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Optimization of the Conditions for Submerged Fermentation (SMF) of the *Thermoactinomyces sacchari* isolated from Azad Jammu and Kashmir Pakistan to Produce Maximum Amylase Enzyme

Muhammad Ayub Jadoon, Tauseef Ahmad,
Malik Mujadd Ur Rehman, Afzal Khan and Abdul Majid

Department of Microbiology,
Hazara University, Mansehra, Khyber Pakhtunkhwa, Pakistan

Abstract: *Thermoactinomycete sacchari* was grown on the AGS medium and the production of amylase was obtained by using submerged fermentation technique.Growth of *Actinomycete* and amylase production was optimized on four different nutrient media. Highest growth and amylase activity (3.882 OD and 132.3 AU, respectively after 72 hours at 60°C) were obtained by using medium-4. Conditions for the maximum production of amylase in submerged culture fermentation were optimized using medium-4. Conditions for the maximum production of amylase in submerged culture fermentation were optimized using medium-4. It was found that by using 5% inoculum of 30 hours age, amylase activity of 140.8 AU was obtained. Optimization of agitation speed (150 rpm), temperature (60°C), pH (7.5), starch (2.5 %) and glucose (3%) increased activity from 140.8 AU to 162.4 AU.

Key words: Actinomycetes · Amylase · AGS Medium · Optimization · Submerged Fermentation

INTRODUCTION

The Actinomycetes group is large, complex and contains more than 60 genera distinguished by a combination of structural and chemical properties. If we look at the industrial application of the extracellular enzymes, amylases come first particularly in the starch processing industry [1,2]. The number of validity described streptomyces species currently stands at nearly 500. calculated that Streptomycetes systematic, notably the delineation of species, is becoming increasingly objective due to the application of the polyphasic taxonomic approach, included enzymes from thermophilic microorganisms, thermozymes, have unique characteristics such as temperature, chemical and pH stability [3]. A few thermophiles can grow at 90°C or above and some have maxima above 100°C. Prokaryotes that have growth optima between 80°C and about 113°C are called hyperthermophiles. Their growth is stunted below 55°C i.e *Pyrococcusobyssi* and *Pyrodictiumoccultum* [4]. In a previous study, a moderately thermostable α- amylase producing actinomycete strain, *Streptomyces erumpens* MTCC 7317 was isolated from a brick kiln soil [5]. The strain showed the optimum incubation period, pH and temperature for α-amylase production in SmF as 36 hrs, 6.0 and 50°C, respectively. The molecular mass of the enzyme was 54.5 kDa [1,2]. The economical method for the production of the enzymes and the breakdown of the starch is SSF than SMF [6].

MATERIALS AND METHODS

Selection of Suitable Growth Media: Growth of Actinomycetes was carried out in four different media in shake flask experiments. The media were autoclaved at 15 psi and 121°C for 15min. Composition of the media are given below:

Medium (M-I): EL-Nakeeb [7].

Starch	10 g
Glycerol	2.5 g
Sodium Chloride	1.0 g
Distilled water	1000 ml
pH of the medium	7.0

Corresponding Author: Tauseef Ahmad, Department of Microbiology, HazaraMansehra, Khyber Pakhtunkhwa, Pakistan. Mob: +92-346-9403966.

Medium (M-2): EL-Nakeeb [7].

Starch	10 g
Peptone	1.0 g
K_2HPO_4	0.3 g
$MgSO_4$. $7H_2O$	0.1 g
Distilled water	1000 ml
pH	7.0

Medium (M-3): EL-Nakeeb [7].

Starch	15 g
Arginine monohydrocholoride	1.0 g
Glycerol	10.5 g
Sodium Chloride (NaCl)	1.0 g
Magnesium Sulfate ($MgSO_4$, $7H_2O$)	0.05 g
Distilled water	1000 ml

Following the submerged fermentation (SMF) technique inoculating with the strains of Actinomycetes the flasks in all the media in triplicate were incubated at 37°C. After 48 hours the growth and amylase production was calculated. Medium in which maximum production was recorded, selected for further study.

Quantitative Test for Amylase Production: The method of Bernfeld (1951) was used for amylase assay.

Reagents: 1% soluble starch in 0.02M Phosphate buffer of pH 7.5.

DNS (Dinitrosalcylic acid) Reagent: Dissolve 1g of DNS in 20 ml 2.0 M sodium hydrochloride and 20 g of potassium sodium tartarate in 100ml distilled water.

Assay Procedure: 1 ml of the enzymes extract of the submerged cultures was incubated with 1 ml of assay medium at 45°C for 1 hour. The enzyme activity was measured on the basis of reducing sugar released during reaction. The amount of reducing sugar released was estimated by adding 1 ml of DNS reagent 1 ml filtrate starch reaction mixture and then determines the absorbance at 450nm using a UV-120-01 spectrophotometer (Shimadzu). Control was prepared in the same way except the DNS reagent was added before incubation.

Unit of Enzyme Activity: One amylase unit is amount of enzyme in 1 ml of filtrate which releases 1.0 mg of reducing sugar from 1.0% starch solution in 1 hour at 60°C and pH 7.5.

Optimization of Inoculum Size: Effect of size of inoculum on the production of amylase by *Thermoactenomycete* was investigated using 1% to 10% inoculum with regular increase of 1%.

Optimization of Age of Inoculum: Studies on the age of inoculum used for fermentation revealed that maximum amylase production can be obtained by using culture of 30 hours.

Optimization of Glucose Concentration: Growth and amylolytic activity were determined by supplementation of the basal media under optimized conditions of temperature (60°C), pH, (7.5), Agitation (150 rpm) and starch (2.5%). Maximum growth was observed at 3% glucose concentration and maximum amylolytic activity was measured at 2.5% glucose concentration.

Optimization of Agitation Speed: Effect on the growth and amylase production was observed at different agitation speeds from 100-180 rpm with regular in cream of ID.

Optimization of Temperature: Temperature of the medium was adjusted from 35°C to 75°C with regular increase of 5 degree. It was observed that maximum growth and amylase production can be achieved at when fermentation in carried out at 60°C.

Optimization of pH: Studies on the effect of pH on the amylase production revealed that maximum growth and amylase production could be achieved when fermentation is carried out at pH value of 7.5.

Optimization of Different Concentrations of Starch: Starch was added in the growth media in different concentrations 0.5-4.0%. It was observed that maximum growth OD (3.683) obtained at 3% starch concentration.

RESULTS

Submerged Culture Fermentation

***Selection of Suitable Medium*:** Selected strain of *Thermoactinomycetesacchari* (W1) was optimized for its growth on four different media. Growth of microorganism on medium 4 was found to be maximum equivalent to 3.882 OD. Production of amylase was also highest (132.3). Amylase unit after 72 hours of incubation at 60°C

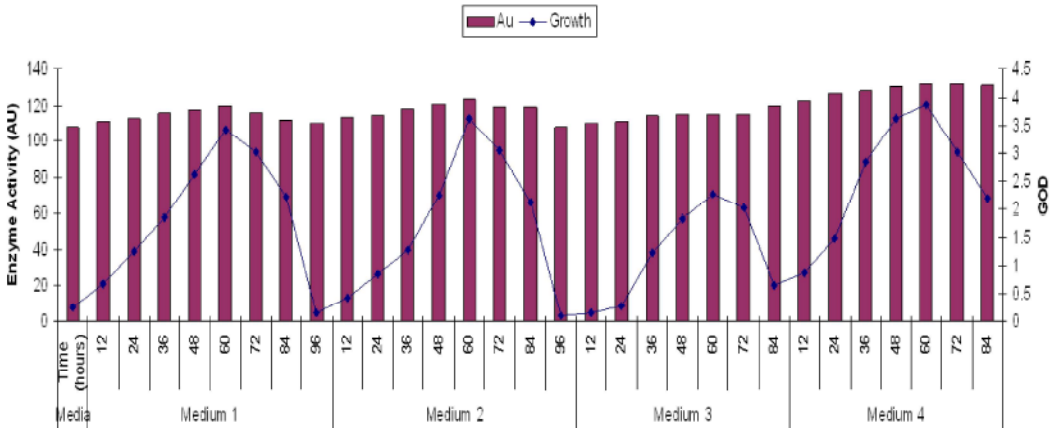

Fig. 4.7: Effect of different media and Amylase production and growth of Thermoactinomycessacchari M1 Au: (AVE: 114.250, STDEVA: 3.689), M2 Au: (AVE: 117.400, STDEVA: 4.247), M3 Au: (AVE: 112.757, STDEVA: 2.822), M4 Au: (AVE: 128.000, STDEVA: 4.465)

(Appendix 2 Figure 4.7). Therefore, medium 4 containing arginine glycerol (12.5 g) dipotassium hydrogen phosphate (K_2HPO_4 = 1.0 g), sodium chloride (NaCl = 1.0 g), Magnesium sulphate ($MgSO_4.7H_2O$ = 0.5 g), Ferrous sulphate ($FeSO_4.5H_2O$ = 0.01 g), Copper sulphate ($CuSO_4.5H_2O$ = 0.001 g), Zinc sulphate ($ZnSO_4.7H_2O$= 0.001 g), Manganese sulphate ($MnSO_4$ = 0.001 g) and distilled water adjusted at pH = 7.5 was used for further studies.

Optimization of Inoculum Size: Effect of size of inoculum on the production of amylase by thermoactenomycete was investigated using 1% to 10% inoculum with regular increase of 1%. The maximum amylase production (140.8 AU) was observed with 5% inoculum. At higher and lower concentration observed activity was lesser. Results are given in Figure 4.8 and Appendix 3.

Optimization of Age of Inoculum: Studies on the age of inoculum used for fermentation revealed that maximum amylase production can be obtained by using culture of 30 hours. Growth, total proteins and amylolytic activities were found to be 0.891 OD (Optical Density), 33.2 mg/ml and 148.6 AU/ml with change in pH from 7.5 to 8.1 (Appendix 4 Figure 4.9).

Optimization of Glucose Concentration: Growth and amylolytic activity were determined by supplementing of the basal media under optimized conditions of temperature (60°C), pH (7.5), agitation (150 rpm) and starch (2.5%). Maximum growth was observed at 3% glucose concentration and maximum amylolytic activity was measured at 2.5% glucose concentration. Overall it was observed that by supplementing media with glucose, growth of Actinomycetes is increased but enzyme activity in decreased from 162.4 AU to 142.5 AU. At higher concentration both growth and enzyme activity are decreased. Results are given in Appendix 5 and Figure 4.10

Optimization of Agitation Speed: Effect on the growth and amylase production was observed at different agitation speeds from 100-180 rpm with regular in cream of ID. Maximum amylolytic activity was observed at 150 rpm which was 156.3 AU At lower and higher speeds activity was decreased (Appendix 6 Figure 4.11).

Optimization of Temperature: Temperature of the medium was adjusted from 35°C to 75°C with regular increase of 5 degree. It was observed that maximum growth and amylase production can be achieved at when fermentation in carried out at 60°C. Amylolytic activity was increase to 149.2AU. At lower temperature 35, 40, 45 and 50°C growth was quite low i.e. 0.82, 1.12, 1.22 and 1.57 respectively. These results indicate that this Actinomycete is thermoactinomycete (Appendix 7 Figure 4.12).

Optimization of pH: Studies on the effect of pH on the amylase production revealed that maximum growth and amylase production could be achieved when fermentation is carried out at pH value of 7.5. Amylolytic activity observed at pH 7.5 was 149.2 after 72 hours of incubation

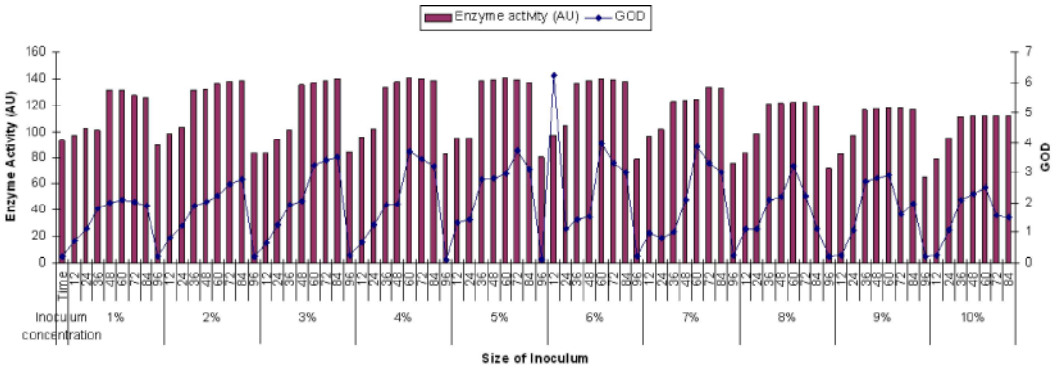

Fig. 4.8: Optimization of inoculum size (AVE: 113.825, STDEVA: 16.687), (AVE: 120.963, STDEVA: 20.171), (AVE: 110.271, STDEVA: 25.826), (AVE: 121.650, STDEVA: 23.204), (AVE: 120.938, STDEVA: 25.165), (AVE: 121.688, STDEVA: 23.749), (AVE: 114.388, STDEVA: 19.571) (AVE: 108.138, STDEVA: 19.241), (AVE: 105.000, STDEVA: 18.644), (AVE: 99.863, STDEVA: 18.591)

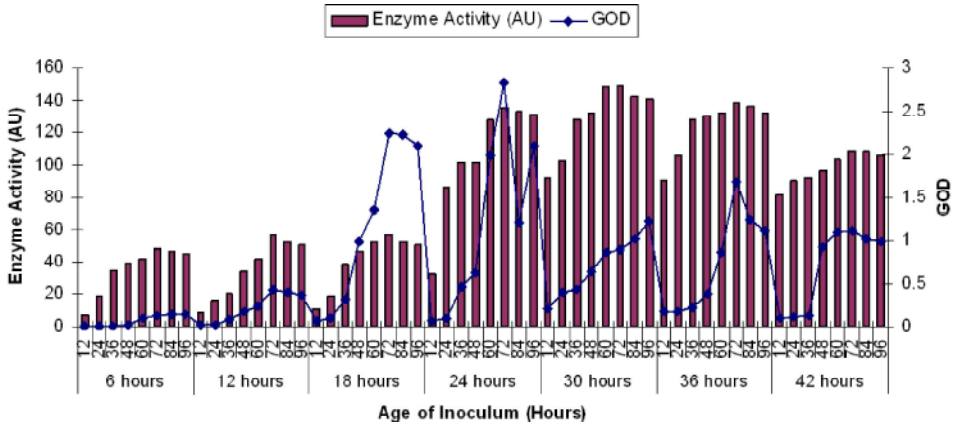

Fig. 4.9: Effect of Age of inoculums (AVE: 35.050, STDEVA: 14.879), (AVE: 34.963, STDEVA: 18.257), (AVE: 40.788, STDEVA: 17.134), (AVE: 106.100, STDEVA: 35.088), (AVE: 129.288, STDEVA: 21.244), (AVE: 124.338, STDEVA: 16.860), (AVE: 98.225, STDEVA: 9.867)

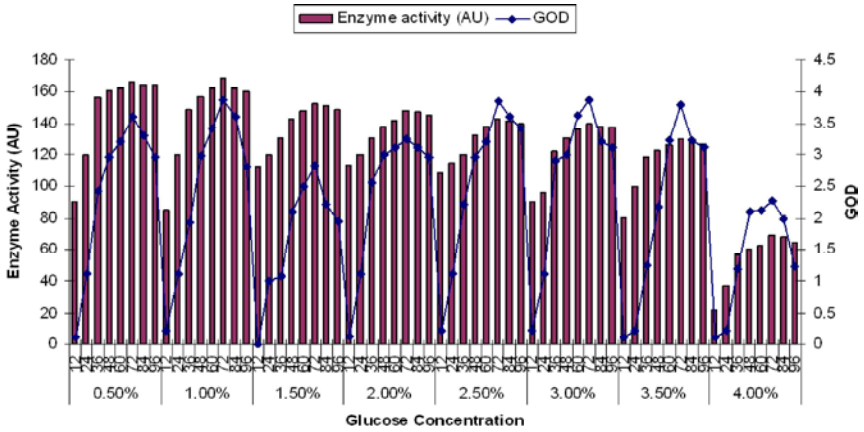

Fig 4.10: Optimization of glucose concentration for the growth and amylolytic activity of T. sacchari (AVE: 148.050, STDEVA: 27.815), (AVE: 145.575, STDEVA: 28.587), (AVE: 138.363, STDEVA: 15.389), (AVE: 135.538, STDEVA: 13.125), (AVE: 129.700, STDEVA: 13.278), (AVE: 123.825, STDEVA: 19.786), (AVE: 116.613, STDEVA: 17.515), (AVE: 55.088, STDEVA: 16.687)

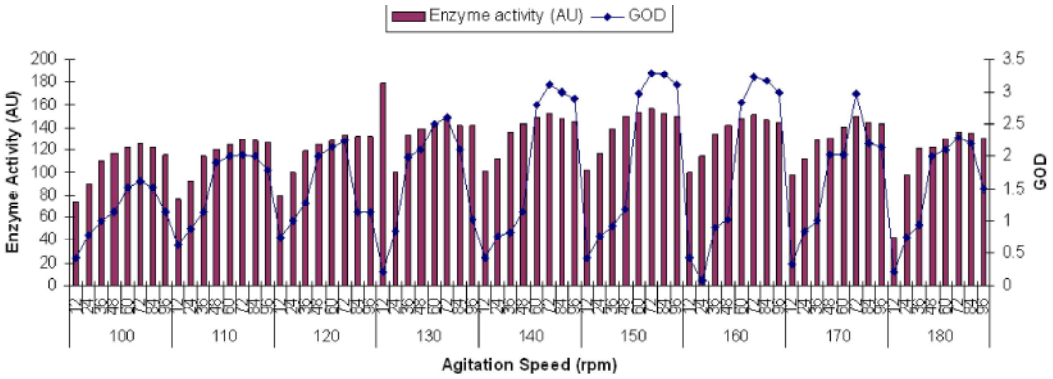

Fig 4.11: Optimization of Agitation speed for the growth and amylolytic activity of Thermoactinomycessacchari (AVE: 109.750, STDEVA: 17.942), (AVE: 114.000, STDEVA: 19.198), (AVE: 118.688, STDEVA: 18.949), (AVE: 140.625, STDEVA: 21.527), (AVE: 136.025, STDEVA: 18.906), (AVE: 140.038, STDEVA: 19.699), (AVE: 135.213, STDEVA: 18.549), (AVE: 130.938, STDEVA: 17.770), (AVE: 114.488, STDEVA: 31.653)

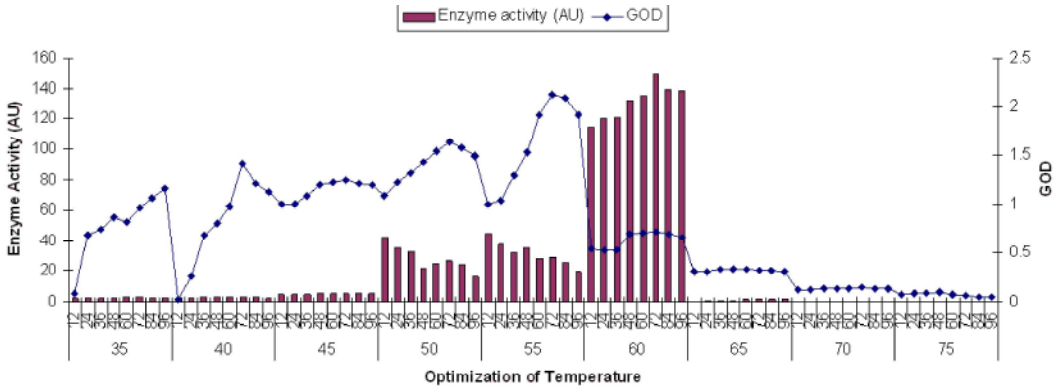

Fig. 4.12: Optimization of temperature for the growth and amylolytic activity of Thermoactinomycessacchari. (AVE: 2.435, STDEVA: 0.337), (AVE: 2.883, STDEVA: 0.283), (AVE: 5.009, STDEVA: 0.315), (AVE: 28.038, STDEVA: 8.267), (AVE: 31.505, STDEVA: 7.516), (AVE: 131.188, STDEVA: 11.603), (AVE: 1.149, STDEVA: 0.482), (AVE: 0, STDEVA: 0), (AVE: 0, STDEVA: 0)

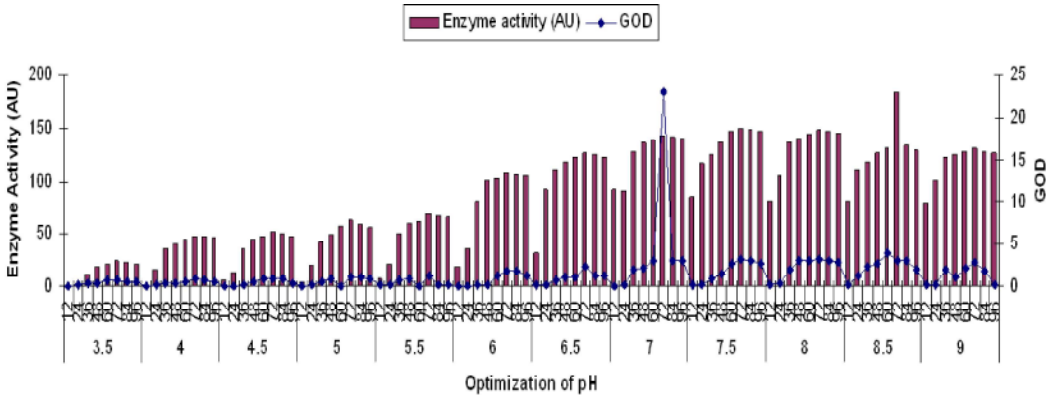

Fig. 4.13: Optimization of pH for the growth and amylolytic activity of T. sacchari (AVE: 15.500, STDEVA: 9.732), (AVE: 35.025, STDEVA: 16.894), (AVE: 37.225, STDEVA: 17.843), (AVE: 44.000, STDEVA: 21.112), (AVE: 50.550, STDEVA: 23.271), (AVE: 82.013, STDEVA: 35.296), (AVE: 105.976, STDEVA: 31.869), (AVE: 126.113, STDEVA: 21.805), (AVE: 131.400, STDEVA: 22.506), (AVE: 130.763, STDEVA: 24.761), (AVE: 120.100, STDEVA: 18.010), (AVE: 117.675, STDEVA: 18.311), (AVE: 126.700, STDEVA: 29.195), (AVE: 117.675, STDEVA: 18.311)

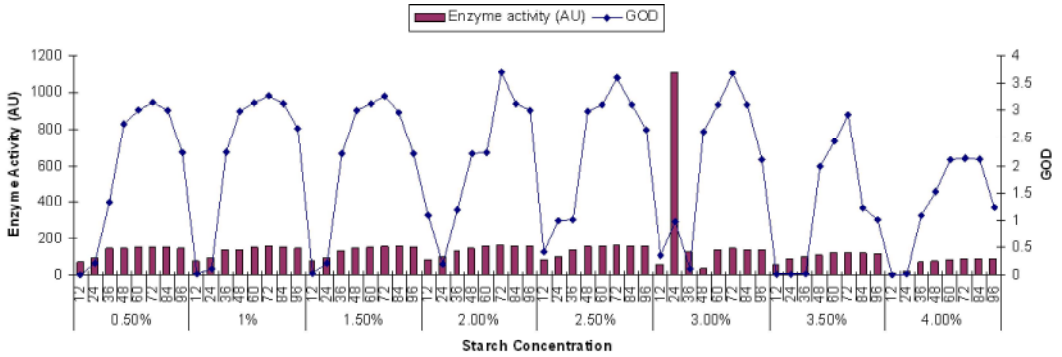

Fig 4.14: Optimization of concentration of starch for the growth and amylolytic activity of T. sacchari (AVE: 132.563, STDEVA: 31.697), (AVE: 132.525, STDEVA: 28.616), (AVE: 134.938, STDEVA: 28.985), (AVE: 138.138, STDEVA: 29.839), (AVE: 140.475, STDEVA: 30.079), (AVE: 113.221, STDEVA: 42.153), (AVE: 106.175, STDEVA: 20.463), (AVE: 68.375, STDEVA: 32.321)

at 60°C. In acidic pH range activity was decreased sharply and gradual increased, when pH was adjusted above 7.0. Results are given in Appendix 8 and Figure 4.13.

Optimization of Different Concentrations of Starch: Starch was added in the growth media in different concentrations 0.5-4.0%. It was observed that maximum growth OD (3.683) obtained at 3% starch concentration however maximum enzyme activity (162.4) was obtained at Au 2.0% starch. Results are given in Appendix 9 and Fig. 4.14.

DISCUSSION

Optimization of the conditions affecting amylase production enhances the production. Medium used for submerged culture fermentation is of prime importance. Medium containing suitable amount of carbon, nitrogen and mineral salts is preferred. For shake flask fermentation medium IV, containing arginine, glycerol, dipotassium hydrogen phosphate, sodium chloride, magnesium sulphate, ferrous sulphate, copper sulphate, zinc sulphate, manganese sulphate and distilled water adjusted at pH-7.5, was selected. This medium was selected to provide sufficient nutrients to the microorganism for its initial growth.

Conditions like inoculum size, age of inoculum, agitation speed, temperature, pH and concentration of starch and glucose were optimized to have maximum yield of amylase. Optimization was carried out to set parameters for maximum production of amylase before using the industrial wastes as source of carbon and nitrogen. Under these optimized conditions production of amylase increased from 132.3 AU to 162.4 AU with the increase in growth from 2.882 to 3.241, as recorded by OD measurement. Studies on inoculum size revealed that at 5% inoculum concentration, maximum growth and amylase activity was observed. At lower and higher concentrations decrease in activity was observed. At lower concentration decrease in amylase production is obviously due to lesser cell counts. At higher inoculum concentration, more population of bacteria and limited nutrients and oxygen lead to poor growth and consequently lower production of amylase (Fig6ure 4.4). Age of incubation, before the addition of microbial culture to growth medium is of vital importance. It may be due to the influence of metabolic activities when inoculum with small and long period of incubation is used. If culture is added when it is in the log phase then we get high yield of metabolites because growth started immediately. If culture has entered lag phase then it takes more time to regain the momentum of growth and therefore we get lesser yield of primary and secondary metabolites. In our study maximum growth of Actinomycetes having 92.981OD and amylolytic activity of 148.6 AU was observed when inoculum of 30 hours was used. Many scientists have reported that inoculum of 18-24 hours old gives optimum yield [8].

In our study optimum results have been obtained by using inoculum of 30 hours age. It may be due to physiological modification in this strain under unique climatic condition of the place of isolation (Hot spring). It is quite interesting to observe growth pattern and yield of secondary metabolites by supplementing growth medium with different carbon and nitrogen sources. For amylase production carbon source is of immense importance as amylase production is stimulated if carbohydrates are present in the medium. In this study medium was supplemented with different concentrations of glucose from 0.5 to 4%. It was observed that there is

noticeable increase in growth of Actenomycete 2.981 to 3.868 OD but amylase production was suppressed from 148.6 AU to 142.5AU. Maximum growth was observed at 3% glucose concentration, however maximum amylase production was observed at 2.5% glucose concentration. Decrease in amylase production is obviously due to the addition of monosaccharide (glucose) which is directly available to the microbes and therefore no stimulation for the production of amylase. As carbon and N source is available in the consumable form from the very beginning and hence supportive to growth. At higher glucose concentration abrupt decrease in growth and amylase production was observed which is due to the dehydration of microorganism [9].

Agitation speed is important for the regular and uninterrupted supply of nutrients and oxygen to the bacterial cells. Growth and Amylase production increased with the increase in agitation up to 150 rpm. At higher agitation speed decrease in growth and amylase production was observed. It might be due to the churning effect that causes disruption of cells at higher speed.

Temperature is critical parameter especially in studies of thermophillic microorganism. Frankena *et al.* [10] reported that synthesis of secondary metabolites and energy metabolism in bacteria in controlled by temperature and supply of oxygen. Higher 1growth (2.860 OD) and Amylolytic activity (159.2 AU) were found to be at 60°C. Therefore, the strain was recognized as Thermoactenomycete. Sharp decrease in Amylase production has been observed at temperatures lower than 45°C and higher than 65°C. Growth and protein contents also decreased at lower and higher temperatures indicating lower production of all primary and secondary metabolites due to poor growth. These studies revealed that Actenomycetes are more tolerant to high temperature than bacillus species as most of the researchers have reported 35-50°C temperature for optimum growth and production of amylases by different strains [11]. Assuming that the thermostabilityof the enzyme results from the unique structure of the protein molecule, two conjectures about its structure were suggested:

The enzyme exists as a randomly coiled molecule in the native state and consequently it no longer loses activity by heating. Thermostability is the result of a rigid structure which is not denatured easily by the change of an external parameter such as temperature or the nature of the solvent. Specialized proteins known as 'Chaperonins' are produced by these organisms which help after their denaturation to refold the proteins to their native form and restore their functions [12].

The pH of growth medium plays key role for the physiological activities of cells and transport of protein and other metabolites across the cell membrane [13]. Initial pH of the culture medium was adjusted from 3.5 - 9.0 and maximum growth and amylolytic activity were observed at pH 7.5. The maximum production of amylase at pH 7.5 is due to the maximum growth of microorganism at this pH. Results also indicate stability of amylase at slightly alkaline pH, however at pH 3.5 - 6.5 growth and amylolytic activity both are quite low. Activity increased abruptly from pH 6.5 - 7.5 and was observed to increase from 126.2 AU to 159.2 AU. So pH range of 7.0 - 8.0 can be recommended for optimal growth of Actenomycete. During fermentation pH was increased upto 8.5 showing alkaline nature of amylase and other metabolites. Narayama and Vijayalakshmi in 2008 reported optimum pH of 7.0 for *Streptomyces albidoflavus*. However wide range of pH from 5-10 has been reported by different species of Actenomycetes and Bacillus [14].

Competing Interest: None Declares

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