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# Production optimization of thermostable bacterial alpha amylase by solid state fermentation of agro-byproducts

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**ABSTRACT**

The production of extracellular α-amylase by thermotolerant *Bacillus amyloliquefaciens* was studied under solid state fermentation (SSF). Various agro- byproducts namely Wheat flour, Barley flour, Corn flour, Gram flour, Moong husk, Arhar husk, Mustard oil cake, coconut oil cake, Banana peel, Potato peel, Sweet Potato peel, Soybean hull, Wheat bran, Rice bran, and Sugarcane baggase were examined for α- amylase production. Wheat flour was found to be best substrate for amylase production (145.56 IU/ml) in phosphate buffer as extracting medium. Further, the appropriate incubation period, moisture level, incubation temperature and inoculum concentration was determined. Maximum yields of 149.62 IU/ml, 144.64 IU/ml, 173.28 IU/ml, 164.48 IU/ml were achieved by employing wheat flour as substrates at temperature 37°C, pH 7, moisture content 80% and incubation period 72 h. The inoculums concentration 4ml ($10^6$cfu/ml) and phosphate concentration 0.03M were found to enhance α-amylase yield. Media supplementation with carbon source as maltose in SSF medium increased amylase enzyme yield (167.44 IU/ml). Organic nitrogen (tryptone) and inorganic source (ammonium chloride) supplementation showed a higher enzyme production 169.16 IU/ml and 167.11 IU/ml respectively.



## Introduction

Amylases are starch degrading enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes having approximately 25% of the world enzyme market **(Reddy *et al.*, 2003; Rajagopalan and Krishnan, 2008)**. They degrade starch and related polymers to yield products characteristic of individual amylolytic enzymes. Initially the term amylase was used originally to designate enzymes capable of hydrolyzing α-1, 4- glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Myrback and Neumuller, 1950; Bernfeld, 1955; Fisher and Stein, 1960). Alpha amylases (endo-1,4-α-D-glucan glucanohydrolase, E.C. 3.2.1.1) are extracellular endo enzymes that randomly cleave the 1,4-α linkage between adjacent glucose units in the linear amylose chain and ultimately generate glucose, maltose and maltotriose units.

Alpha-Amylases have potential application in a wide number of industrial processes such as starch saccharification, textile, food, brewing, fermentation, paper, detergent and fine chemical industries. However, with the advances in biotechnology, its application has expanded in many other fields such as clinical, medicinal and analytical chemistry. **(Pandey *et al.*, 2000; Gupta *et al.*, 2003; Kandra, 2003).**

The microbial amylases have a broad spectrum of industrial applications due to greater stability as compared to plant and animal α-amylases **(Tanyildizi *et al.*, 2005)**. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics. Enzymes from fungal and bacterial sources have dominated applications in industrial sectors however commercial applications α-amylase is mainly derived from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus amyloliquefaciens* and finds potential application in a number of industrial processes **(Pandey *et al.*, 2000; Konsoula *et al.*, 2007)**. *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase that have been currently investigated to improve industrial processes of starch degradation **(Gomes *et al.*, 2003; Stamford *et al.*, 2001; Asgher *et al.*, 2007; Prakash and Jaiswal, 2009)**.

SSF has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the organism **(Singhania *et al.*, 2009; Pandey, 2003)**. Traditionally, amylase has been produced by submerged fermentation (SmF) but in recent years solid state fermentation (SSF) process have been increasingly utilized for the production of this enzyme **(Kunamneni *et al.*, 2009)**.

SSF offers numerous oppurtunities in processing of agro-industrial residues and SSF reproduces the natural microbiological processes like composting and ensiling. On one hand by utilizing the low cost agricultural residues SSF adds on to economic feasibility of the process and on other hand it solves the problem of its disposal which otherwise cause pollution **(Singhania *et al.*, 2009; Pandey, 2003)**. SSF has many advantages over SmF, including superior productivity, simple technique, low capital investment, low energy requirement and less water output, better product recovery and lack of foam build up and reported to be the most appropriate process for developing countries **(Tanyidizi *et al.*, 2007)**.

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The use of agricultural wastes makes SSF an attractive alternative method. The major factors that affect microbial synthesis of enzymes in a SSF system include selection of a suitable substrate and microorganism, particle size of the substrate, inoculum concentration and moisture level of the substrate **(Anto *et al*., 2006; Pandey *et al*., 1999)**.

The aim of the present study was to evaluate the feasibility of easily available agro byproducts in SSF for the production of α-amylase by *Bacillus amyloliquefaciens* MTCC 610. In this study, the effects of incubation time, incubation temperature, extraction medium, inoculum level, initial pH, supplementation with various carbon, nitrogen, and phosphorous concentration were investigated with wheat flour.

## Materials and Methods

### Place of Work

The present study was conducted in the Department of Microbiology and Fermentation Technology, Jacob School of Biotechnology and Bioengineering, Sam Higginbottom Institute of Agriculture, Technology & Sciences (Deemed to be University), Allahabad (U.P).

### Procurement and Maintenance of Culture

***Bacillus amyloliquefaciens* (MTCC 610)** used in the present study was obtained from the Microbial Technology Culture Collection, Institute of Microbial Technology (IMTECH), Chandigarh (Punjab), India. The strain was grown on nutrient agar slants and maintained at 4°C.

### Substrates and its pretreatment

Various agro by-products and their residues viz. Wheat flour, Barley flour, Corn flour, Gram flour, Moong husk, Arhar husk, Mustard oil cake, coconut oil cake, Banana peel, Potato peel, Sweet Potato peel, Soybean hull, Wheat bran, Rice bran, and Sugarcane baggase were used as substrate for solid state fermentation (SSF). These were obtained from local market of Allahabad city, India. Agro residues like bran, peel and baggase were chopped and dried (70°C) for 16 h. The dried residues were then ground to powder form (40 mm mesh) and stored in polythene bags at room temperature (30±2°C) till used as substrate for alpha amylase production **(Asghar *et al*., 2002)**.

### Preparation of Inoculum

For the preparation of inoculum, a volume of 50 ml of nutrient broth was inoculated with a loopfull of cells from a 24h old slant and kept at 37°C in a rotary shaker (100 rpm). After 18 h of incubation, 1 ml of this nutrient broth culture of *B. amyloliquefaciens* was used as the inoculum for solid state fermentation **(Gangadharan *et al*., 2006)**.

### Solid state fermentation medium for production of Alpha amylase

Five grams of each of the dried substrate were placed in 250 ml Erlenmeyer flasks and then moistened with mineral salt solution ($K_2HPO_4$, 2g/L; $NH_4NO_3$,10g/L ; NaCl,1g/L ; $MgCl_2$, 1g/L). Distilled water was added to the mineral salt solution in order to maintain the concentration of mineral elements in the medium and to adjust the required moisture level **(Gangadharan *et al*., 2006)**

### Shake flask studies

The fermentation media in the flasks were autoclaved at 121°C for 20 minutes and cooled to about 30°C. The flasks were inoculated with 1% inoculum of *B. amyloliquefaciens* and the contents of the flask were mixed thoroughly to ensure uniform distribution of the inoculum. The flasks were incubated at 37°C for 24 h in a shaking incubator operated at speed of 100 rpm. All the experiments were run parallel in triplicates **(Gangadharan *et al*., 2006)**.

### Extraction of enzyme

After fermentation, the fermented matter in each flask was extracted by the addition of different extraction medium like distilled water, 0.2M Phosphate buffer (pH 7±0.2), 0.1% Tween-80 and Triton-X-100 to a total extract volume of 200 ml. The entire content was mixed thoroughly at 30°C for 1 h in rotary shaker at 100 rpm and filtered using a Whatman filter paper no.1. The suspensions were then centrifuged at 8000 rpm at 4°C for 10 minutes. The supernatant was carefully collected and used as crude enzyme extract for the estimation of alpha amylase activity.

### Estimation of alpha amylase

The alpha amylase assay was carried out according to the method of **Okolo *et al*. (1995)**. The reaction mixture consisted of 1.25 ml of 1 % soluble starch, 0.5 ml of 0.2 M phosphate buffer (pH=7), and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50°C, 3 ml of 3, 5-dinitrosalicylic acid (DNS) reagent was added and boiled for 5 minutes to stop the reaction. The liberated reducing sugars (glucose equivalents) were estimated by **glucose standard curve** using the 3, 5-dinitrosalicylic acid (DNS) **(Miller, 1959)**.The colour developed was read at 510 nm using a UV- spectrophotometer. The blank contained 0.75 ml of 0.2 M phosphate buffer (pH=7) and 1.25 ml of 1 % starch solution. One unit (IU) of α- amylase was defined as the amount of enzyme releasing one µmol of glucose equivalents per minute under the assay conditions". The enzyme activities used for representations were the average values of three independent experiments.

$$\text{Enzyme activity (U/ml)} = \frac{\text{Amount of reducing sugars (X)} \times \text{Dilution factor}}{\text{Molecular weight of glucose} \times \text{time of incubation} \times \text{volume of enzyme}}$$

### Evaluation of various agrobased substrates for production of alpha amylase by using *Bacillus amyloliquefaciens*

In an attempt to choose a potential substrate for solid state fermentation which supports amylase production, various agro byproducts like Wheat flour, Moong husk, Barley flour, Corn flour, Gram flour, Arhar husk, Mustard oil cake, Coconut oil cake, Rice bran, wheat bran, Potato peel, Sweet potato peel, Banana peel, Sugarcane baggase, Soybean husk were evaluated individually. SSF was carried out by taking 5 grams of dried substrate in a flask to which mineral salt solution and distilled water was added to adjust the required moisture level. The contents of the flask were mixed and autoclaved at 121°C for 20 minutes. The flasks were inoculated using 1 ml of culture broth and incubated at 37°C for 24 h. Crude enzyme was extracted by mixing a known quantity of fermented matter with extraction medium. The suspension was then centrifuged at 8000 rpm at 4°C for 10 minutes. The supernatant so obtained after centrifugation was used for enzyme assay. Among the various substrates screened for SSF, the substrate showing highest enzyme activity was considered potential substrate for production of alpha amylase.

### Optimization of fermentation process parameters for production of alpha amylase

Fermentation media play an important role in the growth of the microorganism as well as in alpha amylase production. Fifteen different substrates were evaluated for enzyme production by using two strains namely *Bacillus amyloliquefaciens* in shake flasks. Best substrate was employed for further optimization of production process parameters namely initial moisture content (55, 60, 65, 70, 75, 80, 85 and 90 %), incubation time (24, 48, 72, 96, and 120 h), incubation

temperature (35, 40, 45, 50, and 55°C), initial pH (4, 5, 6, 7, 8, 9, and 10) of the medium, inoculums size (0.5, 1, 2, 4, 6, and 8 x $10^6$ CFU/ml).

**Optimization of medium components for production of alpha amylase in SSF**

The optimization of medium components is of primary importance in any fermentation process. The best substrate was employed for further optimization of nutrient supplementation such as inorganic nitrogen sources (0.15M) (Ammonium nitrate, Ammonium chloride and Ammonium sulphate), 1% organic nitrogen sources (peptone, tryptone, yeast extract, soybean meal). Added phosphate ($KH_2PO_4$) concentration (0.01M, 0.02M, 0.03M and 0.04 M) were also optimized for production of alpha amylase. To study the efficacy of various inducers the medium was supplemented independently with 1% glucose, lactose, maltose and soluble starch. Distilled water, 0.2M phosphate buffer (pH 7), Tween 80 and Triton X100 were used independently to find the best extraction medium for the enzyme.

**Results and Discussion**

**Evaluation of various agrobased substrates for production of Alpha amylase by *Bacillus amyloliquefaciens***

Fermentative production of alpha amylase using *Bacillus amyloliquefaciens* from different agrobased substrates was investigated. Fifteen different substrates were screened for the alpha amylase production. From the set of experiments, the highest alpha amylase enzyme yield (145.56 IU/ml) was observed from wheat flour when extracted with phosphate buffer. Barley flour gave high amylase enzyme yield (138.64 IU/ml) under Triton X-100 extraction solvent followed by Tween80 medium (131.48 IU/ml). Wheat flour also gave significant amylase enzyme production (134.64 IU/ml) in distilled water as an extracting medium. Among all the substrates, soybean husk was found to give lowest enzyme yield (40.44 IU) when extracted with distilled water **(Table 4.1, Fig 4.1)**.

**Table 1: Production of α- amylase by *Bacillus amyloliquefaciens* in different agrobyproducts and extraction medium**

| S.No | Substrate (Agrobyproducts) | Enzyme Activity(IU/ml) in different extraction medium | | | |
|---|---|---|---|---|---|
| | | Distilled water | Phosphate Buffer | Tween 80 | Triton X-100 |
| 1. | Wheat flour | **134.64** | **145.56** | 114.36 | 112.66 |
| 2. | Moong husk | 59.33 | 51.38 | 45.24 | 58.28 |
| 3. | Barley flour | 72.45 | 128.10 | **131.48** | **138.64** |
| 4. | Corn flour | 60.39 | 92.44 | 62.22 | 52.58 |
| 5. | Gram flour | 95.72 | 83.47 | 53.74 | 59.64 |
| 6. | Arhar husk | 53.38 | 78.14 | 54.72 | 46.44 |
| 7. | Mustard oil cake | 48.24 | 60.82 | 70.40 | 60.48 |
| 8. | Coconut oil cake | 51.26 | 96.71 | 50.25 | 75.50 |
| 9. | Rice bran | 76.76 | 93.62 | 98.35 | 98.44 |
| 10. | Wheat bran | 92.07 | 108.05 | 109.13 | 116.67 |
| 11. | Potato peel | 53.73 | 86.60 | 87.23 | 91.87 |
| 12. | Sweet potato peel | 60.95 | 95.92 | 89.45 | 90.15 |
| 13. | Banana peel | 75.40 | 133.44 | 95.56 | 99.17 |
| 14. | Sugarcane baggase | 63.05 | 104.78 | 64.89 | 102.34 |
| 15. | Soybean husk | 40.44 | 60.72 | 45.27 | 70.54 |

Due to Substrate: $F_{(cal)} = 9.88 > F_{(tab)} = 1.93$ (S) at 5%;

Due to Extraction medium: $F_{(cal)} = 8.04 > F_{(tab)} = 2.82$ (S)

The investigation revealed that alpha amylase production was responded by the *Bacillus amyloliquefaciens* in all the agrobased substrate tested. Different workers **(Losane and Ramesh 1990; Haq *et al.*, 2003; Gangadharan *et al.*, 2006; Anto *et al.*,2006; Balkan and Ertan *et al.*,2007)** conducted on amylase production using different microbes and reported wheat bran as best substrate for enzyme synthesis. Irfan *et al.*(2011) reported wheat bran as best substrate among other substrates such as rice bran and cottonseed meal.

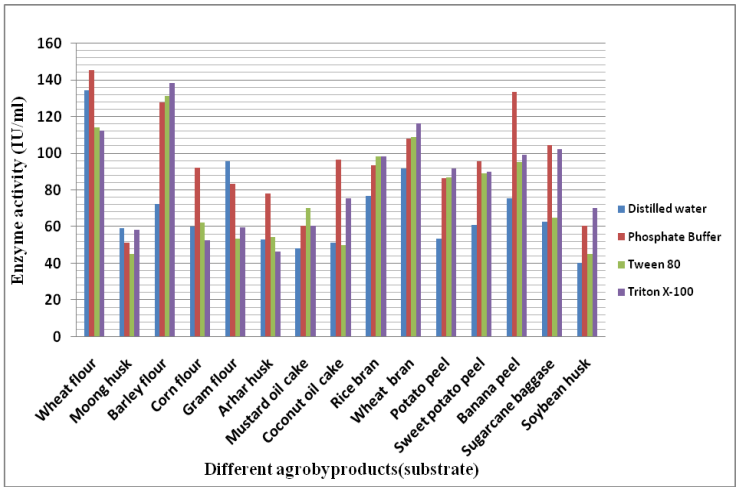


**Fig 4.1: Yield of α- amylase by *Bacillus amyloliquefaciens* using various agro based substrates**

In the present study mustard oil cake gave higher amylase production in comparison to wheat bran, rice bran. Similar findings were also observed in another study conducted by **Saxena and Singh (2011)** for alpha amylase production. **Sodhi *et al.* (2005)** also reported higher amylase enzyme yield in case of wheat bran in comparison to rice bran and corn bran. **Tanyildizi *et al.* (2007)** also reported alpha amylase production from corn gluten meal (CGM) by using *B. amyloliquefaciens*. In the present study microorganism showed good production of alpha amylase when used with chopped banana peel as substrate and results are in lined with those of **Kokab *et al.* (2003)**. From the investigation conducted by **Umamaheshwari *et al.* (2010)** wheat bran was recorded to have higher efficiency in amylase production in comparison to rice bran which supports the present study. **Unakal *et al.* (2012)** also observed alpha amylase activity by using *Bacillus subtilis* when banana peel was used in the medium. **Kaur and Vyas (2012)** also found wheat bran to be suitable natural source for maximum production of amylase when compared with rice flakes. **Sivramkrishnan *et al.* (2007)** reported higher yield of alpha amylase when wheat bran was used with different oil cakes.

**Optimization of process parameters for α- amylase production by using *Bacillus amyloliquefaciens* under solid state fermentation**

**Effect of inoculum size on α- amylase production**

Production of α-amylase under SSF condition was conducted in 250 ml capacity Elenmeyer flasks in rotary shaker incubator at 100rpm at 37°C. Varying inoculum concentrations from $0.5x10^6$ to $8x10^6$ CFU/ml were used for the production of α-amylase during solid state fermentation. Inoculum concentration was an important factor for the production of α-amylase enzyme. Lower inoculum size upto $1x10^6$ CFU/ml resulted in lower enzyme yield (98.55 IU/ml). The maximum α-amylase enzyme yield (164.16 IU/ml) was obtained when 4 ml of inoculum containing $1x10^6$ CFU/ml was used. After that there was a gradual decrease in enzyme production and it reduced upto 84.76 IU/ml as inoculum concentration was increased to $8x10^6$ CFU/ml. **(Table 2, Fig 2)**.

**Table 2: Production of α- amylase by *Bacillus amyloliquefaciens* under different Inoculum sizes**

| S.No | Inoculum size($10^6$CFU/ml) | Enzyme activity(IU/ml) |
|---|---|---|
| 1. | 0.5 | 62.64 |
| 2. | 1 | 98.55 |
| 3. | 2 | 144.88 |
| 4. | 4 | **164.16** |
| 5. | 6 | 132.84 |
| 6. | 8 | 84.76 |

**r= 0.149 $t_{cal}$ =0.303 < $t_{tab}$ = 2.77 at 5% (NS), Y = 107.61 + 1.96 x**

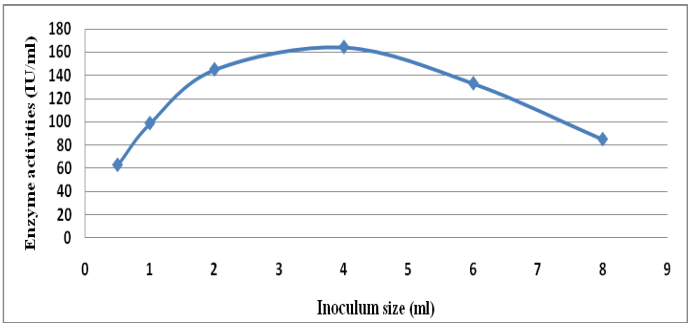


**Fig 2: Yield of alpha amylase under different Inoculum sizes of *Bacillus amyloliquefaciens***

The present research showed α- amylase production under varying inoculums sizes of *Bacillus amyloliquefaciens*. Increase in inoculum size was found to have an adverse affect on α-amylase production. This may be due to the limiting nutrients at higher cell density. The free excess liquid present in an unadsorbed form give rise to an additional diffusional barrier together with that imposed by solid nature of the substrate and this leads to a decrease in growth of bacterium and enzyme production. Lower inoculum level results in lower number of cells in the production medium. This requires a longer time to grow to an optimum number to utilize the substrate and form the desired product. Thus 2 ml was used as inoculum for further optimization studies. The findings were similar to those of several other workers **(Ramachandran *et al*., 2004; Haq *et al*., 2010)**. **Gangadharan *et al*. (2006)** reported that alpha amylase production under solid state fermentation decreased with the increase of inoculum size. **Riaz *et al*. (2003)** studied the different level of inoculum size from 1-8% for the production of alpha amylase and reported maximum enzyme production at 4% level of inoculum and beyond this level, production of enzyme was decreased. **Anto *et al*. (2006)** reported optimum enzyme production at 10% inoculum size and found similar decrease in enzyme production as the inoculum size increased from 10-40%.

**Effect of moisture content on α- amylase production**

As the moisture content of the medium changes during fermentation due to evaporation and metabolic activities, adjusting the optimum moisture level of substrate during SSF is therefore most important. Low and high level of moisture level of substrate affect the growth of microorganism resulting lower enzyme production. In the present study, a gradual increase in the enzyme production was obtained with increase in moisture content from 55-80%. Highest α- amylase yield (173.28 IU/ml) was obtained when the moisture content was maintained at 80% followed by the yield (154.16 IU/ml) at 75% moisture content. Further enzyme production was decreased with the increase of 85% and 90% moisture level of substrate. The lowest α-amylase enzyme titre (62.66 IU/ml) was obtained at 55% moisture content of substrate **(Table 3; Fig 3)**.

**Table 3: Production of α- amylase by *Bacillus amyloliquefaciens* under different moisture content of medium**

| S.No | Moisture content (%) | Enzyme activity(IU/ml) |
|---|---|---|
| 1. | 55 | 62.66 |
| 2. | 60 | 141.24 |
| 3. | 65 | 144.78 |
| 4. | 70 | 149.85 |
| 5. | 75 | 154.16 |
| 6. | 80 | **173.28** |
| 7. | 85 | 128.13 |
| 8. | 90 | 98.34 |

**r= 0.226, $t_{cal}$ =0.569 < $t_{tab}$ = 2.44 at 5% (NS) , Y = 0.65 x - 84.25**

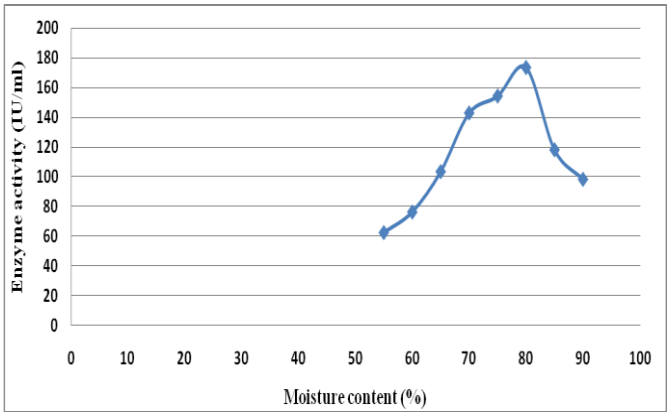


**Fig 3: Yield of α- amylase by *Bacillus amyloliquefaciens* under different moisture content of medium**

The present investigation showed alpha amylase production under different moisture level fermentation medium ranginging from 55% to 90%. Higher moisture level decreases porosity, changes in structure of the substrate particles, promotes development of stickness and lower oxygen transfer. If the quantity of water become insufficient and does not allow a good diffusion of solutes and gases, the cell metabolism slows down or it can stop completely because of the lack of substrates or due to too high conentration of inhibitor metabolites in or near the cell. **Ramachandran *et al*. (2004)** reported maximum amylase enzyme yield at 68% and yield was decreased with further increase in moisture level. Similar findings were reported by **Ramesh and Losane (1990)**. **Anto *et al*. (2006)** reported maximum alpha amylase production by thermophilic *B.coagulans* using a high level of moisture at 1:2.5 ratio of substrate and moisture content. **Gangadharan *et al*. (2006)** also reported maximum enzyme production when the substrate moisture was set at 85%. **Asgar *et al*. (2002)** studied α- amylase production under SSF of banana peels at different moisture levels and reported that enzyme activity increased upto 70% moisture and decreased thereafter with 80% moisture. **Krishna and Chandrasekran (1996)** also reported 70% moisture level as optimum for alpha amylase production by *Bacillus subtilis* in SSF of banana stalk. **Babu and Satyanarayan(1995)** recorded highest production of alpha amylase at the ratio of 1:2.5 of substrate and moisture level.

**Effect of pH on α- amylase production**

Initial pH is one of the critical parameters which correlate with the microbial growth because the concentration of hydrogen ion plays an important role by inducing morphological changes in the organism and in enzyme secretion. The influence of pH on α-amylase production was investigated in the present research. The maximum production of α-amylase was observed when initial medium pH was 7.0 which yielded 144.64 IU/ml. The enzymatic activity sharply increased from pH 6 to pH 7. A

gradual decrease in the enzyme yield was obtained from pH 8 to 10 because increase in pH of the medium beyond 7.0 did not favour the secretion of enzyme by the bacterium **(Table 4; Fig 4)**.

**Table 4: Alpha amylase production by *Bacillus amyloliquefaciens* under different pH of SSF medium**

| S.No | pH of Fermentation medium | Enzyme activity (IU/ml) |
|---|---|---|
| 1. | 4 | 65.96 |
| 2. | 5 | 88.90 |
| 3. | 6 | 124.56 |
| 4. | 7 | **144.64** |
| 5. | 8 | 112.58 |
| 6. | 9 | 64.70 |
| 7. | 10 | 56.68 |

**r= -0.20, $t_{cal}$ =-0.578 < $t_{tab}$ = 2.30 at 5% (NS), Y = 116.05 - 3.15 X**


**Fig 4: Yield of Alpha amylase by *Bacillus amyloliquefaciens* under different pH of SSF medium**

The present investigation showed variations of alpha amylase production under different pH of the solid state fermentation medium. They are the indicators of changes in metabolic activity. The variation in pH results from the substrate consumption (i.e. Protein hydrolysis) and metabolic production (i.e. Organic acids). Similar variation in enzyme production was observed with increase and decrease in pH by several co-workers **(Gangadharan *et al.*, 2006; Anto *et al.*, 2006)**. **Haq *et al.* (2003)** reported pH 7.5 – 8.0 to be the best for α-amylase production by *Bacillus subtilis*. The study conducted by **Singh *et al.* (2009)** and **Alva *et al.* (2007)** are in accordance with the present study in which maximum production of α-amylase was reported at pH 5.8 -6.0. **Asgar *et al.* (2002)** also reported a gradual decrease in enzyme yield from pH 7 to 9. The results are in contrast with those of **Keating and Kelly (1996)** who got maximum α-amylase activity at pH 5. **Irfan *et al.* (2011)** recorded maximum α-amylase production at pH 7.5 in SSF medium by using *Bacillus subtilis*. **Kokab *et al.* (2003)** also found maximum production of amylase enzyme at pH 7 and comparatively lower production was observed by at pH 8 when *Bacillus subtilis* was used in SSF of banana peels.

**Effect of incubation temperature on α- amylase production**

Fermentation temperature is an important criterion for solid state fermentation since growth and production of a metabolite are usually sensitive to temperature. In this present study, the influence of different incubation temperatures varying from 30°C to 55°C on α- amylase production by *Bacillus amyloliquefaciens* were investigated. The results revealed that highest α- amylase production (149.62IU/ml) was recorded at 37°C. The enzyme production however decreased at higher temperatures. The production of amylase enzyme was found least (75.54 IU/ml) at temperature (30°C). The amylase activity also decreased about upto 122.82 IU/ml and 110.36 IU/ml of maximum yield at temperatures 40°C and 45°C respectively **(Table 5; Fig 5)**.

**Table 5: Alpha amylase production by *Bacillus amyloliquefaciens* under different Incubation temperature of SSF**

| S.No | Incubation Temperature($^{o}$C) | Enzyme activity(IU/ml) |
|---|---|---|
| 1. | 30 | 75.54 |
| 2. | 37 | **149.62** |
| 3. | 40 | 122.82 |
| 4. | 45 | 110.36 |
| 5. | 50 | 89.36 |
| 6. | 55 | 76.84 |

**r=-0.32, $t_{cal}$ =0.676 < $t_{tab}$ = 2.77 at 5% (NS) , Y = 148.77 - 1.06 X**

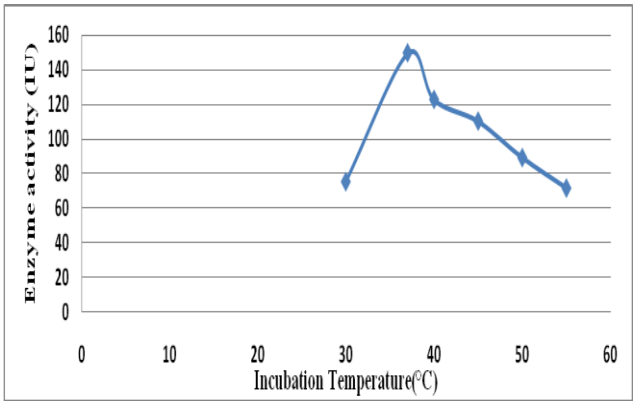


**Fig 5: Effect of different incubation temperature on Alpha amylase production by *Bacillus amyloliquefaciens* under Solid state fermentation**

The production of alpha amylase by using *Bacillus amyloliquefaciens* was examined at varying temperatures ranging from 30°C to 55°C. Similar study was conducted by **Gangadharan *et al.* (2006)** in which 37°C found to be optimum for biosynthesis of alpha amylase. **Tanyildizi *et al.* (2007)** have reported 33°C for the highest production of α-amylase from corn gluten meal. **Sajitha *et al.* (2011)** optimized incubation temperature for the production of α- amylase by using *Bacillus megatarium* and found maximum at 35°C. **Ashraf *et al.* (2005)** have reported maximum enzyme yield at 40°C when *Bacillus licheniformis* was used. It has been reported that the metabolic heat generated during microbial cultivation in SSF exerts harmful effects on the microbial acivity **(Pandey, 1990)**. **Asghar *et al.* (2002)** also reported highest amylase enzyme yield at 35°C by using *Bacillus subtilis*. Similar observation was also recorded by **Riaz *et al.* (2003)** obtained maximum production by using *Bacillus subtilis* at 40°C. **Haq *et al.* (2010)** conducted fermentation by using mutant strain of *Bacillus amyloliquefaciens* EMS-6 in stirred fermenter and noticed better yield when the incubation temperature of the medium was adjusted at 37°C. **Irfan *et al.* (2011)** reported the optimum production of α- amylase by *B. subtilis* at 35°C. **Castro *et al.* (1992)** reported the continuous production of amylase from *B. amyloliquefaciens* at 36°C. **Kokab *et al.* (2003)** found optimum temperature at 35°C for the production of alpha amylase from banana stalk by *Bacillus subtilis* and also observed decrease in enzyme yield with the variation of incubation temperature. **Haq *et al.* (2009)** noticed maximum α- amylase production by the mutant strain of *Bacillus licheniformis* EMS-200 at 37 °C. It might be due to the fact that 37°C is optimum temperature of growth of bacterial culture and subsequently for enzyme production. In addition, high temperature might have reduced the moisture contents of the fermentation medium and growth of the organism resulting in the decreased enzyme production

**(Markkanen and Suihko, 1974)**. The thermal characteristics of substrate and the low moisture content in SSF are especially difficult conditions for heat transfer. Heat removal is inadequate for dissipating metabolic heat due to the poor thermal conductivity of most solid substrates and result in temperature gradient **(Raimbault, 1998).**

**Effect of incubation period on alpha amylase production**

The incubation time is governed by characteristic of the culture and also based on growth rate. In the present study the optimum time course of fermentation was investigated. Each flask of fermented broth was harvested at regular interval of 24 hrs upto 120 hrs. It was observed from experiments that there was gradual increase in α-amylase production through 24hrs (73.64 IU/ml), 48hrs (143.96 IU/ml) and maximum production (164.48 IU/ml) was found at 72 hrs of incubation. The enzyme yield showed sharp decrease on further extension of fermentation period. The alpha amylase yield reduced about 25.74% of maximum enzyme yield at 96 hrs and further amylase yield was found minimum (104.86 IU/ml) at 120 hr of fermentation period.**(Table 6; Fig 6)**.

**Table 6: Production of α- amylase by *Bacillus amyloliquefaciens* under different Incubation period of SSF**

| S.No | Incubation Time(hr) | Enzyme activity(IU/ml) |
|---|---|---|
| 1. | 24 | 73.64 |
| 2. | 48 | 143.96 |
| 3. | 72 | **164.48** |
| 4. | 96 | 122.14 |
| 5. | 120 | 104.86 |

**r= 0.183, $t_{cal}$ =0.322 < $t_{tab}$ = 3.182 at 5% (NS), Y = 109.63 - 0.169 X**

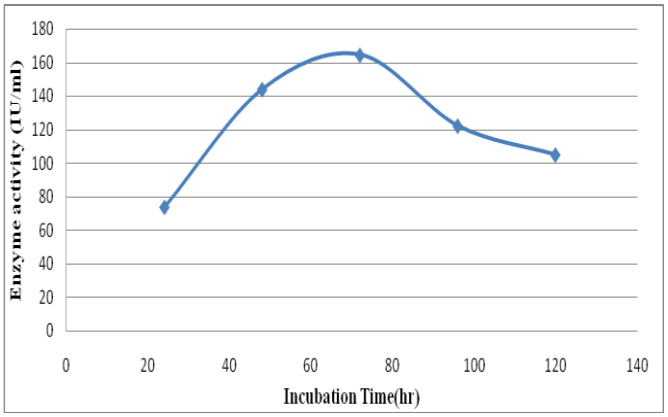


**Fig 6: Variation in α-amylase production by *Bacillus amyloliquefaciens* under different Solid state fermentation period**

The present investigation was performed for alpha amylase production under different fermentation periods. The decrease in enzyme yield after optimum level might be due to denaturation and decomposition of alpha amylase because of interaction with other components in the medium or substrate inhibition. In a similar study conducted by **Gangadharan *et al.* (2006)** in which fermentation period of 72 h was found optimum for alpha amylase production by *Bacillus amyloliquefaciens*. In another research **Anto *et al.* (2006)** observed maximum enzyme yield after 72 h by using *B. cereus* with wheat bran as substrate and decreased with further incubation. **Tanyildizi *et al.* (2007)** showed alpha amylase production with incubation time and reported that the *B. amyloliquefaciens* utilized the corn gluten meal effectively with highest yield after 24 h. **Ashraf *et al.* (2005)** also observed maximum enzyme production from wheat bran after 48h when *Bacillus licheniformis* was used. **Asghar *et al.* (2002)** found 48 h best incubation time for production of alpha amylase by using *Achraniotus sp.* in waste bread medium. **Irfan *et al.* (2011)** recorded 48h of fermentation period as best for the alpha amylase secretion. **Riaz *et al.* (2003)** found maximum production after 48hrs by using *Bacillus subtilis* with pearl millet as substrate. **Kokab *et al.* (2003)** optimized solid state flask culture for production of α-amylase by *B. subtilis* using banana peel as a substrate and found maximum yield at 24h. **Tunkova *et al.* (1993)** produced α- amylase by *B. licheniformis* 44MB 82-G using glucose as carbon source and optimum enzyme activity of culture medium was recorded after 96 hrs. These variations in the fermentation period might be due to the different microbial strains and different substrate used during fermentation processes. **Haq *et al.* (2010)** also obtained highest enzyme yield in 48 h by using *B. amyloliquefaciens* EMS-6 in stirred fermenter. **Sodhi *et al.* (2005)** carried out solid state fermentation with bacillus sp. PS-7 in flasks and trays with wheat bran and the maximum enzyme yield was attained after 48 and 72 h with flasks and trays respectively.

**Optimization of Medium Parameters for alpha amylase production by *Bacillus amyloliquefaciens* under solid state fermentation**

**Evaluation of additional Carbon sources (1%) on alpha amylase production**

Alpha amylase is an inducible enzyme, which is generally induced in the presence of starch or its hydrolytic product. In the present examination different sugars such as glucose, lactose, maltose and starch were supplemented in the fermentation medium for the production of α- amylase. The sugars were added in the medium at 1% level. Highest α-amylase production was observed in maltose supplemented medium (167.44 IU/ml) followed by starch (151.46 IU/ml). In the presence of other sugars however the production of enzyme was reduced. In the present study lowest amylase enzyme yield (128.56 IU/ml) was obtained when Lactose was used as additional carbon source **(Table 7; Fig 7)**.

**Table 7: Production of α- amylase production by *Bacillus amyloliquefaciens* under various carbon sources supplementation**

| S.No | Carbon source | Enzyme activity(IU/ml) |
|---|---|---|
| 1. | Control | 145.90 |
| 2. | Glucose (1%) | 136.37 |
| 3. | Lactose (1%) | 128.56 |
| 4. | Maltose (1%) | **167.44** |
| 5. | Soluble starch (1%) | 151.46 |

(Due to carbon source) $F_{cal}$ =480.56 > $F_{tab}$ = 5.31, (S)

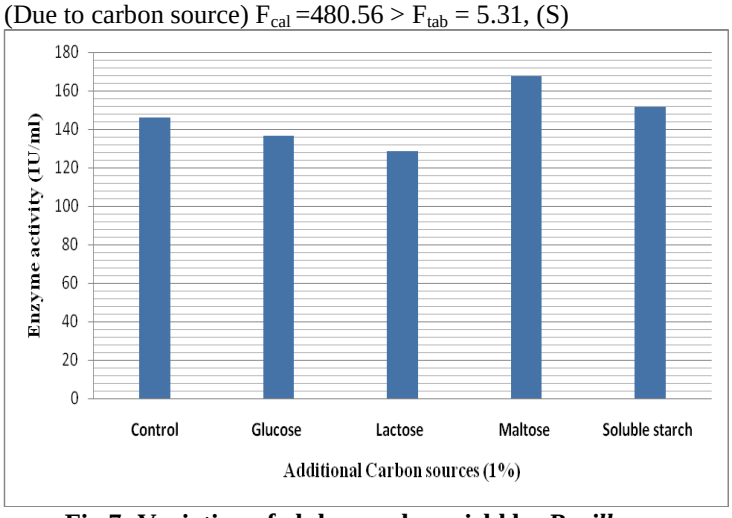


**Fig 7: Variation of alpha amylase yield by *Bacillus amyloliquefaciens* with different Carbon sources (1%) supplementation**

The present examination for the production of alpha amylase was conducted in different flasks supplemented with

different sugars and the result showed maximum amylase production with maltose followed by starch, lactose and glucose. Similar finding was obtained by **Gangadharan *et al.* (2006)** in which highest enzyme yielded by the addition of starch followed by maltose. Maximum alpha amylase yield also reported by **Ashraf *et al.* (2005)** when starch was supplemented to the medium at 1% level. A synthetic analogue of maltose, alpha methyl D-glucoside, resulted in 3-fold higher α- amylase production than inducers such as starch and maltose supplemented medium **(Goto *et al.*, 1998)**. **Haq *et al.* (2010)** found soluble starch as best for maximum production of α-amylase and medium containing lactose gave relatively less production of α-amylase. **Mamo and Gessesse (1999)** observed good growth and amylase production in the presence of starch and maltose. Also very little enzyme production was observed in media containing glucose which is in accordance with the present investigation. According to **Sato and Yamamoto (1975)**, polysaccharides like starch, glycogen induced α-amylase formation when *Bacillus licheniformis* was used for α- amylase production. **Anto *et al.* (2006)** reported marginal increase in α-amylase production by *B. cereus* using wheat bran under solid state fermentation and observed highest production with glucose. In contrast a study conducted by **Babu and Satyanarayan (1995)** reported that carbon sources such as glucose, maltose and starch did not enhance α-amylase production by thermophilic *B. coagulans* using wheat bran under solid state fermentation. **Aiyer (2004)** investigated different soluble sugars at 1% level for α-amylase production by using *B. licheniformis* and reported higher enzyme yield in fructose and maltose supplemented medium. **Sodhi *et al.* (2005)** reported higher enzyme production by using *Bacillus sp.* PS-7 with glycerol supplemented medium followed by Maltose and soluble starch. Maximum enzyme activity was obtained by **Ashwani *et al.* (2011)** by using *Bacillus mirini* in the production medium supplemented additionally with starch whereas minimum enzyme activity was recorded in the presence of dextrose. **Konsoula and Kryiakides (2007)** studied effect of carbon sources such as soluble starch, lactose, glucose, maltose and maltotriose on production of α-amylase by using *Bacillus subtilis* and found maximum production in starch supplemented medium followed by lactose and maltose. **Asghar *et al.* (2007)** also observed a decrease in growth and enzyme production when glucose was added to the fermentation medium. The addition of 1% glucose to the culture medium along with waste potato starch was found to repress the growth of the *B.subtilis* JS-2004 and synthesis of α-amylase. **Varalakshmi *et al.* (2008)** reported highest amylase enzyme yield when soluble starch was supplemented followed by glucose. *A. oryzae* was induced by maltose and starch, which was reported by **Shivramkrishan *et al.* (2006)**. In the present study the production of α-amylase by *B.amyloliquefaciens* was greatly suppressed when bacterium was grown on readily metabolizable sugars since a very low activity was detected in the culture medium in the presence of glucose.

**Effect of different Organic Nitrogen sources on alpha amylase production**

The source and concentration of organic nitrogen in the fermentation medium has an impact on microbial growth and enzyme production. The α-amylase production by *Bacillus amyloliquefaciens* was studied by adding different organic nitrogen sources in basal medium that was supplemented with peptone, tryptone, yeast extract and soy peptone at 1% level in the fermentation medium containing wheat flour. A high level of α- amylase (169.16 IU/ml) produced when tryptone was used as nitrogen source followed by peptone (151.56 IU/ml) and yeast extract (123.45 IU/ml). Compared to all the organic nitrogen sources, amylase enzyme production was found lowest (119.32 IU/ml) in media containing soy peptone **(Table 8; Fig 8)**.

**Table 8: Variation in production of α- amylase production by *Bacillus amyloliquefaciens* with different organic nitrogen supplementation**

| S.No | Nitrogen source | Enzyme activity(IU/ml) |
|---|---|---|
| 1. | Control | 144.36 |
| 2. | Peptone (1%) | 151.56 |
| 3. | Tryptone (1%) | **169.16** |
| 4. | Yeast extract (1%) | 123.45 |
| 5. | Soya peptone (1%) | 119.32 |

(Due to organic nitrogen source) $F_{cal}$ =236.85 > $F_{tab}$ = 5.31, (S)

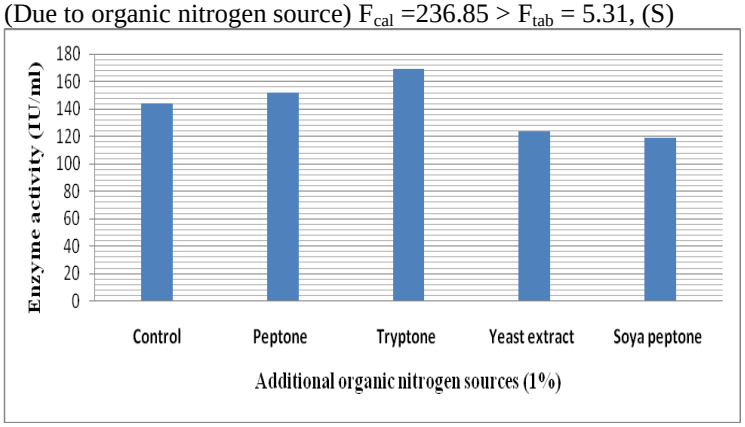


**Fig 8: Effect of different Organic Nitrogen sources supplementation on alpha amylase production by *Bacillus amyloliquefaciens***

The present research investigated the production of alpha amylase by using *Bacillus amyloliquefaciens.* The wheat flour medium supplemented with different nitrgen sources namely peptone, tryptone, yeast extract and soy peptone were fermented under solid state condition. The present investigation showed maximum amylase production in tryptone supplemented medium followed by peptone and yeast extract. Similar findings were reported by **Tanyildizi *et al.* (2007)** in which higher enzyme activity was obtained by supplementing yeast extract as a nitrogen source in the medium. **Asghar *et al.* (2002)** also reported enhanced production of alpha amylase by using *Bacillus subtilis* when peptone (0.3%) was additionally supplemented. The present investigation is in accordance with **Krishna and Chandrasekran (1996)** in which maximum α-amylase enzyme activity was observed by *Bacillus* sp. with 0.5% peptone additional nitrogen source. **Haq *et al.* (2009)** observed lesser α- amylase production in the cultural medium supplemented with bactopeptone or yeast extract as sole source. **Mamo and Gessesse (1999)** found high level of amylase enzyme yield when protease peptone and tryptone were used as nitrogen source. **Gangadharan *et al.* (2006)** recorded a marginal increase in enzyme yield with the addition of peptone into the medium. This indicates that any of the sources namely peptone, yeast extract and tryptone can be alternatively used. **Kobab *et al.* (2003)** also reported maximum enzyme yield in the medium supplemented with 0.2% peptone as nitrogen source. The present research is in agreement with the report of **Mulimani and Ramalingam (2000)** in which decrease in α-amylase enzyme production under solid state fermentation was pronounced when using soy meal as medium supplements. **Anto *et al.* (2006)** also reported decreased alpha amylase production with the supplementation of organic nitrogen sources like urea and yeast extract by using *Bacillus cereus.* **Aiyer (2004)**

investigated that the peptone was best organic source followed by meat extract for the production of alpha amylase by using *B.licheniformis* SPT 27. Supplementation of various organic nitrogen sources also stimulated the amylase production by Bacillus sp. PS-7 in the conducted by **Sodhi *et al.* (2005). Ashwani *et al.* (2011)** reported enhanced production of alpha amylase by using *Bacillus sp. marini* with the addition of yeast extract in the production medium. **Asghar *et al.* (2007)** found that the growth and synthesis of α- amylase *B. subtilis* JS-2004 was favoured by yeast extract. Maximum production of α-amylase was obtained using *Bacillus subtilis* in medium containing tryptone **(Konsoula and Kyriakides, 2007)**. Peptone (1%) when supplemented individually gave an increase in enzyme yield in SSF using coconut oil cake **(Ramachandran *et al.*, 2004)**. **Swain *et al.* (2006)** also reported efficient enzyme production in medium containing peptone and yeast extract.

**Evaluation of different Inorganic nitrogen sources on alpha amylase production**

The present investigation conducted for the production of α-amylase by varying inorganic nitrogen sources into the fermentation medium. Various inorganic nitrogen sources such as ammonium nitrate, sodium nitrate, ammonium chloride and ammonium sulphate at the level of 0.15 M were supplemented into production media. The fermentation was conducted fermentation medium containing wheat flour supplemented with inorganic nitrogen sources and using *Bacillus amyloliquefaciens.* The maximum enzyme production was found when the media was supplemented with ammonium chloride (167.11 IU/ml). Other inorganic nitrogen additives (ammonium nitrate, ammonium sulphate, ammonium chloride) exerted negative effect on the microbial activity and resulted in lower enzyme titres. The present study showed that the enzyme secretion was favoured by ammonium chloride supplementation **(Table 9; Fig 9)**.

**Table 9: Variation of α- amylase production by *Bacillus amyloliquefaciens* with different Inorganic nitrogen sources supplementation**

| S.No | Inorganic Nitrogen source | Enzyme activity(IU/ml) |
|---|---|---|
| 1. | Control | 142.43 |
| 2. | Ammonium nitrate(0.15M) | 128.59 |
| 3. | Sodium nitrate(0.15M) | 112.75 |
| 4. | Ammonium chloride(0.15M) | **167.11** |
| 5. | Ammonium sulphate(0.15M) | 124.49 |

(Due to Inorganic nitrogen source) $F_{cal}$ =210.20 > $F_{tab}$ = 5.31, (S)

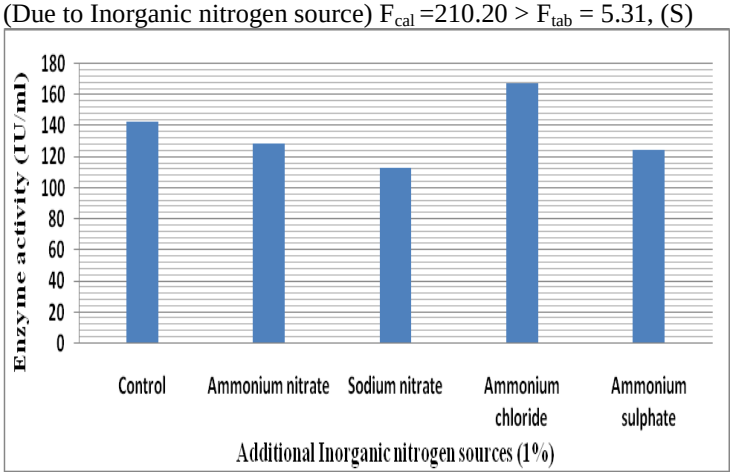


**Fig 9: Production of α- amylase production by *Bacillus amyloliquefaciens* with different Inorganic nitrogen sources supplementation**

The present study revealed that ammonium chloride (0.15M) was found suitable for maximum production of alpha amylase. In a similar research conducted by **Gangadharan *et al.* (2006)** also found increase in amylase enzyme yield when ammonium chloride was supplemented but decrease in amylase enzyme yield when ammonium sulphate and sodium nitrate were used as an additional supplement in the medium. **Aiyer (2004)** observed lowest enzyme production by using *Bacillus licheniformis* SPT 27 when ammonium sulphate was used additionally in the medium. **Mamo and Gessesse (1999)** also reported no growth of *Bacillus* sp. WNII when $NO_3^+$ and $NH_4^+$ was used as nitrogen sources. In contrast addition of ammonium chloride did not enhance the production of alpha amylase production by using *Bacillus cereus* as reported by **Anto *et al.* (2006)**. **Sodhi *et al.* (2005)** also observed lower level of alpha amylase enzyme yield with ammonium chloride supplemented medium which is in contrast with the present study. **Kaur and Vyas (2012)** evaluated various inorganic sources and obtained lower enzyme yields in ammonium sulphate as well as sodium nitrate supplemented medium. **Zar *et al.* (2013)** reported lower enzyme yield with ammonium sulphate and sodium nitrate supplemented medium which is in agreement with the present research.

**Effect of different concentrations of Phosphate on alpha amylase production by *Bacillus amyloliquefaciens***

Phosphate serves as the construction material of cellular components such as cyclic AMP, nucleic acids, phospholipids, nucleotides and coenzymes. α- amylase synthesis was found to be stimulated by phosphate concentration. The different $KH_2PO_4$ concentrations 0.01 M, 0.02 M, 0.03 M, and 0.04 M were supplemented to production medium for the production of alpha amylase. The present investigation revealed that α- amylase production was enhanced by addition of $KH_2PO_4$ upto 0.03M level and gave relatively higher enzyme yield (165.18 IU/ml) in comparison with the control (146.38 IU/ml) **(Table 10, Fig 10)**.

**Table 10: Effect of different concentrations of Phosphate on alpha amylase production by *Bacillus amyloliquefaciens***

| S.No | Phosphate concentration | Enzyme activity(IU/ml) |
|---|---|---|
| 1. | Control | 146.38 |
| 2. | 0.01M | 142.82 |
| 3. | 0.02M | 154.53 |
| 4. | 0.03M | **165.18** |
| 5. | 0.04M | 114.34 |

r= -0.34, tcal =- 0.641 < $t_{tab}$ = 3.18 at 5% (NS), **Y = 152.99 - 417.2 X**

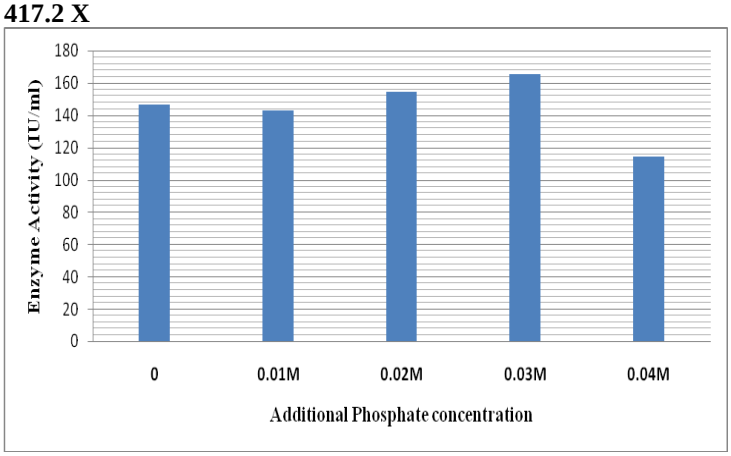


**Fig 10: Variation in α- amylase production by *Bacillus amyloliquefaciens* due to different concentrations of Phosphate supplemented to SSF**

The enhanced amylase enzyme production with phosphate supplementation in the present study is in agreement with the studies of **Gangadharan *et al.* (2006)**. **Markkanen and Enari (1972)** reported production of α- amylase by *Bacillus subtilis* NCIB8646 and recorded maximum α-amylase production at

phosphate supplementation at 0.033M level. **Kobab *et al*. (2003)** reported maximum amylase yield with 0.1% $KH_2PO_4$. **Unakal *et al*. (2012)** reported maximum α- amylase activity with 0.04% $KH_2PO_4$ in the optimum SSF of banana peel medium. **Bajpai *et al*. (1992)** observed that 0.1% $KH_2PO_4$ in cheese whey medium produced maximum α- amylase production by *Bacillus subtilis*. **Asghar *et al*. (2002)** studied varying levels of $KH_2PO_4$ on production of α- amylase in growth medium containing waste bread by using *Arachineotus* sp. and found optimum yield with 0.2% $KH_2PO_4$.

**Statistical Analysis**

The obtained data were analyzed using ANOVA and Correlation.

**Conclusion**

The results obtained in the present study indicated *Bacillus amyloliquefaciens* MTCC 610 as a potential strain for α-amylase production using solid-state fermentation with wheat flour as substrate. Commercial α-amylase production is usually produced by submerged fermentation; however, SSF appears promising due to the natural potential and advantages they offer. Based on the present study, it appears that wheat flour, which is readily available agricultural byproduct, could replace the commercial and more expensive substances in the development of a suitable economic fermentation medium for obtaining high yields of α-amylase. However, the present study was entirely a laboratory-scale study, and it has to be further improved for a large-scale SSF.

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