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Production and kinetics of amylase from starch using mutant strain of *Bacillus Sp* MTCC 1434

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ABSTRACT

Amylases are enzymes that hydrolyze starch into simple sugars like Glucose, fructose and dextrin. In this work amylase was produced by *Bacillus sp* 1434. Five mutant strains were developed from the wild strain of *Bacillus sp.* using UV irradiation technique by varying the exposure timings. The mutant strain 3 gave the maximum enzyme activity of 1.15 mg/ml per hour. The effect of product at different physical parameters like temperature, pH, substrate concentration, were studied. The optimum substrate is found to be soluble starch, the temperature 37°C is optimum for maximum enzyme activity. The maximum enzyme activity is found at pH 2 where usually the microbial growth arrests due to acidic pH. Monod model for growth kinetics and Michaelis Menton for product formation were found to represent the experimental data.

KEY WORDS: UV irradiation, exposure, amylase, enzyme.

1. INTRODUCTION

1.1. Microbial Amylases: Amylases are one of the most important enzymes and are of great significance in present day of biotechnology. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries (Paula, 2010). Although they can be derived from several sources, such as plants, animals and microorganisms, the enzyme from microbial sources generally meet industrial demands. Microbial amylase could be potentially useful in the pharmaceutical and fine chemical industries if enzymes with suitable properties could be prepared. Amylase (a term that refers here α-amylase, β-amylase and glucoamylase) is of great significance due to its wide area of potential application. Moreover, microbial amylases have a broad spectrum of industrial applications as they are more stable with great genetic diversity, high enzymatic activity in a wide range of conditions (extreme pH, temperature, osmolarity, pressure etc.), simple and cost effective production and easy manipulation to obtain enzymes of desired characteristics (Vijayalakshmi, 2012; Tanyildizi, 2005; Vidyalakshmi, 2009).

Bacteria and fungi secrete amylases to the outside of their cells to carry out extra cellular digestion. When they have break down insoluble starch, the soluble and products such as (glucose and maltose) are absorbed in to their cells. Amylases are extra-cellular enzymes produced by microorganisms. Amylase can be classified based on how it breaks down starch molecules. Three important classes of amylases are, i) α– amylase, ii) β– amylase, iii) glucoamylase. α-amylase reduced the viscosity of starch by breaking down the bonds at random, there force producing varied sized chains of glucose. β-amylase breaks the glucose – glucose bonds by removing two lactose units at a time, there by producing maltose. Glucoamylase breaks successive bonds from the non-reducing end of the starch chain producing glucose. Many microbial amylases usually contain a mixture of these amylases. Among the microbes, fungi and bacteria are the major sources for amylase production. *Bacillus* is a genus of gram positive bacteria which have many amylase producing species. The cultivation of bacteria could be conveniently done in a suitable media and the amylase is extracted from the production or fermentation broth. In aerobic condition, the starch breakdown rapidly occurs by various *Bacillus* spices. Indeed, 60% of commercially available enzymes are obtained from different species of *Bacillus* i.e. *B. subtilis, B. stearothermophilus, B. licheniformis and B. amyloliquefaciens* (Burhan, 2003).

1.2. Nature of amylases: Amylase hydrolyzes starch molecule to give products such as dextrins and progressively smaller polymers composed of glucose units. In another way amylase can be classified into two categories as liquefying and saccharifying. Bacteria amylase that attacks 1only the α-1, 4 bonds belongs to liquefying category. Amylase belongs to the Saccharifying category attacks polysaccharides from the non-reducing end. The substrate for amylase is starch, a polysaccharide consisting of two fractions, amylose and amylopectin (Tester, 1990; Jana, 1995; Greet, 1996). Amylose is a straight chain polysaccharide in which glucose units are joined by α-1, 4 glycosidic bonds and the chain length can be up to 350 glucose units long. Amylopectin on the other hand, has branching points joining to other linear chains through alpha 1, 6 glycosidic linkage resulting in much shorter linear regions of approximately 30 glucose units. α-amylase also referred to as the liquefying amylase, hydrolyses the splitting of alpha 1,4 bonds of amylose and amylopectin, in a random manner. It is unable to cleave the alpha 1, 6 linkages in amylopectin resulting in the production of low molecular weight dextrin (Saeed, 2010; Leloup, 1991).

1.3. Starch hydrolysis by amylase: There are two forms of amylase alpha-amylase and beta amylase. Both are required for the hydrolysis of starch or glycogen and main action is to convert the substances into disaccharides maltose for the complete hydrolysis of the polysaccharide, the enzyme maltose is required in addition to alpha-amylase and beta amylase.

1.4. Factors affecting synthesis of extra cellular enzyme: The composition and concentration of media greatly affect the growth and production of extracellular amylase in bacteria (Viswanathan, 2014; Srivastava, 1986). The factors which influence the synthesis of enzyme by microorganisms are of following. i) Composition of the medium ii) General medium constituents. Low carbon nitrogen, nitrogen ratio favors amylase production. There is an optimum C: N ratio varies from 5-9 of alpha-amylase production by *bacillus subtilis* strain. Nitrogen requirement: usually growth and enzyme formation are stimulated by supplying complex forms of nitrogen such as amino acid mixtures, protein digests, nucleic acid digests etc. Carbohydrate requirement: the production of extracellular enzyme requires energy and for most microorganisms this is usually as metaboilsable carbohydrate though most bacteria can also are amino acids. The carbon sources may also fulfill the role of inducer, example, for inducible starch is used for amylase production. Trace metals: mg and k are essential for the acclivity and stability of many extracellular enzymes.

2. MATERIALS AND METHODS

2.1. Maintenance of culture: The wild type strain of *Bacillus sp* 1434 procured from Microbial Type culture collection, Institute of microbial Technology, Chandigarh, India. It was used in the present investigation.

2.2. Preparation of mutant strain: The above wild strain of the yeast, Saccharomyces cereveisiae was used in the fermentation studies of ethanol production. The mutagenesis experiments were carried out using UV irradiation technique. Forty eight-h-old yeast strain prepared in nutrient broth medium was centrifuged aseptically for 10min. The spores were suspended in 50mL saline water. The suspensions were then diluted up to 10^3 to 10^5 times; 10mL of it was transferred to petriplates. The petriplates were then placed under an UV lamp for different exposure times (5, 10, 15, 20 and 25 min). After completion of exposure time, 0.5mL of the suspension was transferred to petriplates containing growth medium and plates were placed in the incubator at 25^{o}c for 3 days. The mutant cultures were inoculated in 20mL of sterilie nutrient broth and the medium was autoclaved and inoculum was added to it. Incubation was performed for 12h at a speed of 150 rpm maintaining temperature of 30^{o}C in an incubated shaker.

2.3. Culture Used: The microorganism used throughout the study was mutant strain 3 (i.e. 15 minutes exposure *Bacillus sp.*

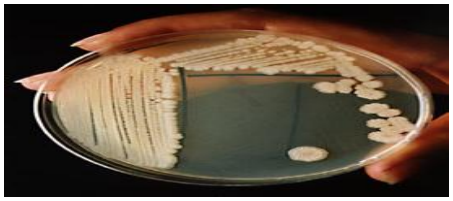

Fig.1.Mutated *Bacillus sp* (15 minutes exposure time)

2.4. Preparation of *Bacillus sp* Growth Medium: The nutrient agar medium was used for the maintenance and preservation of strains. Composition of the Media, Beef Extract 3.0g, Peptone 10.0g, Sodium Chloride 5.0g, Agar 20.0g, pH 7.0, Distilled Water 1000ml.The medium was sterilized for 30minutes at 121ºC and cooled. A loopful of 24 hours culture of *Bacillus sp*. was inoculated into the above medium and incubated for 42 hours in an incubator cum shaker at room temperature.

2.5. Production Medium: The following medium components were used for amylase production.

Composition	Percentage
Starch	1-5%
Peptone	2-10%
Potassium Chloride	0.5%
Magnesium Sulphate hepta hydrate	0.5%

The 100ml production medium was dispensed in each of 250ml conical flasks. These were sterilized for 15minutes at 121ºC cooled and inoculated with liquor culture. All flasks were incubated at 30ºC in an incubator cum shaker. Then at suitable time interval samples were withdrawn for analysis of amylase production using UV spectrophotometer method.

2.6. Extraction of Amylase: The enzyme was extracted from the flask culture broth by means of centrifugation. The flask culture broth collected during the incubation period was transferred into a centrifuge tube. The culture was centrifuged at 5000rpm for 20minutes. He pellet obtained was discarded and supernatant was collected as the enzyme extract. Until the demonstration of enzyme activity, the crude extract was stored in a freezer at 0ºC.

2.7. Spectrophotometer analysis: Spectrophotometer analysis is extremely selective, sensitive and colored substances absorb in the visible range. Variation of the colour of the solution with charge in concentrations from the basis of spectrophotometer analysis.

2.8. Enzyme Assay: The extracted enzyme solution was used for the amylase assay by the DNS (dinitro salicylic acid) method described below. Enzyme activity may be defined as amount of glucose produced per ml of reaction mixture per unit time. Steps involved are,

a) 1ml of 1% soluble starch solution was taken into the test tube.
b) 1ml of crude enzyme extract was added to above tubes.
c) The mixture was kept up adding 1ml water to the 1ml starch solution.
d) A blank was setup by adding 1ml of water to the 1ml starch solution.
e) After incubation, the reaction as stooped by adding 2ml of DNS (dinitro salicylic acid) reagent.
f) The mixture was boiled for 5minutes in a water bath at 95ºC.
g) The colour developed was read at 540nm as optical density values using a colourimeter.

2.9. Estimation of enzyme activity: Enzyme activity may be defined as the amount of glucose produced per ml in the reaction mixture per unit time. From the standard graph optical density values obtained in the colourimetric method were converted in to glucose concentration. From this enzyme activity can be calculated by the equation,

$$Enjyme\ activity = \frac{Amount\ of\ glucose\ produced}{Volume\ of\ enzyme\ \times Assay\ time}$$

2.10. Amylase Production in Batch fermentation: Amylase production was studied in a batch fermentation method. Suitable conditions for the amylase production were applied. During the incubation period, samples were withdrawn and following parameters amylase, glucose, biomass concentrations were studied respectively.

2.11. Determination of Glucose Concentration in the Medium: 5ml of broth was taken then the broth was centrifuged at 15000rpm for 5minutes. The pellet was removed and supernatant was taken for the detection of amount of glucose produced in the medium. The amount of glucose produced in the medium could be detected by DNS (dinitro salicylic acid) method. Steps involved are

a) 1ml of above centrifuged solution was taken.
b) 2ml of DNS was added to the above solution.
c) A blank was setup by centrifuging the sample collected before inoculation of the culture. 1ml of this solution and 2ml of DNS (dinitro salicylic acid) was taken as the blank.
d) The test tubes were heated at 95ºC in a water bath for 5minutes.
e) The colour developed was read at 540nm using a spectrophotometer

From the standard curve glucose produced can be found out.

2.12. Determination of Cell Biomass: Centrifuge tubes were washed and dried in an oven to remove all the moisture. Weights of empty dry centrifuge tubes were found using electronic balance. For estimation of cell mass, 10ml of sample was taken in centrifuge tubes, centrifuged for 20minutes. The settled biomass was made free of water and it was kept in oven to remove all moisture. The weights of centrifuge tube with the biomass were taken using the electronic balance. The difference between the weights gave the weight of cell mass.

3. RESULTS AND DISCUSSION

3.1. Starch Hydrolysis Test: A clear zone was observed around the growth of the organism. This clear zone indicates that the organism is an amylase producing one (*Bacillus sp.*) The bacterial growth in starch agar is shown in the figure 2.

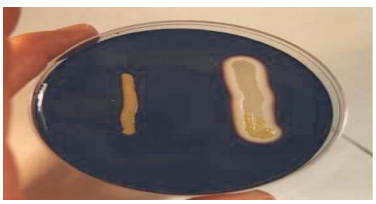

Figure.2.Bacterial growth in the starch agar medium

3.2. Effect of initial Starch Concentration on the Production of Amylase: The effect of starch concentration on the production of amylase was studied at different initial starch concentration of 1%, 2%, 3%, 4%, and 5% of starch level and keeping the composition of all other media components constant. The medium was inoculated by 24 hours fresh culture of *Bacillus sp* and kept for incubation at room temperature in a incubator cum shaker. As the initial starch concentration increases the amylase production also increases up to 4% starch level. Further increase in the initial substrate concentrations results in lesser yield of amylase. This may be due to substrate inhibition on *Bacillus sp.* The maximum yield of amylase (activity) is 1.28mg/ml.hr was obtained for the initial starch concentration of 4% in the production medium. After 48 hours, the activity of amylase at different starch concentrations of 1%, 2%, 3%, 4%, and 5% were found to be 0.56mg/ml.hour, 0.8mg/ml. Hour.0.90mg/ml. Hour, 1.28mg/ml. Hour and 1.14mg/ml.

Hour respectively. The optimum starch concentration which favors the production of amylase with maximum activity was found to be at 4% initial starch concentration.

3.3. Effect of initial pH on the Production of Amylase: The effect of initial pH on the production of amylase studied at different initial pH (1, 2, 4, 6, 7, 8 and 9) and keeping composition of all other media components constant. The medium was inoculated by 24 hours fresh culture of *Bacillus sp* and kept for production at room temperature in an incubator cum shaker. The maximum yield of amylase was obtained for the initial pH of 2. The activity of amylase at different pH of, 1, 2, 4, 6, 7, 8 and 9 was found. The optimum pH for the production of amylase was found to be 2.0 the results are shown in Figure 3.

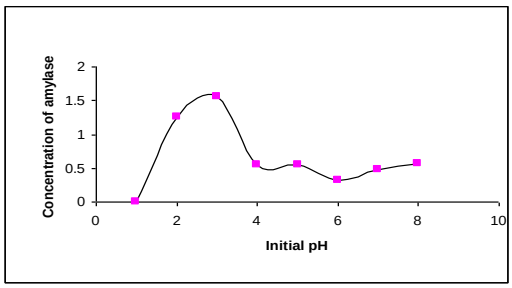

Figure.3.Effect of initial pH on the production of amylase

3.4. Effect of temperature on the production of amylase: The effect of temperature on the production of amylase was studied at different temperature, 20ºC,37ºC,50ºC and keeping the composition of all other media components constant. The medium was inoculated by 24 hours fresh culture of *Bacillus sp*. The maximum yield of amylase was obtained at temperature of 40ºC.

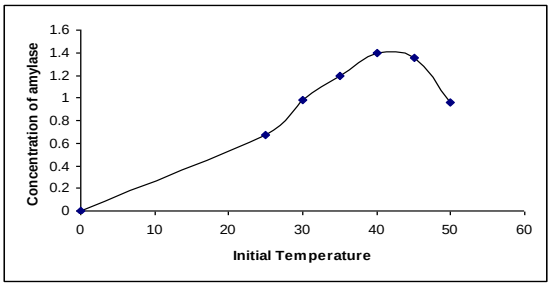

Figure.4 Effect of Initial Temperature on the Production of Amylase

3.5. Amylase Production in batch fermentation: Using the optimized media, amylase production was studied in flask culture. The composition of the optimized media used includes Starch (4%), Peptone - 6%, Potassium chloride- 0.5%, Magnesium sulphate hepta hydrate- 0.5%. At temperature 40ºC and pH 2.0 of starch 4% amylase was produced in culture broth obtained by centrifugation. At various time intervals, the samples were withdrawn and amylase activity was found. The biomass production and glucose were also calculated

Table.1.Time course of amylase production on starch

Time (hours)	Volume of starch reaction mixture(ml)	Volume of amylase added (ml)	Optical density	Concentration of glucose corresponding to OD (mg)	Enzyme activity (mg/ml hr)
12.0	1.0	1.0	0.33	0.46	0.23
24.0	1.0	1.0	0.57	0.78	0.39
36.0	1.0	1.0	0.95	1.30	0.65
48.0	1.0	1.0	1.14	1.54	0.77
60.0	1.0	1.0	1.79	2.46	1.23
72.0	1.0	1.0	1.66	2.28	1.14

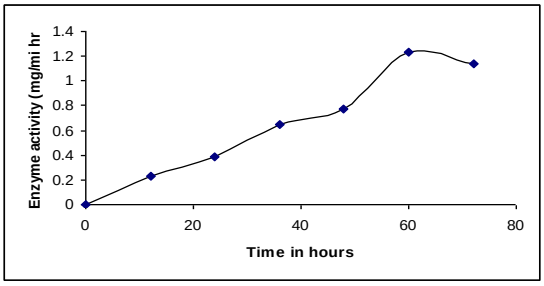

Figure.5.Effect of enzyme activity on the substrate

Table.2.Time course of glucose production on the substrate

Time (hours)	Volume broth solution (ml)	Volume DNS reagent (ml)	Optical density (A°)	Concentration of glucose corresponding to OD values (mg/ml)
12.0	1.0	2.0	0.14	0.18
24.0	1.0	2.0	0.29	0.37
36.0	1.0	2.0	0.43	0.58
48.0	1.0	2.0	0.56	0.76
60.0	1.0	2.0	0.74	0.99
72.0	1.0	2.0	0.69	0.96

A graph was drawn with concentration of glucose along the Y-axis and incubation time along the X-axis. From the graph it was clear that, the concentration of glucose produced in the medium increases with time, reaches a level maximum level and then decreases.

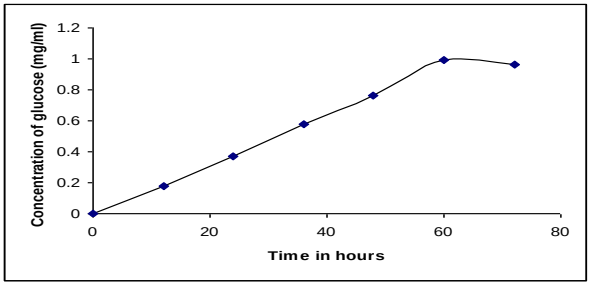

Figure.6.Effect of glucose concentration on the substrate

Table.3.Time course of biomass production on the substrate

Time (hours)	Weight of centrifuge tube before centrifugation, X (g)	Weight of the centrifuge after centrifugation, Y (g)	Biomass produced in the medium (Y-X)g
0.0	10.31	10.40	0.09
12.0	10.37	10.49	0.11
24.0	10.38	10.50	0.12
36.0	10.38	10.52	0.14
48.0	10.38	10.54	0.16
60.0	10.38	10.54	0.16
72.0	10.38	10.54	0.16

A graph was drawn between incubation time (X-axis) and bio mass produced (Y-axis).it was clear that, the bio mass produced in the medium increases with time and reaches a constant.

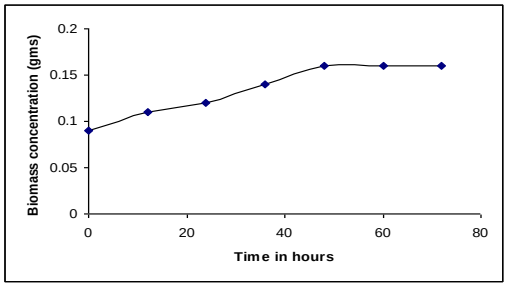

Figure.7.Effect of biomass concentration on the production medium

Amylases are enzymes having many industrial uses. It is present in plants, animals and microbes, but the enzyme from microbial source meet industrial demands. The study of production of amylase from *Bacillus sp.* was done experimentally, using different carbon, nitrogen, pH and temperature. Fermentation was monitored up to 72 hrs and carried out in the presence of starch as carbon source. Amylase is produced by *Bacillus sp* only when the production media is devoid of glucose and a starch act as inducer molecule. From the study it was clear that, a carbon and nitrogen ratio of 2:3 favors amylase production. Effect of pH on the production was studied and found that acidic pH enhances the production of amylase. Temperature has positive effect on production of amylase, as the temperature increase the production of amylases reached peak level up to 40ºC. But further increase of temperature; decrease the production due to feedback inhibition. Similarly amylase production was move in acidic medium then in neutral and alkaline medium. Amylase produced from *Bacillus sp* is of stable at high temperature.

3.6. KINETICS

3.6.1. Monad model for growth kinetics: A linear relationship between the specific growth rate and cell mass concentration should be considered as a specific case and it may not be valid for all the strains. Using the monad

model maximum specific rate and saturation constants were determined. The growth rate can be described by the equation

$$dX/dt = \mu X$$

Where the specific growth rate μ is given by the monad type model as

$$\mu = \mu_{max} S/Ks + S$$

3.6.2. Michaelis Menton model for product formation: A carbon source such as starch is used to form cell material and metabolic product as well as the maintenance of cells, therefore the substrate consumption can be described by the equation (Bharathiraja and Jayamuthunagai, 2008).

$$V = Vmax\ S/Km + S$$

The reciprocal form of the above equation has been used to find the kinetic parameters Vmax and Km.

$$1/V = Km/Vmax + 1/Vmax$$

3.6.3. Kinetic parameters for growth and product formation:

Monod model	μ max = $0.97h^{-1}$
	Ks = 24.5 g/l
Michaelis Menton Model	Vmax = 1.12 g/l
	Km = 2.85 g/l s

4. CONCLUSION

From this work, the production of amylase from the mutated strain in batch culture was studied. The production of amylase was maximum when the starch concentration was 4%. The initial substrate concentration was varied from 1% to 5% by keeping all other conditions constant and UV mutation of 15 minutes exposure time. The activity of amylase at starch concentration is 1.15mg/ml.hr. The optimum pH was 2.0 with the maximum activity of 1.15mg/ml.hr at 4% starch concentration. The effect of optimum temperature for the production of amylase was also studied at 40ºC.Using the optimized media, amylase production was studied in the batch fermentation method. In future the same method has to be adopted in the continuous fermentation technology.

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