Optimization of alpha amylase production from B. amyloliquefaciens using Solid State Fermentation

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ABSRACT: ***The wide spectrum of the amylase enzyme in the industries to creating the demand and persist to produce more amount of enzymes. The present work has been initiated with a goal of optimization of solid state fermentation condition for amylase using agro waste and microbial strain like B. amyloliquefaciens (MTCC 610). In this study the productivity of amylase, fermentation has been carried with the presence of calcium (Ca^{+2}), Nitrate (NO_3), and chloride ions (Cl^-) as well as in the presence of D-inositol and mannitol. Amylase needs calcium ion for the preservation of its structure, activity and stability that proves beneficial also for amylase production using solid state fermentation. The addition of sugars in the SSF media is protect the amylase from thermal decay at various incubation periods at 37°C.***

Keywords: Amylases, Bacillus amyloliquefaciens, Solid State Fermentation, starch hydrolase

Introduction

Amylases are a group of hydrolases that can specifically cleave the O-glycosidic bonds in starch. Two important groups of amylases are glucoamylase and a-amylase. Glucoamylase (exo-1,4-a-D-glucan glucanohydrolase hydrolyzes single glucose units from the nonreducing ends of amylose and amylopectin in a stepwise manner .Whereas a-amylases (endo-1,4-a-D-glucan glucohydrolase, are extracellular enzymes that randomly cleave the 1,4-a-D-glucosidic linkages between adjacent glucose units inside the linear amylose chain.(Muhammad IRFAN,2012).

Most of the amylases are metalloenzyme requiring Ca for their activity, structural integrity, and stabilization [**Burha 2003, Levine 1982, Klee 1982**]. At least three calcium binding sites have been located on barley alpha-amylase isoform that is also visible for plants, mammals, fungi, and bacteria [**Kadziola 1994, MacGregor 1988**]. For *B. amyloliquefaciens*, the calcium binding site is contributed by three conserved regions of the polypeptide chain comprising residues Gly^{97} -Ala^{109}, Ile^{217} -His^{235} and Ser^{314} –Ser. Depletion of calcium ion from the binding site abolishes amylase activity. Similar stabilization effect has been provided by chloride and nitrate ions as reported by Aghajari et al. [18].
The microbial amylases meet industrial demands and a large number of them are available commercially; although many microorganisms produce this enzyme and the most commonly used for their industrial application are *Bacillus licheniformis, Bacillus amyloliquifaciens* (**Vidyalakshmi, 2009**). *Bacillus amyloliquefacien s,* a potent a-amylase producer, has been used to study the mechanism of enzyme secretion as well as from the standpoint of industrial production of enzymes (**QIXIAN ZHANG, 1983**).

Agro wastes like wheat bran, rice bran, and coconut oil bran have replaced the high cost media generally used in submerged fermentation for alpha-amylase preparation because of their simplicity, low cost, easy availability, better productivity, and lesser water output. Additionally it solves the pollution problem occurring due to their disposal in the surrounding [**Stredansky, 1999**]. High starch content of almost all agro wastes (60–70% by weight) can be effectively utilized as a major nutrient source by microorganisms like bacteria, fungi, and so forth, for the synthesis of inducible alpha-amylase which is under the control of catabolic repression.

From the prior knowledge the primary solid state fermentation culture condition, the present study was initiated using wheat bran as a prime source of nutrient and *B. amyloliquefaciens* (MTCC 1270) as the producer organism at pH 7 to increase the alpha-amylase yield through media optimization. Earlier reports are also in agreement with the fact that most of the Bacillus species, namely, *Bacillus licheniformis* and *Bacillus stearothermophilus*, are the most effective producers of alpha-amylase [Mulimani *et al.*, 2000, Shuklaand 2006, Vijayabaskar 2012, Baysal 2003, Mukherjee 2009, Sodhi 2005, Soni 2003]. This study aimed to optimization of various parameters in the fermentation media to stimulate alpha-amylase yield from SSF.

Materials and Methods

Microorganism *Bacillus amyloliquefaciens* (MTCC 610, IMTECH, Chandigarh) was used as working strain for solid state fermentation (SSF) extraction of alpha-amylases. All the reagents are of analytical grade (SRL).

Preparation of inoculum and Solid State Fermentation (SSF).

Wheat bran was collected from local market and solid state fermentation has been carried out with 4 gm dry wheat bran in a 100 mL Erlenmeyer flask. The moisture level of the wheat bran was adjusted to 50% (w/w) with autoclaved distilled water. The contents of the flask were autoclaved prior to the solid state fermentation. 25 mL of nutrient broth was taken in a 100 mL flask and was inoculated with a loop full of *Bacillus amyloliquefaciens* cells from a 24-hour-old slant and kept at 37°C in a shaker. After 16 hours of growth, 1 mL inoculum (1.5–2 × 10 cfu/mL) from this broth culture was added in the WB. It was fermented for various fermentation periods (24 and 48 hours) at different temperatures (30°, 33°, 37°, and 42°C).

Enzyme Extraction

After 24 and 48 hours of fermentation, the fermented media containing wheat bran were mixed with 25mL 20 mM phosphate buffer (pH = 7.0) for 30 minutes at 4°C in a rotary shaker at150rpm.The suspension was then centrifuged at 8000 rpm for 15 min at 4°C. The supernatant has been collected and used for amylase assay.

Amylase Assay

Alpha-amylase activity of the extract was measured by DNS method [19]. In brief the reaction mixture containing 1% soluble starch, 20 mM phosphate buffer (pH = 7), and fermented extract was taken and incubated at 37°C for 20 minutes followed by the addition of 3,5dinitrosalicylic acid (DNS). The amount of the reducing sugar liberated during assay was estimated by measuring color development at 540 nm by UV-VIS spectrophotometer. 1U of amylase activity is defined as the amount of enzyme that liberated micromole of maltose per minute under standard assay condition.

Protein Estimation.

The protein content of the extract was determined following Lowry's method [Lowry *et al* 1951].

Starch Hydrolysis

A 2% starch agar plate (beef extract—0.3%, soluble starch—1%, and agar—2%) has been prepared and streaked from a 24-hour-old culture of *Bacillus amyloliquefaciens*. The plate was grown for 48 hours in 37°C. To check the starch hydrolysis property of alpha-amylase the plate was flooded with iodine solution.

Optimization of Media

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) and 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) and Triton X100 were used independently to find the best extraction medium for the enzyme. The alpha-amylase activity has been calculated according to DNS method [Miller 1959].

Statistical Analysis

Effect of each parameter was studied in triplicate and graphically represented as the mean ± SD (n=3) using Origin 5.

RESULTS AND DISCUSSION

Amylase identification by starch hydrolase

The starch agar plate was inoculated with *B. amyloliquefaciens* (MTCC 610) and kept for 48 hours at 37°C. The plate was flooded with iodine and clear zone of starch hydrolysis has been observed (Figure 1). This ensures that this microorganism secretes amylase that is capable of starch hydrolysis.

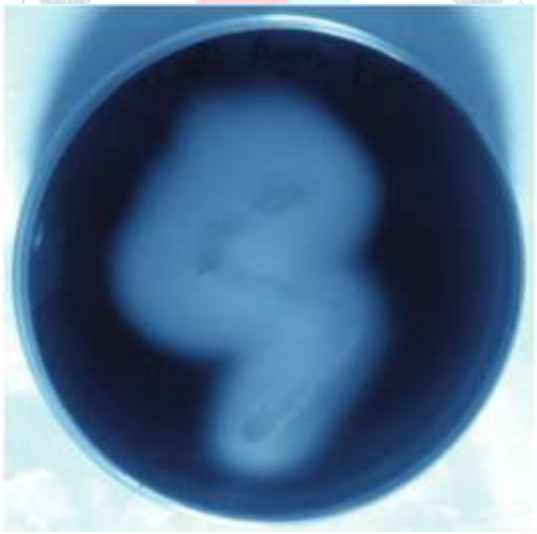

Figure 1.1. Starch hydrolysis performed on a 2% starch agar plate using

***B. amyloliquefaciens* (MTCC 610)**

Production of Alpha-Amylase from *B. amyloliquefaciens* (MTCC 610) Using Solid State Fermentation

To optimize the appropriate fermentation period for high yield alpha amylase production, the study had been initiated with wheat bran and *B. amyloliquefaciens* (MTCC 610) for 24, 48, and 72 hours. The values of specific activity of alpha-amylase were 73.64 ± 0.25 U/mg, 143.96 ± 0.24 U/mg, 164.48± 0.24, 122.14± 0.24 and 104.86 ± 0.75 U/mg, respectively, after 24, 48,72, 96,and 120 hours using SSF under identical fermentation conditions (Figure 2.1). Fermentation conducted for longer period of time was accompanied with decline in the alpha-amylase activity caused by denaturation and degradation of enzyme products.

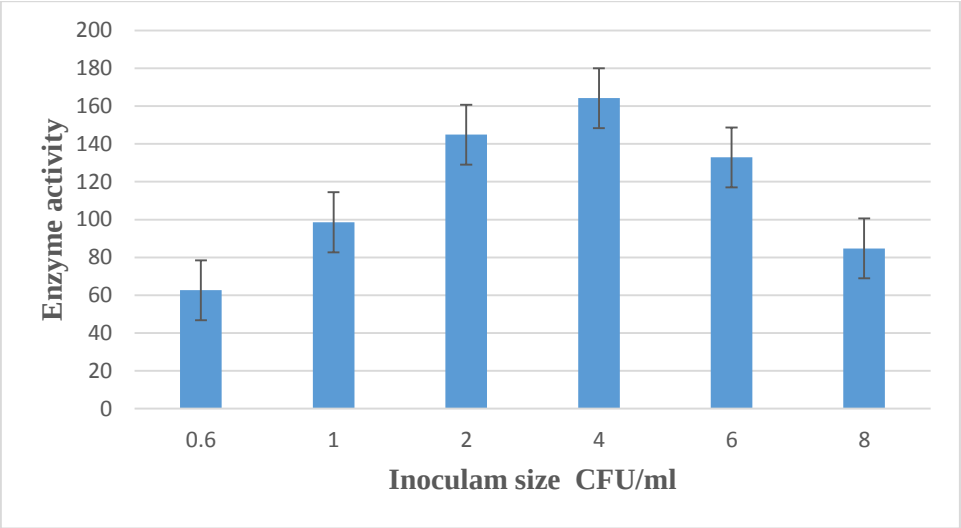

Figure 2.1. Different size of inoculam used for alpha amylase production

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) fermentation period of 72 h was found optimum for alpha amylase production by *Bacillus amyloliquefaciens*.

Effect of incubation temperature on α-amylase production

Fermentation temperature is an important criterion for solid state fermentation. The influence of different incubation temperatures varying from 30°C to 55°C on α- amylase production by *Bacillus amyloliquefaciens* (MTCC 610) were investigated. The results revealed that highest α-amylase production (149.62 IU/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 17.57% (122.82 IU/ml) and 25.9% (110.36 IU/ml) of maximum yield at temperatures 40°C and 45°C, respectively (Figure 2.2).

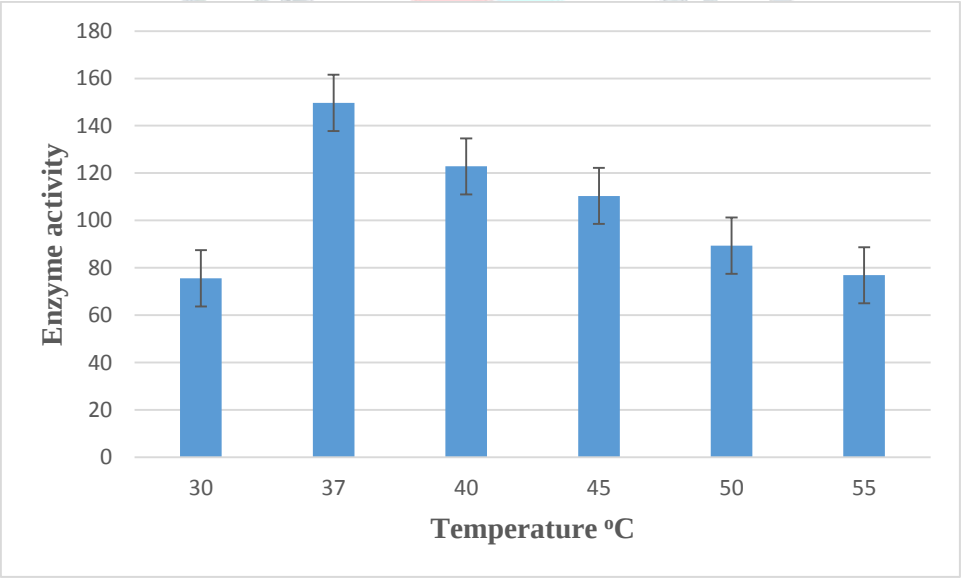

Figure 2.2. Effect of temperature on *α*-amylase production using *Bacillus amyloliquefaciens*

The production of alpha amylase by using *Bacillus amyloliquefaciens* (MTCC 610) 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.

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 very important. Low and high level of moisture level of substrate affect the growth of microorganism resulting in 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 (Figure2.3). No significant effect of moisture content of the medium on amylase production was observed ($P > 0.05$).

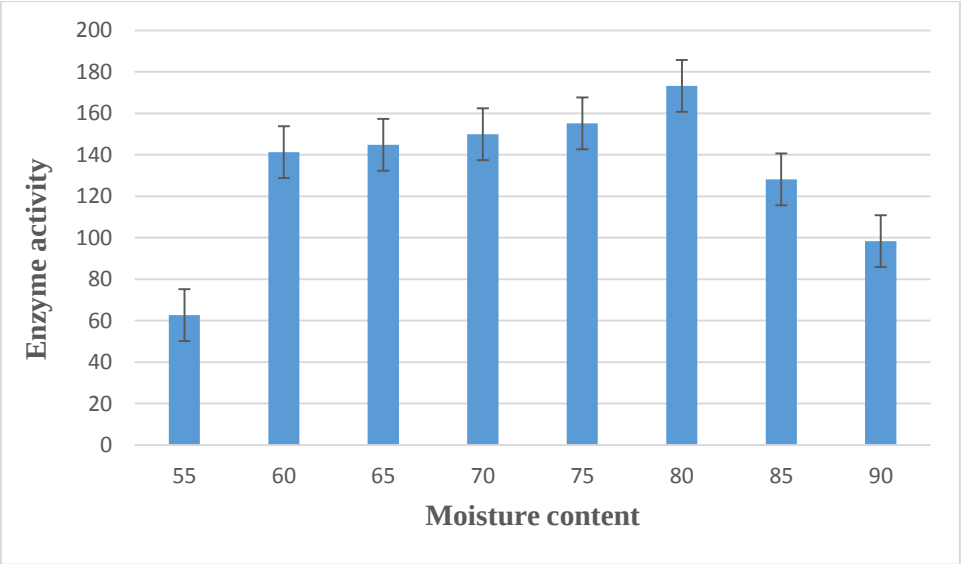

Figure 2.3. Effect of moisture content on α-amylase production using *Bacillus amyloliquefaciens*

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% which decreased with further increase in 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 5 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 (Figure 2.4). The effect of pH of the fermentation medium on amylase enzyme production was found to be statistically non-significant (P> 0.05).

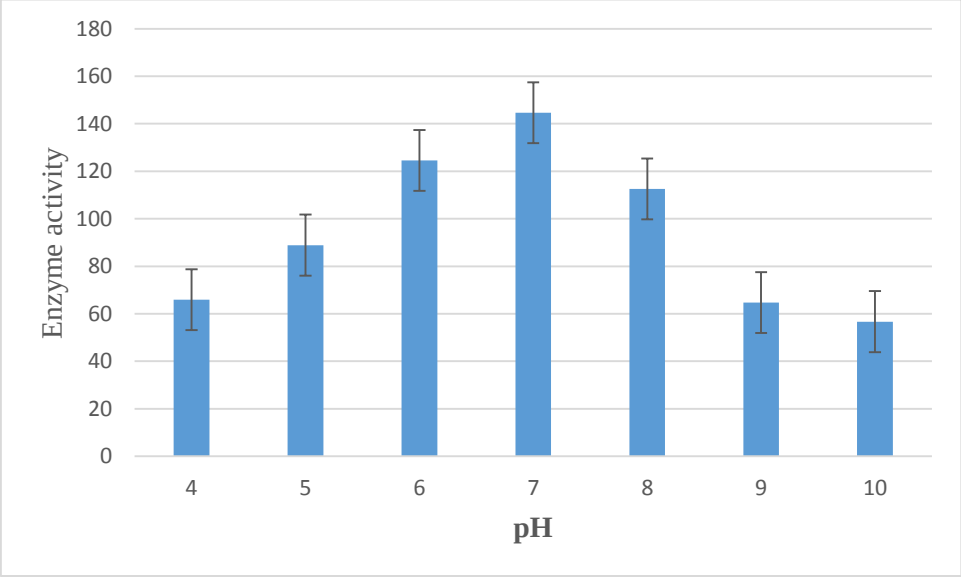

Figure 2.4. Effect of pH on α-amylase production using *Bacillus amyloliquefaciens*

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).

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 h upto 120 h. It was observed from experiments that there was sharp increase in α- amylase production through 24 h (73.64 IU/ml), 48 h (143.96 IU/ml) and maximum production (164.48 IU/ml) was found at 72 h of incubation. The enzyme yield showed sharp decrease on further extension of fermentation

period. The alpha amylase yield reduced about 25.7% of maximum enzyme yield at 96 h and further amylase yield was found 102.86 IU/ml at 120 h of fermentation period (Figure 2.5). However, the effect of incubation time on amylase production was found statistically non-significant (P>0.05).

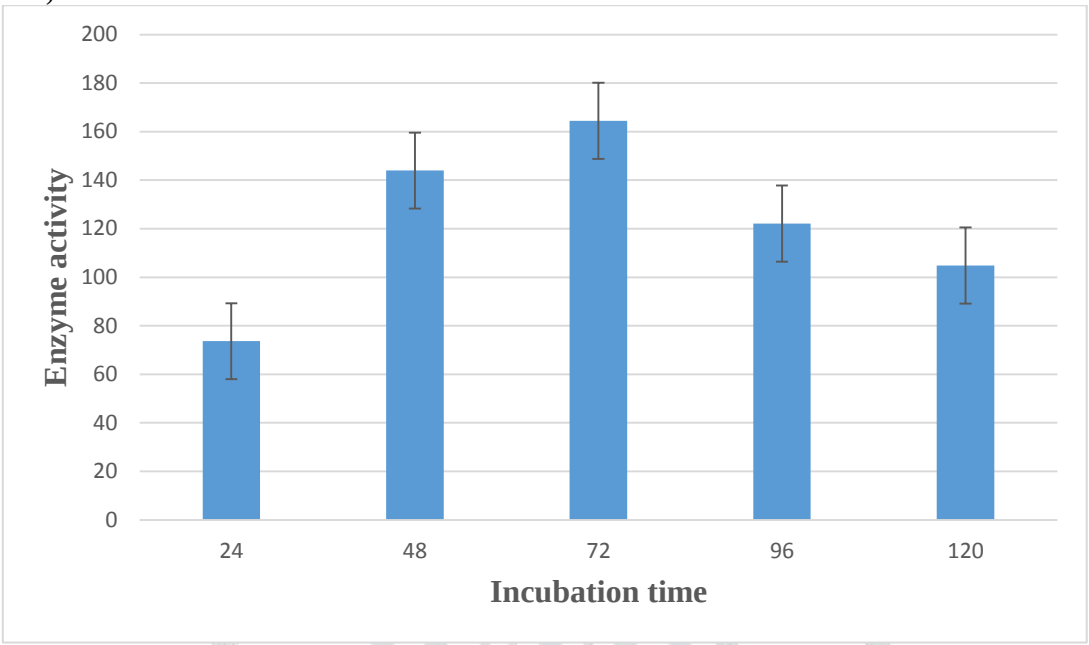

Figure 2.5. Effect of incubation period on *α*-amylase production using *Bacillus amyloliquefaciens*

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) 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.

Optimization of Medium Parameters for α-amylase production by *Bacillus amyloliquefaciens* (MTCC 610) 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 was obtained when glucose was used as additional carbon source (Figure 2.6). The data obtained was analysed using correlation and the carbon source was found to have significant (P< 0.05) importance on alpha amylase production.

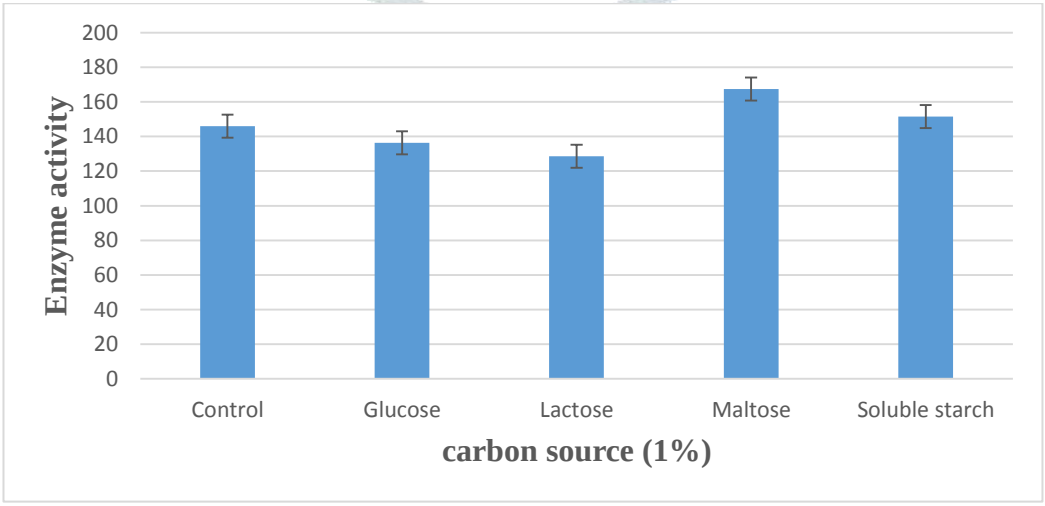

Figure 2.6. Effect of incubation period on *α*-amylase production using *Bacillus amyloliquefaciens*

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

was also reported by Ashraf *et al*. (2005) when starch was supplemented to the medium at 1% level.
This study concluded that the production of a-amylase by *B. amyloliquefaciens* has been examined by using wheat bran in solid state fermentation process. The optimum yield of a-amylase enzyme was obtained from 4CFU/ml of inoculum, 37^{o}C temperature, 80% moisture content, pH 7, 72hr of incubaton and 1% of maltose.

Conflict of interest
Acknowledgement

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