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Research Article

Optimization of fermentation medium components to improve α-amylase production by submerged fermentation technology

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Abstract: The fermentation medium composition and fermentation design greatly affects both the growth and productivity of extracellular enzymes from microorganisms. The objective of the research is to improve the α-amylase production by *Bacillus licheniformis* ATCC 6346 by optimizing fermentation medium compositions in submerged fermentation. The fermentation medium contained (L^-) 2.0 g soluble starch, 1.0 g $(NH_4)_2SO_4$, 2.0 g peptone, 1.0 g NaCl, 0.005 g $FeCl_3$, 0,005 g $MgCl_2.6H_2O$, 0.005 g $CaCl_2.2H_2O$, 1.0 g KH_2PO_4 and 2.5 g K_2HPO_4 α. Production of α-amylase in the fermentation medium was 20.1 UmL^{-1}. Optimizing the concentration of the components to (L^-) 4 g soluble starch, 5 g $(NH_4)_2SO_4$, 7.5 g K_2HPO_4, 4.0 g KH_2PO_4, 6 g peptone, 0.01g $CaCl_2.6H_2O$, 0.01 g $MgCl_2.6H_2O$ and 0.01 g $FeCl_3$ have improved the α-amylase production from 20.1 to 44.1 UmL^{-1}.
Keywords: *Bacillus licheniformis,* α-amylase, submerged fermentation.

INTRODUCTION

α-Amylase (EC 3.2.1.1, 1,4-α-D glucanohydrolase, endoamylase) hydrolyses starch, glycogen and related polysaccharides by randomly cleaving internal α-1,4-glucosidic linkages. Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis*, *B. stearothermophilus*, *B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications [1]. The composition and concentration of the medium play an important role in the growth and production of extracellular amylase by bacteria, yeast and *Aspergillus* sp [2]. In particular, amylolytic enzymes are used in processes where rapid hydrolysis of starch is required or the high viscosity of starch must be lowered, such as in the textile, glucose syrup, confectionary, brewing, paper and alcohol industries [3]. Different studies were made to improve α-amylase production by bacteria [4], yeast and fungus [5].

Industrial enzyme traditionally obtained from Solid State and Submerged fermentation technology production [6]. The use of the submerged culture is advantageous because of the ease of sterilization and process control is easier to engineer in these systems. Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production pattern [7]. In this study, it was aimed to improve the α-amylase production by *Bacillus licheniformis* ATCC 6346 by optimizing media compositions in submerged fermentation.

MATERIALS AND METHODS

Bacillus licheniformis ATCC 6346 from Heriot-Watt University U.K was used in this study. The nutrient agar medium used in the study contained (L^-) 25.0 g nutrient agar, 3.0 g soluble starch. The activation medium used in the study contained (L^-) 25.0 g nutrient broth, and 3.0 g soluble starch at pH 7.0. The fermentation medium contained (L^-) 2.0 g soluble starch, 2.0 g $(NH4)_2SO_4$, 2 g peptone; 0.005g $FeCl_3$; 0.005 g $MgCl_2.6H_2O$; 0.005 g $CaCl_2.2H_2O$; 1.0 g of KH_2PO4, and 2.5 g of K_2HPO_4 at pH 7.0. A loopful of *Bacillus licheniformis* ATCC 6346 grown in nutrient agar slants with 0.3 % soluble starch at 37 ^{o} C for 24 h was transferred to 10 mL activation medium and incubated at 42 ^{o} C in a rotary shaker (100 rpm) for 12 h and used as inoculum.

Production of α-amylase in fermentation medium

To 100 mL of fermentation medium, 20 % (v/v) inoculum was added and incubated at 42 ^{o}C in an orbital shaker (100 rpm). Samples were taken at different time intervals. After measuring the OD at 600 nm, samples were centrifuged. The supernatant was used for α-amylase activity measurement [8].

Optimization of medium for improved α-amylase production

To optimize the soluble starch different amounts of soluble starch from 0.2 to 1.0 g were taken. Inoculum of *B. licheniformis* ATCC 6346 was transferred and allowed to grow at 42^{o}C while shaking at 100 rpm. The medium containing 0.2 g soluble-starch was used as control. Samples (5 mL) were withdrawn from each flask at different time intervals and growth and α-amylase activity were measured. The amount of soluble starch required for the maximum α-amylase production was optimized.

To optimize the $(NH_4)_2SO_4$ different amounts of $(NH_4)_2SO_4$ from 0.2 to 0.9 g were taken. Inoculum of *B. licheniformis* ATCC 6346 was added and allowed to grow at 42 ^oC and at 100 rpm. The medium containing 0.2 g $(NH_4)_2SO_4$ was used as control. Samples (5 mL) were taken at different time intervals from the spent medium and used for α-amylase activity and growth measurements. The amount of $(NH_4)_2SO_4$ required for the maximum production of α-amylase was optimized.

To optimize K_2HPO_4 different amount of K_2HPO_4 from 0.05 to 0.95 g were taken. Inoculum of *Bacillus licheniformis* ATCC 6346 was transferred and allowed to grow at 42 ^oC while shaking at 100 rpm. The medium containing 2.5 g K_2HPO_4 was used as control. Samples (5mL) were withdrawn from each flask at different time intervals and optical density and α-amylase activity were measured. The amount of K_2HPO_4 required for the maximum production of α-amylase was optimized.

To optimize KH_2PO_4 different amount of KH_2PO_4 from 0.05 to 0.90 g were taken. Inoculum of *Bacillus licheniformis* ATCC 6346 was transferred and incubated at 42^oC while shaking at 100 rpm. The medium containing KH_2PO_4 1.0 g was used as control. Samples (5 mL) were withdrawn from each flask at different time intervals and growth and α-amylase activity were measured. The amount of KH_2PO_4 required for the maximum production of α-amylase was optimized.

To optimize peptone different amount of peptone varying from 0.2 to 1.2 g containing medium were taken. Inoculum of *B.licheniformis* ATCC 6346 was added and incubated at 42 ^oC and at 100 rpm. The medium containing peptone 0.2 g was used as control. Samples (5 mL) were taken at different time intervals from the spent medium and used for α-amylase activity and growth measurements. The amount of peptone required for the maximum production of α-amylase was optimized.

To optimize NaCl different amounts of NaCl from 0.0 to 0.9 g were taken. Inoculum of *Bacillus licheniformis* ATCC 6346 was transferred and incubated at 42 ^oC while shaking at 100 rpm. The medium containing NaCl 0.1 g was used as control. Samples (5 mL) were withdrawn from each flask at different time intervals and growth and α-amylase activity were measured. The amount of NaCl required for the maximum production of α-amylase was optimized.

To optimize $CaCl_2.2H_2O$ different amounts of $CaCl_2.2H_2O$ from 0.0005 to 0.0045 g were taken. Inoculum of *Bacillus licheniformis* ATCC 6346 was added and incubated at 42 ^oC and at 100 rpm. The medium containing 0.0005 g $CaCl_2.2H_2O$ was used as control. Samples (5 mL) were taken at different time intervals from the spent medium and used for α-amylase activity and growth measurements. The amount of $CaCl_2.2H_2O$ required for the maximum production of α-amylase was optimized.

To optimize $MgCl_2.6H_2O$ different amounts of $MgCl_2.6H_2O$ from 0.0005 to 0.0045 g were taken. Inoculum of *Bacillus licheniformis* ATCC 6346 was added and incubated at 42 ^oC and at 100 rpm. The medium containing 0.0005 g $MgCl_2.6H_2O$ was used as control. Samples (5 mL) were taken at different time intervals from the spent medium and used for α-amylase activity and growth measurements. The amount of $MgCl_2.6H_2O$ required for the maximum production of α-amylase was optimized.

To optimize $FeCl_3$ different amount of $FeCl_3$ from 0.0005 to 0.0045 g were taken. Inoculum of *Bacillus licheniformis* ATCC 6346 was added and incubated at 42 ^oC and at 100 rpm. The medium containing 0.0005 g $FeCl_3$ was used as control. Samples (5 mL) were taken at different time intervals from the spent medium and used for α-amylase activity and growth measurements. The amount of $FeCl_3$ required for the maximum production of α-amylase was optimized.

RESULTS AND DISCUSSION

Optimization of fermentation medium components to improve α-amylase production

Effect of different concentrations of soluble starch on α-amylase production

Amylase production is known to be induced by a variety of carbohydrates, nitrogen compounds and minerals [9]. When the concentration of starch in the fermentation medium was changed from 2.0 to 4.0 gL^{-1} highest growth (1.521, 600 nm) was obtained in the medium containing 5 gL^{-1} soluble starch at 12 hours and the O.D of 1.501 was obtained when the starch concentration was 4 gL^{-1} at 12 hours. When the concentration of starch in the fermentation medium was changed from 2.0 to 4.0 gL^{-1} the production of α-amylase by *B.licheniformis* ATCC 6346 was increased from 20.08 to 28.64 UmL^{-1} at 33h and 42 ^oC (100 rpm). Highest α-amylase activity (28.64 UmL^{-1}) was obtained in the medium containing 4 gL^{-1} starch (Figure 1). At 33 h of fermentation in 10 gL^{-1} starch containing medium 5.6 UmL^{-1} of α-amylase was produced. This may be due to substrate inhibition. Hence 4.0 gL^{-1} of starch was chosen for further studies. In the production of amylase from *Aspergillus niger* AM07, the enzyme production increased with the increase in starch concentration from 1 to 3 % [10]. In *Bacillus sp.* AK-2, increasing the concentration of starch up to 0.3 % increased α-amylase production [11].

Highest growth was obtained at 12 h but maximum α-amylase activity was obtained at 33 h. Therefore the α-amylase production was maximum, when the cell population entered into late stationary phase, suggesting the enzyme secretion is not growth associated. Similar

findings have been recorded for *Bacillus thermooleovorans* NP 54 [12, 13, 14].

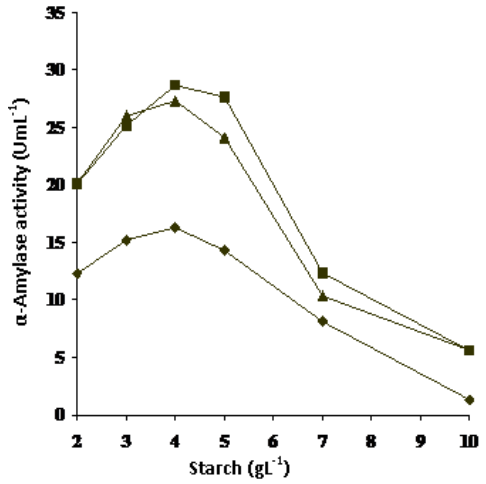

Figure 1: Production of α-amylase at (♦), 24; (■), 33 and (▲), 48 h by *Bacillus licheniformis* ATCC 6346 in medium having different concentrations of Starch, at 42 °C and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85 °C and pH 7.0 using 20 gL^{-1} starch as substrate.

Effect of different concentrations of $(NH_4)_2SO_4$ on α-amylase production

Nitrogen sources are essential for the bacterial growth because nitrogen is an important constituent of proteins, nucleic acid and vitamins, etc. Nitrogen can be obtained from organic and inorganic substances. Various inorganic nitrogen sources such as ammonium sulphate, ammonium chloride and ammonium hydrogen phosphate were tested for growth and enzyme production by bacteria [15]. In this study, peptone was used as organic nitrogen source with $(NH_4)_2SO_4$ as the inorganic nitrogen source. The experiment was carried out with different concentrations of $(NH_4)_2SO_4$ to find the optimum concentration for maximum α-amylase production by the bacteria. Increase in $(NH_4)_2SO_4$ concentration from 2-9 gL^{-1} has slightly increased the growth of *B.licheniformis* ATCC 6346. Highest growth (at 600 nm) of 1.567 was obtained in the medium containing 5 gL^{-1} $(NH_4)_2SO_4$ at 12 h. Growth of this organism in medium containing 7 and 9 gL^{-1} $(NH_4)_2SO_4$ was less than that in the medium containing 5 gL^{-1} $(NH_4)_2SO_4$. This showed that high concentrations of $(NH_4)_2SO_4$ have inhibited the growth of *B.licheniformis* ATCC 6346.

When the amount of $(NH_4)_2SO_4$ in the medium was varied from 2 to 9.0 gL^{-1}, the α-amylase production in control medium, which contained 2 gL^{-1} $(NH_4)_2SO_4$ was 35.72 UmL^{-1}. α-Amylase activity produced in the medium containing 5 gL^{-1} $(NH_4)_2SO_4$ was the highest (39.6 UmL^{-1}) at 48h (Figure 2) and it was 1.10 fold higher than that of control. When Carbon to Nitrogen (Total) ratio was 9:5, highest α-amylase production was obtained. Under the conditions the Carbon to Inorganic nitrogen and Organic nitrogen to Inorganic nitrogen ratios were 8:10 and 1:5 respectively. Production of α-amylase in the media containing 7 and 9 gL^{-1} $(NH_4)_2SO_4$ were 31.52 and 30.06 UmL^{-1} respectively at 48h. Higher concentrations of $(NH_4)_2SO_4$ above 5 gL^{-1} (Carbon to Total nitrogen, Carbon to Inorganic nitrogen and Organic nitrogen: Inorganic nitrogen ratios were 9:11, 4:7 and 1:7 respectively) have inhibited α-amylase production. In this experiment $(NH_4)_2SO_4$ showed positive effect on α-amylase production and 5 gL^{-1} $(NH_4)_2SO_4$ was selected for further studies.

Highest growth was obtained at 12 h while maximum α-amylase activity at 48 h which is in the late stationary phase. Therefore increase in $(NH_4)_2SO_4$ has only increased the α-amylase production but not the rate of enzyme production.

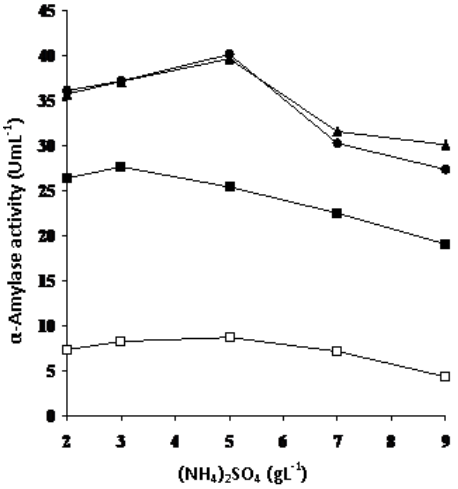

Figure 2: Production of α-amylase at (□), 24; (■), 33; (▲), 48 and (●), 60 h by *Bacillus licheniformis* ATCC 6346 in medium having different concentrations of $(NH_4)_2SO_4$, at 42 °C and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85 °C and pH 7.0 using 20 gL^{-1} starch as substrate.

Effect of different concentrations of K_2HPO_4 on α-amylase production

The phosphate ions are critical for the growth of microbes, to utilize glucose in Embden-Meyerhof-Parnas pathway [16]. Therefore concentration of K_2HPO_4 was optimized for α-amylase production. No detectable differences in the growth of *B.lichenifrmis* ATCC 6346 were observed when the concentration of K_2HPO_4 in the medium was varied from 0.5 to 9.5 gL^{-1}. Growth in the media containing 2.5 gL^{-1} and 7.5 gL^{-1} K_2HPO_4 was 1.555 and 1.573 respectively at 12h. Growth in the medium containing 0.5 gL^{-1} K_2HPO_4 was least and could be insufficient for the metabolic activities of *B.licheniformis* ATCC 6346.

When the concentration of K_2HPO_4 in the

fermentation medium was varied from 0.5 to 9.5 gL^{-1}, production of α-amylase was increased from 26.2 to 42.66 UmL^{-1} at 60 h. At 60 h, 43.19 UmL^{-1} enzyme activity was obtained in the medium containing 7.5 gL^{-1} K_2HPO_4, (42 ^oC and 100 rpm, Figure 3), while 40.81 UmL^{-1} enzyme activity was obtained at 48 h. In the control medium which contained 2.5 gL^{-1} K_2HPO_4, 34.9 UmL^{-1} α-amylase activity was produced. Therefore 1.17 fold increase in α-amylase production was observed when the K_2HPO_4 was increased from 2.5 to 7.5 gL^{-1} at 48 h. When the concentration of K_2HPO_4 was 7.5 gL^{-1} the α-amylase activity obtained at 48 and 60 h were 40.81 and 43.19 UmL^{-1} respectively and the difference in α-amylase activity obtained was 2.38 UmL^{-1}. Therefore 48 h of fermentation was suitable for this optimization study. The α-amylase production by *Bacillus thermooleovorans* NP54, rose steadily with increasing concentrations up to 0.1 % K_2HPO_4 [17]. In this experiment KH_2PO_4 needed to enhance the production of α-amylase was optimized.

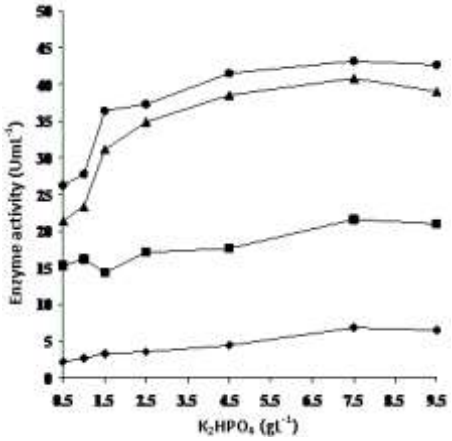

Figure 3: Production of α-amylase at (♦), 24; (■), 36; (▲) 48 and (●), 60 h by *Bacillus licheniformis* ATCC 6346 in fermentation medium having different concentrations of K_2HPO_4, at 42 ^oC and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85^oC and pH 7.0 using 20gL^{-1} starch as substrate.

Effect of different concentrations of KH_2PO_4 on α-amylase production

Here changes in KH_2PO_4 concentration showed no influence on the growth. Growth of this bacteria at low (0.5 gL^{-1}) and high (9 gL^{-1}) concentrations of KH_2PO_4, showed more or less same growth (1.534 and 1.56 respectively) at 12hours. When the amount of KH_2PO_4 in the medium was varied from 0.5 to 9.0 gL^{-1}, the α-amylase produced in the medium containing 4.0 gL^{-1} KH_2PO_4 was the highest (41.4 UmL^{-1}) at 36 h, 42 ^oC and 100 pm (Figure 4) while that in the control medium which contained 1.0 gL^{-1} KH_2PO_4 was 37.54 UmL^{-1} . When the ratio of K_2HPO_4 : KH_2PO_4 was 15:8, highest α-amylase production was obtained. At 36 h in the media with 0.5, 1.0 and 2.0 gL^{-1} KH_2PO_4, α-amylase production was almost the same (36.22, 37.54 and 38.1 UmL^{-1} respectively).

When the amount of KH_2PO_4 in the media were 6 (35.98 UmL^{-1}) and 9 gL^{-1} (31.06 UmL^{-1}), the production of α-amylase was inhibited. This may be because the ratio between K_2HPO_4: KH_2PO_4 were 5:4 and 5:6 which should have increased the pH to 7.26 and 7.24 respectively. This increased pH value of the medium was adjusted to 7.0. Thus the ratio between K_2HPO_4 and KH_2PO_4 would have changed by the addition of acid and hence would have lead to only the increase in the HPO_4^{2-} concentration. Therefore increase in HPO_4^{2-} seems to be inhibiting the α-amylase production. Thus 4.0 gL^{-1} of KH_2PO_4 was selected for further studies. Optimized KH_2PO_4 showed 1.1 fold higher α-amylase activity than control. In *Bacillus amyloliquefaciens* when the KH_2PO_4 concentration was varied from 0.005 to 0.03 M, maximum α-amylase activity was obtained with 0.01 M concentration of KH_2PO_4 [18]. After optimizing the concentration of KH_2PO_4 as 4 gL^{-1} the concentration of peptone was optimized to increase the production of α-amylase.

Effect of different concentrations of peptone on α-amylase production

The nature and relative concentration of carbon and nitrogen sources are important on the production of amylase. In the media containing different concentrations of peptone, highest growth (1.987, 600 nm) was obtained with that containing 12 gL^{-1} peptone at 24 hours, while 1.685 of growth was obtained in the medium with 6 gL^{-1} peptone. When the production of α-amylase was maximum at 48 h (Figure 5), the growth was 0.777 (at 600 nm) in the medium containing 6 gL^{-1} peptone. With 12 gL^{-1} peptone the growth (at 600 nm) was increased to 1.111 at 48 h. Growth of *B.licheniformis* ATCC 6346 was increased with increasing concentration of peptone; because peptone; (Oxoid; L34) contains 13.3 % (w/w) total nitrogen, 2.3 % (w/w) amino nitrogen and amino acids (Compositions of Neutralized bacteriological peptone (The OXOID Manual)) which would induce the bacterial growth. Highest concentration of peptone inhibited the production of α-amylase. This may be due to some heavy metal ions (Compositions of Neutralized bacteriological peptone (The OXOID Manual)) present in the peptone or some amino acids present in the peptone.

When the amount of peptone in the medium was varied from 1.0 to 6 gL^{-1}, and the other components of the medium were kept constant, production of α-amylase was increased from 37.33 to 43.15 UmL^{-1} and highest α-amylase activity (43.15 UmL^{-1}) was obtained in the medium containing 6 gL^{-1} peptone at 48 h (Figure 5).

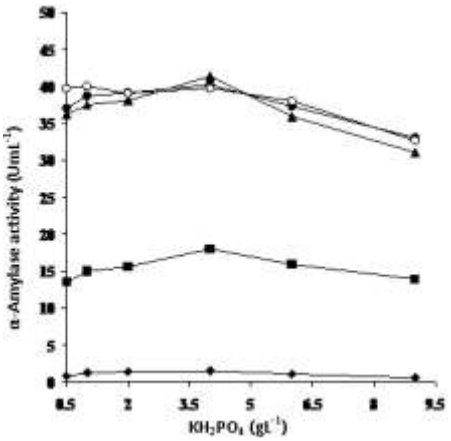

Figure 4: Production of α-amylase at (♦), 12; (■), 24; (▲) 36; (●), 48 and (O), 60 h by *Bacillus licheniformis* ATCC 6346 in fermentation medium having different concentrations of KH_2PO_4, at 42°C and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85 °C and pH 7.0 using 20 gL^{-1} starch as substrate.

In control medium containing 2 gL-1 peptone, 40.76 UmL-1 α-amylase activity was obtained at 48h. Therefore 6 gL-1 of peptone was selected for further studies. The Carbon to Nitrogen (Total) ratio in the media containing 1 to 12 gL-1 peptone were 3:2, 18:13, 9:8, 1:1, 9:11 and 9:12 respectively. Highest α-amylase production was obtained in the media having Carbon to Nitrogen (Total), Organic nitrogen to Inorganic nitrogen and Carbon to Organic nitrogen ratios of 1:1, 4:5 and 9:4 respectively. Higher concentrations of peptone above 6 gL-1 (Carbon to Nitrogen (Total), Organic nitrogen to Inorganic nitrogen and total Carbon to Organic nitrogen ratios were 9:11, 6:5 and 3:2 respectively) have inhibited α-amylase production.

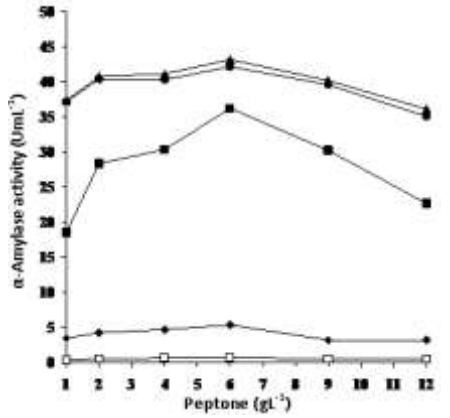

Figure 5: Production of α-amylase at (□), 12; (♦), 24; (■) 36; (▲), 48 and (●), 60 h by *Bacillus licheniformis* ATCC 6346 in fermentation medium having different concentrations of peptone, at 42 °C and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85 °C and pH 7.0 using 20 gL^{-1} starch as substrate.

Under optimized peptone concentration 1.1 fold higher α-amylase activity was obtained than in the control medium. The optimum Carbon to Nitrogen (Total) ratio reported for α-amylase production was 1:1 by *Bacillus licheniformis* SPT 27 [19]. Increased concentration of peptone has inhibited the α-amylase production this could be due to the presence of some undefined Ca ion in peptone.

Effect of different concentrations of NaCl on α-amylase production

When the concentration of NaCl in the fermentation medium was varied from 0.0 to 4.0 gL^{-1} maximum growth (1.663, at 600 nm) was observed in the absence of NaCl at 24 h. When the NaCl concentration was 4 gL^{-1}, the growth obtained at 24 h was 1.57.

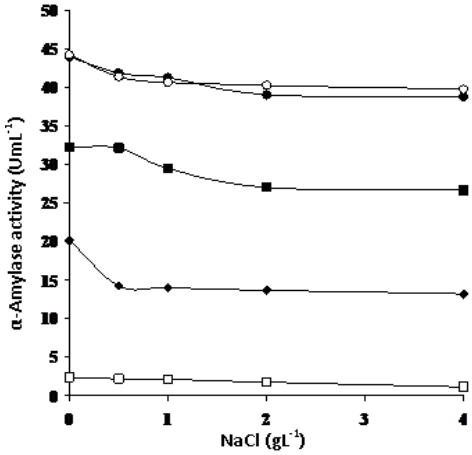

Figure 6: Production of α-amylase at (□), 12; (♦), 24; (■) 36; (▲), 48 and (●), 60 h by *Bacillus licheniformis* in fermentation medium having different concentrations of NaCl, at 42 °C and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85 °C and pH 7.0 using 20 gL^{-1} starch as substrate.

Similar to growth α-amylase production by *B.licheniformis* ATCC 6346 was highest in the medium which had no NaCl (43.96 UmL^{-1}) at 48 h (Figure 6). In the control medium, which containd 1 gL^{-1} NaCl, 41.26 UmL^{-1} of enzyme activity was obtained at 48 h, (at 42 °C and 100 rpm). With increase in the concentrations of NaCl from 0.5 to 4 gL^{-1}, production of α-amylase decreased from 41.82 and 38.77 UmL^{-1} at 48 h. With increase in NaCl concentration, the osmotic pressure of the medium might have increased and would have lead to exosmosis / death of the cells. Thus the growth and α-amylase production were inhibited. The NaCl present in peptone (Compositions of Neutralized bacteriological peptone (The OXOID Manual)) must be sufficient for the enzyme production. Hence it was decided to not to use NaCl for further studies.

Effect of different concentrations of $CaCl_2.2H_2O$ on α-amylase production

Highest growth (1.754, 600 nm) was obtained in the medium containing 0.02 gL^{-1} $CaCl_2.2H_2O$ at 24 h and the OD obtained was 1.738 when the concentration of $CaCl_2.2H_2O$ was 0.01 gl^{-1} at 24 h. Between the concentrations of 0.005 to 0.045 gL^{-1} of $CaCl_2.2H_2O$ no significant change in the growth of *Bacillus licheniformis* ATCC 6346 was observed. This shows that Ca^{2+} has no influence on the growth of *B.licheniformis* ATCC 6346.

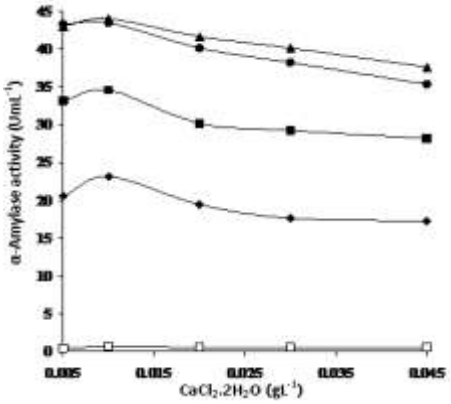

Figure 7: Production of α-amylase at (□), 12; (♦), 24; (■) 36; (▲), 48 and (●), 60 h by *Bacillus licheniformis* in fermentation medium having different concentrations of $CaCl_2.2H_2O$ at 42 °C and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85 °C and pH 7.0 using 20 gL^{-1} starch as substrate.

When the amount of $CaCl_2.2H_2O$ in the medium was varied from 0.005 to 0.045 gL^{-1} α-amylase production was highest (44.01 UmL^{-1}) in the medium containing 0.01 gL^{-1} $CaCl_2.2H_2O$ at 48 h, (at 42 °C and 100 rpm, Figure 7). In control medium, which contained 0.005 gL^{-1} $CaCl_2.2H_2O$, 43.05 UmL^{-1} enzyme activity was obtained at 48 h, under the same conditions. At high concentrations of $CaCl_2.2H_2O$ turbidity was observed in the medium because Ca^{2+} was precipitated as $Ca_3(PO_4)_2$.

Effect of different concentrations of $MgCl_2.6H_2O$ on α-amylase production

Highest growth (1.836, 600 nm) was obtained in the medium containing 0.01 gL^{-1} $MgCl_2.6H_2O$ at 24 h. Between the concentrations of 0.005 to 0.045 gL^{-1} of $MgCl_2.6H_2O$ there was no significant change in the growth.

When the amount of $MgCl_2.6H_2O$ in the medium was varied from 0.005 to 0.045 gL^{-1}, α-amylase production (45.01 UmL^{-1}) was highest in the medium which contained 0.01 gL^{-1} $MgCl_2.6H_2O$ at 48 h, (at 42 °C and 100 rpm Figure 8). In control medium, which contained 0.005 gL^{-1} $MgCl_2.6H_2O$, α-amylase produced was 43.24 UmL^{-1} at 48 h.

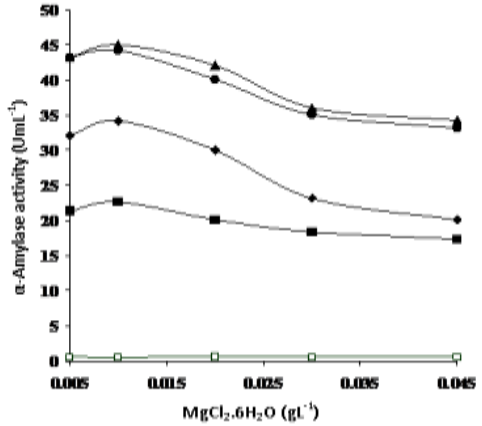

Figure 8: Production of α-amylase at (□), 12; (■), 24; (♦) 36; (▲), 48 and (●), 60 h by *Bacillus licheniformis* in fermentation medium having different concentrations of $MgCl_2.6H_2O$ at 42 °C and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85 °C and pH 7.0 using 20 gL^{-1} starch as substrate.

Effect of different concentrations of $FeCl_3$ on α-amylase production

When the amount of $FeCl_3$ in the medium was varied from 0.005 to 0.045 gL^{-1}, while the other components of the medium were kept constant, highest growth (1.842, 600 nm) was obtained in the medium containing 0.02 gL^{-1} $FeCl_3$ at 24 h. The growth was 1.841 when 0.01 gL^{-1} $FeCl_3$ was used. Between the concentrations of 0.005 to 0.045 gL^{-1} $FeCl_3$ there was no significant change in the growth of *B.licheniformis* ATCC 6346. Highest α-amylase activity was produced in the medium containing 0.01 gL^{-1} $FeCl_3$ (44.10 UmL^{-1}) at 48 h (Figure 9). In the control medium (0.005 gL^{-1} $FeCl_3$), 43.05 UmL^{-1} of enzyme activity was obtained at 48 h, (at 42 °C and 100 rpm).

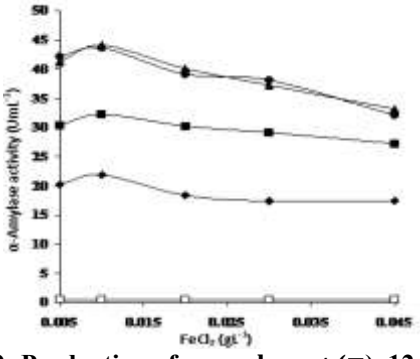

Figure 9: Production of α-amylase at (□), 12; (♦), 24; (■) 36; (▲), 48 and (●), 60 h by *Bacillus licheniformis* in fermentation medium having different concentrations of $FeCl_3$ at 42 °C and pH 7.0 (100 rpm). The medium was inoculated with 20 % (v/v) of inoculum and α-amylase activity was measured at 85 °C and pH 7.0 using 20 gL^{-1} starch as substrate.

CONCLUSION

In result of current study the composition of the fermentation medium was optimized as follow (L^-) 4.0 g soluble starch, 5.0 g $(NH4)_2SO_4$, 6 g peptone, 0.01g $FeCl_3$, 0.01 g $MgCl_2.6H_2O$, 0.01g $CaCl_2.2H_2O$, 4.0 g of KH_2PO4 and 7.5 g of K_2HPO_4 at pH 7.0. The highest activity on the optimized medium was 44.1 UmL^{-1} at 48h (42 ^oC and 100 rpm). The enzyme production was improved by 2.2 fold from further detailed study on culture conditions are necessary to improve α-amylase production.

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