes. As [39] mentioned, amylases consist of at least one $Ca^{2+}$ ion in their structure, which is responsible for integrity and stability of the enzyme. Therefore, presence of $Ca^{2+}$ might also be a reason for elevated thermostability observed from these organisms.

Even though low thermostability was observed from *B. megaterium*, compared to the negative control it was able to show a thermostability in a broad range of temperatures, yet there are only a few studies have been carried out on thermostability of *α*-amylase of *B. megaterium* in comparison to the *α*-amylase of *B. licheniformis* and *B. amyloliquefaciens*.

### 3.5. Effect of Carbon Source on Extracellular *α*-Amylase Production

The nature and the amount of carbon source in growth media influence the production of *α*-amylase in microorganisms. In this study, effect on extracellular amylase production was investigated using three different carbon sources: cassava, sweet potato and corn starch. Soluble starch was used as the positive control. Negative control was conducted without adding any carbon source.

All three *Bacillus* species were able to yield maximum enzyme activity, when cassava was present as the sole carbon source in the fermentation medium (**Figure 3**). Maximum enzyme activity, 0.467 ± 0.024 $U{\cdot}mL^{-1}$ was obtained from *B. amyloliquefaciens*. This was followed by 0.166 ± 0.007 $U{\cdot}mL^{-1}$ of *B. licheniformis*

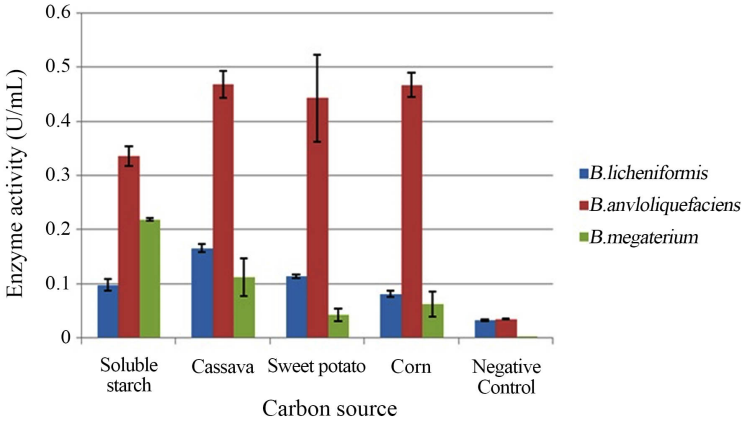


**Figure 3.** Effect of carbon source on extracellular *α*-amylase production of *B. licheniformis*, *B. amyloliquefaciens and B. megaterium*. Each value is an average of three parallel replicates. Error bars indicate the SE of mean values.

and 0.112 ± 0.035 U·mL$^{-1}$ of *B. megaterium*, with cassava starch as the carbon source. When compared with the negative control, all carbon sources have shown a significantly high stimulating effect on extracellular amylase production. The Tukey comparison revealed that, there is no significant difference in amount of amylase produced by different carbon sources; in most of the cases. Especially sweet potato and cassava have not shown any significant difference in amylase production, when used as the sole carbon source.

Our results correlate with the results obtained by [40] for *A. niger.* They revealed cassava starch as the best carbon source for extracellular enzyme production when tried with corn and sorghum starch. According to the [41], they also carried out similar study for *B. alvei and* revealed that sorghum starch gave the maximum enzyme yield. It was also revealed in this study that cassava can also utilize as a good carbon source, for amylase enzyme production.

In case of both *B. licheniformis and B. amyloliquefaciens*, natural carbon sources were able to induce high enzyme production than soluble starch. This might be due to that availability of other nutrient compounds that have favorable effect on *α*-amylase production.

### 3.6. Effect of Carbon Concentration on Extracellular *α*-Amylase Production

Five concentrations of cassava starch ranging between 0.5% - 2.5% were used to obtain the optimum *α*-amylase producing concentration. Maximum extracellular amylase activity 0.693 ± 0.010 U·mL$^{-1}$ was given by 2% of cassava starch from *B. amyloliquefaciens* (**Figure 4**). It was followed by 0.2 ± 0.014 U·mL$^{-1}$ of *B. licheniformis* at 1.5% of cassava starch and 0.092 ± 0.010 U·mL$^{-1}$ of *B. megateriu*mat 1% cassava starch. All the three organisms show significantly high amylase activity in above mentioned optimum carbon concentrations according to the Tukey pairwise comparison under ANOVA test than any other carbon concentration. It was observed that gradual increase in extracellular amylase activity up to the optimum value and then it was gradually decreased.

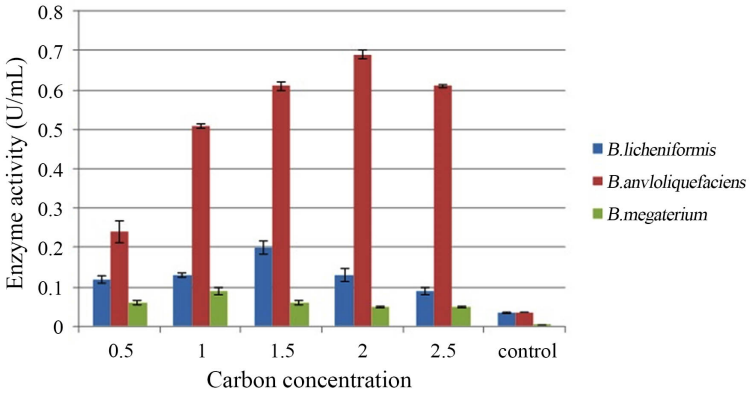


**Figure 4.** Effect of carbon concentration on extracellular $\alpha$-amylase production of *B. licheniformis*, *B. amyloliquefaciens and B. megaterium*. Each value is an average of three parallel replicates. Error bars indicate the SE of mean values.

Enzyme activity at all starch concentrations in all three organisms was significantly higher than negative control. Therefore, it can be assumed that there were no negative effects by increase of starch concentration.

Results obtained from this study were supported by [20]. They also revealed that maximum enzyme activity was obtained for *B. amyloliquefaciens* in 2% of potato starch waste. Nevertheless, according to [19] 0.5% of soluble starch has given the maximum enzyme activity for *B. subtilis* IP 5832.

Very high starch concentrations are not suitable for enzyme production. It can be attributed to the high viscosity, caused by high starch concentrations. It may interfere with $O_2$ transfer and this will lead to a deficiency in dissolved $O_2$ for growth of the microorganisms [19].

### 3.7. Effect of pH on Extracellular $\alpha$-Amylase Production

Initial pH of the growth medium plays a crucial role in the enzyme production. To identify the optimum pH value for the amylase production pH study was conducted in a pH range of 5.0 - 9.0, with an interval of 0.5. In this study maximum amylase activity, 0.671 ± 0.035 $U{\cdot}mL^{-1}$ was obtained at pH 7 in the case of *B. amyloliquefaciens*. Then it was followed by, *B. licheniformis* 0.205 ± 0.006 $U{\cdot}mL^{-1}$ and *B. megaterium* 0.110 ± 0.001 $U{\cdot}mL^{-1}$ which were obtained at pH 6.0 and 7.5, respectively (**Figure 5**).

From the Tukey pairwise comparison under ANOVA test, it was observed that both *B. amyloliquefaciens and B. licheniformis* show significantly high amylase activity at their optimum pH values than any other pH values tested. However, *B. megaterium* was not able to show a significant difference in its optimum pH value. Enzyme activity in pH 6.0 to 8.0 did not show any significant difference.

Previous studies have revealed that optimum initial pH value of the growth medium for bacteria is between 6 to 7. It has been also reported that *B. amyloliquefaciens* MIR-41 deliver high amylase production in pH of 6.8 [39] [42]. *B.*

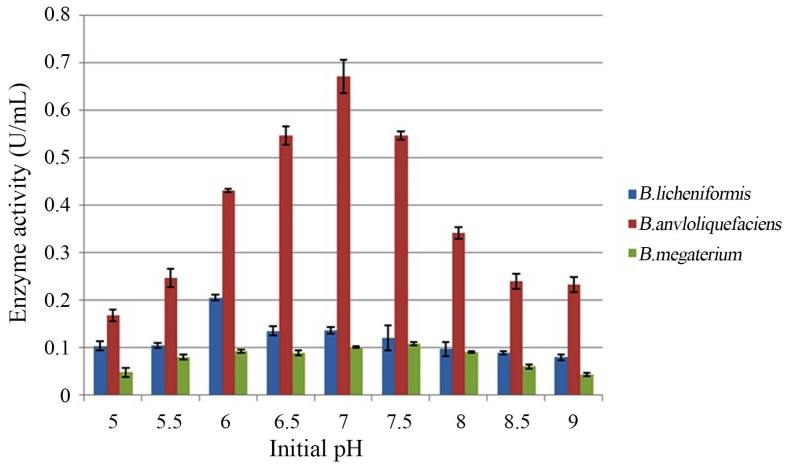


**Figure 5.** Effect of initial pH of the growth medium on extracellular *α*-amylase production of *B. licheniformis*, *B. amyloliquefaciens and B. megaterium*. Each value is an average of three parallel replicates. Error bars indicate the SE of mean values.

*megaterium* was obtained pH 7 as the optimum pH value for amylase production [18]. Also, [43] reported pH 7 as the most suitable pH value for enzyme production. Hence, it can be considered that results obtained in this experiment are in accordance with the results of previous studies.

### 3.8. Effect of Nitrogen Source on Extracellular *α*-Amylase Production

Effect of different nitrogen sources was tested by adding 0.5 % w/v of organic and inorganic nitrogen sources to the medium as the sole nitrogen source. Nitrogen is an important element for growth and enzyme production of microorganisms. An organic nitrogen source, tryptone gave the highest enzyme activity 0.294 ± 0.010 U·mL$^{-1}$ and 0.300 ± 0.020 U·mL$^{-1}$ for both *B. amyloliquefaciens* and *B. megaterium* respectively (**Figure 6**). According to [11] and [44] reported that tryptone was the best nitrogen source for *α*-amylase production. For *B. licheniformis* highest activity, 0.129 ± 0.04 U·mL$^{-1}$ was given by $KNO_3$.

From Tukey pairwise comparisons, it was observed that in *B. licheniformis*, yeast extract did not show any significant effect on enzyme production as a nitrogen source, when compared with the control. In *B. amyloliquefaciens* yeast extract has shown a negative effect on enzyme production. Nevertheless, in *B. megaterium* yeast extract had a significantly high effect on *α*-amylase production. According to [45], they also reported that *B. subtilis* IMG22 did not show any specific increase of enzyme production, when yeast extract was added as the sole nitrogen source, which in accordance with the results obtained for *B. amyloliquefaciens* and *B. licheniformis*, but in contrast to the data obtained for *B. megaterium.* It has been reported that yeast extract also served as good organic nitrogen source for *α*-amylase synthesis from *B. amyloliquefaciens* according to [14] and [46].

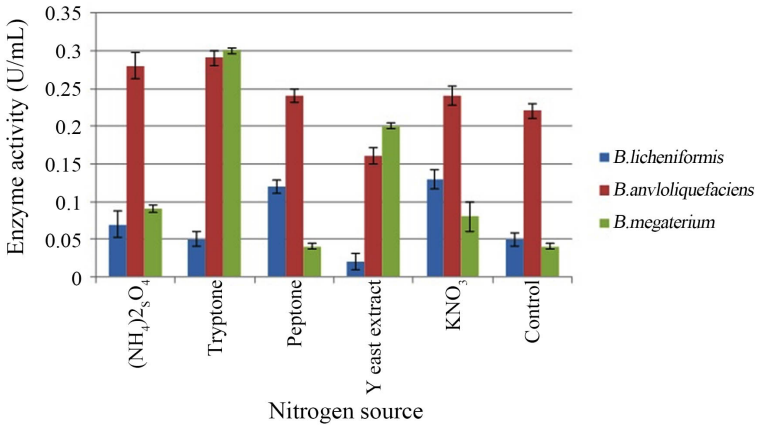


**Figure 6.** Effect of nitrogen source on extracellular *α*-amylase production of *B. licheniformis*, *B. amyloliquefaciens and B. megaterium*. Each value is an average of three parallel replicates. Error bars indicate the SE of mean values.

It was as previously reported that organic nitrogen sources are more suitable for amylase production than inorganic nitrogen sources [1]. Since, tryptone has given the highest enzyme production for both *B. amyloliquefaciens* and *B. megaterium*, it can be considered that results of this study correlate with that of previous studies.

Some of the previous reports also have mentioned that some amylase producing organisms prefer complex nitrogen sources such as tryptone and peptone over inorganic simple nitrogen sources such as various salts of ammonia and potassium [47]. In this experiment unprecedented amylase production was observed from *B. megaterium*, when tryptone and yeast extract were used as sole nitrogen sources. It can be assumed, that in case of *B. megaterium*, there is an essential requirement of some nutrient compounds which are only present in these two organic compounds. Tryptone and peptone consist of various amino acids and vitamins, which might be responsible for this result. However, reports about amino acids and vitamins on amylase production are highly variable. It can also be that organism prefers protein as a nitrogen source for growth rather than for extracellular enzyme formation [48].

Similar situation has also been observed by [49]. According to that study, amylase production of *B. amyloliquefaciens* ATCC 23350 was increased by a factor of 300, when glycine was present in the fermentation medium as a nitrogen source. It was also discovered that glycine can also play a role of pH controller in the fermentation medium. However, there are also some reports that these amino acids can act as inducers for amylase synthesis and secretion [50]. Nevertheless, to identify what cause this unprecedented result in our study, further experiments have to be carried out.

The difference in the requirement of nitrogen sources can be attributed to the difference in their genetics. The organisms might have different mechanisms to metabolize their nutrient requirements [51].

## 4. Conclusions

The fermentation conditions and composition of the media for optimal production of thermostable extracellular $\alpha$-amylases by *B. amyloliquefaciens* strain ATCC 23350, *B. licheniformis* strain ATCC 14580 and *B. megaterium* strain ATCC 14581 have been developed in this study. From this study, it can be concluded that enzyme synthesis can be affected by conditions of fermentation such as growth temperature, incubation period and components of media such as carbon source, carbon concentration, nitrogen source and initial pH of the growth medium.

From the data obtained we can come to conclusions that both *B. amyloliquefaciens* and *B. licheniformis* produce thermostable $\alpha$-amylases. Among them, $\alpha$-amylase of *B. amyloliquefaciens* was moderately thermostable and $\alpha$-amylase of *B. licheniformis*is highly thermostable. *B. megaterium* also was able to show thermostability in a broad range of temperatures, but enzyme activity was lowest, when compared with other two organisms.

Optimum enzyme activity for *B. licheniformis* was obtained at 85˚C, where as optimum enzyme production and growth were obtained at 37˚C. Optimum enzyme activity for *B. amyloliquefaciens* was found at 75˚C. In the case of *B. amyloliquefaciens* optimum enzyme production and growth were reported at two different temperatures, 37˚C, and 45˚C respectively. In *B. megaterium* optimum enzyme activity was reported at 45˚C, where as optimum enzyme production and growth were reported at 30˚C.

In most of the experiments, the highest enzyme production was obtained from *B. amyloliquefaciens*, which followed by *B. licheniformis* and *B. megaterium*. The highest enzyme production was reported as 48 h for all three organisms. After 48 h gradual decrease in enzyme production was observed. This may be due to the depletion of nutrients and accumulation of protease enzymes.

Cassava was found to be the best starch source to induce $\alpha$-amylase production. It was even able to yield higher amylase production than soluble starch when used as the sole carbon source. This might be due to the presence of other nutrients such as minerals, which induce the production of $\alpha$-amylases.

According to the results obtained, three different initial pH values were obtained as the optimum values. All three *Bacillus* sp. show acceptable amylase production in most of the pH. Therefore, we can come to a conclusion that these three organisms can be active on a broad range of pH values and can produce amylase enzyme.

The highest enzyme production for *B. megaterium* was reported, when tryptone was used as the nitrogen source. For yeast extract also significantly high amylase production was obtained. It can be assumed that this unprecedented result was due to an introduction of vitamin or amino acid to the fermentation medium with the nitrogen source. To exactly identify the reason for this sudden increase further experiments are required to carry out.

Even though most of the previous studies have reported that organic nitrogen

sources are as the most suitable nitrogen sources for *α*-amylase production, in the case of *B. licheniformis* maximum yield was obtained from inorganic nitrogen source of $KNO_3$. It can be assumed that complex nature of organic nitrogen sources could make it harder to metabolize for some microbes. These different nutrient requirements can be attributed to their genetic variations.

According to the results obtained, it can be concluded that both *B. amyloliquefaciens* strain ATCC 23350, *B. licheniformis* strain ATCC 14580 can be utilized as potential thermostable extracellular *α*-amylase producers. They have shown high thermostability and high amylase production than *B. megaterium* strain ATCC 14581 in most of the situations. Those selected strain could found various applications in industrial biotechnology. Due to importance of these findings, further studies have to be carried out in order to commercialize the production process.

## Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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