Global Journal of Science Frontier Research: C
Biological Science
Volume 14 Issue 5 Version 1.0 Year 2014
Type : Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4626 & Print ISSN: 0975-5896

Study on Enzyme Activity in the Production and Optimization of High Temperature Alkaline α-Amylase Enzyme by Bacillus Lichenoformis using Low Cost Medium Derived from Agricultural byproducts

By M. Sathiyamoorthy & Dr. S. Theneshkumar
Himalayan University, India

Abstract- Production of **α**-amylase enzyme by Bacillus Lichenoformis using stirred tank fermentor (BIOSTAT – E) was carried out. The strain was obtained from National Chemical Laboratory, Pune, India. Corn starch is used as the substrate. The enzyme production was studied by changing the various parameters like temperature, pH, rpm and substrate concentration. The enzyme activity shows maximum at a temperature of 350C – 370C, pH 8 and 300 rpm. The maximum enzyme production was achieved, for 1% concentration of cornstarch at 35.60C and pH 9 using the fermentation medium contains yeast extract and peptone and the enzyme activity was found to be 55.93 DUN/ml.

*Keywords: **α**-amylase, bacillus lichenoformis, low cost medium, agricultural by products, fermentation, alkaline enzyme.*

GJSFR-C Classification : FOR Code: 860399

STUDYONENZYMEACTIVITYINTHEPRODUCTIONANDOPTIMIZATIONOFHIGHTEMPERATUREALKALINEAMYLASEENZYMEBYBACILLUSLICHENOFORMISUSINGLOW COSTMEDIUMDERIVEDFROMAGRICULTURALBYPRODUCTS

Strictly as per the compliance and regulations of :

Study on Enzyme Activity in the Production and Optimization of High Temperature Alkaline α-Amylase Enzyme by *Bacillus Lichenoformis* using Low Cost Medium Derived from Agricultural byproducts

M. Sathiyamoorthy [α] & Dr. S. Theneshkumar [σ]

Abstract- Production of α-amylase enzyme by Bacillus Lichenoformis using stirred tank fermentor (BIOSTAT – E) was carried out. The strain was obtained from National Chemical Laboratory, Pune, India. Corn starch is used as the substrate. The enzyme production was studied by changing the various parameters like temperature, pH, rpm and substrate concentration. The enzyme activity shows maximum at a temperature of 35^0C – 37^0C, pH 8 and 300 rpm. The maximum enzyme production was achieved, for 1% concentration of cornstarch at 35.6^0C and pH 9 using the fermentation medium contains yeast extract and peptone and the enzyme activity was found to be 55.93 DUN/ml. Since the cost of yeast extract and peptone is very high, so the further work was done using some low cost carbon and nitrogen sources like defatted cotton seed, defatted soya flour and mustard seed which are extracted from agricultural byproducts. The enzyme activity for using the low cost medium was found to be nearly triple such as 121.49 DUN/ml. The enzyme production reaches the steady phase at 24 hours. So it is highly recommended that using the low cost medium for the α-amylase enzyme gives better biomass cell concentration and enzyme activity as well.

Keywords: *α-amylase, bacillus lichenoformis, low cost medium, agricultural by products, fermentation, alkaline enzyme.*

I. Introduction

Enzymes are proteins which catalyze variety of reactions in the biological system. When enzymes were first intensively studied in the last two centuries this chemical nature was obscure and even the reactions catalyzed were frequently ill defined. It was natural and therefore, that individual enzymes were given names by their discoverers. Most enzymes are studied and need to be named before any significant information about their structures exists. Whenever the 'same' enzyme from different organism is studied, it is found that Proteins different in detailed structure (and some times in gross structure) can have essentially the same catalytic properties. In the recommendations of the "International Union of Biochemistry Nomenclature Committee (1984), therefore, an enzyme name does not specify a structure but instead defines the Principal reaction catalyzed.

Enzymes are classified in to six classes. Enzymes in the first three classes all catalyze transfer reactions, with stoichiometry A+B → P+Q, but differ in other respects. Oxidoreductases catalyze reaction in which one or more electronics (usually two) are transferred from a donor (reducing agent) to an acceptor (Oxidizing agent). In many oxidoreductases the oxidized substrate can be regarded as a hydrogen donor, and for these enzymes the term dehydrogenase is preferred. Hydrolases catalyze hydrolytic reaction, i.e. reactions in which water is the acceptor of the transferred group. The transferases thus comprise all enzymes catalyzing transfer reaction that are not oxide reductases or hydrolases. Lyases catalyze elimination reaction, where the bond is broken without oxidoreduction or hydrolysis and in most cases have stoichiometry. A → P+Q.

The six classes are further sub divided in to subclasses, to specify the type of reaction more fully and to indicate the reactants. All the enzymes have a property of either intra cellular or extra cellular in nature. But most of them are extra cellular in nature.

a) Intracellular Enzymes

Enzymes occur in all living cells, where they catalyze and regulate reactions of Biochemical pathways essential to the existence of the living system. In general substrates for these enzymes are small molecular weight molecules, e.g. Sugars, amino acids, carboxylic acids, which are able to permeate the membrane. Their catalytic properties are regulates by conformational changes in their three dimensional structure accomplished by allosteric cofactor molecules.

Author α: Research Scholar, Department of Chemical Engineering, Himalayan University, Arunachal Pradesh, India.
e-mail: sathyachemical@gmail.com
Author σ: Department of Chemical Engineering, SRM University, Kattankulathur, Chennai, Tamilnadu, India.

b) Extracellular enzymes

Extra cellular enzymes were originally defined as enzymes which are external to the cell wall and in contact with surrounding medium. At present we consider transport the membrane as the primary secretion event. Thus for the purpose of this review the term & erection is used to refer to the transmembrane passage of protein and the term extra cellular to those proteins that have undergone this process. The biological function of this kind of enzymes may be seen in the hydrolysis of macro molecules which are too large to be transported in to the cell.

c) Animal tissue Enzyme

Enzymes used in Industry are isolated from animal and plant tissues, as well as from Micro organisms. One of these three sources may be favored for a given enzyme. For example, some proteolytic enzymes isolated from animals may be advantageous in special fields of application. The enzyme chymosin, also known as rennet, is an acid protease used in the milk-clotting step of cheese production. A mixture of chymosin and its zymogen prochymosin, which may be converted chymosin by low pH treatment, are currently obtained from the abo-masum of an unweaned calf. Animal glands, e.g. the pancreas, are sources for hydrolyzing enzymes used as a digestive acids. The pancreas is a very rich sources of enzymes. It contains about 23% of trypsinogen and 10 -14% of chymotrypsinogen. So called pancreatin, a digestive aid, contains several enzymes such as amylase, lipase and protease.

d) Plant tissue enzymes

Plant protease isolated from pineapple (bromelain) and the papaya plant (papain) have been used for meat tenderizing and chill proofing beer. Useful amylolytic enzymes occur in plant tissues such as barely, wheat, rye, Potatoes, sweet potatoes, beans, soy beans, α - amylase, β - amylase, which starts at the non-reducing ends of the outer chains of the starch and proceeds by gradual removal of maltose units and de branching enzyme which hydrolyzes the α -1 - 6 linkages of starch, were detected in these plants.

e) Microbial enzymes

Microorganisms have become increasingly important as producers of industrial enzymes and in fact most enzymes used in industry today are of Microbial origin. Attempts are now being made to replace enzymes which traditionally have been isolated from animal tissue and plant tissues with enzymes from Microorganisms. Examples for partial replacement of plant and animal enzymes in dudes. Amylases and endo - β - glucanases of malted Barley and wheat by enzymes from Bacillus and Aspergillus in the beer, distillery, baking and textile industries. Plant and animal proteases by Aspergillus and Thermoactinomyces protease for meat tenderization and for chill proofing beer.

f) Uses of α – amylase

The enzyme α–amylase is used as a biocatalyst in many small scale and large scale industries some of the uses are.

- The Bacterial α–amylase used in starch hydrolysis industries, Brewing industries, Detergents industries and textile industries.
- The fungal α–amylase used in starch industries and baking industries.
- The α–amylase from Malt used as a digestive aid and supplement to bread.
- The α–amylase from Aspergillus Orygaze is used to produce starch liquefying syrups.
- The α–amylase from Bacillus Subtillis used in Desizing textile industries, Alcohol fermentation industries and glucose producing industries.
- The α–amylase produced from Aspergillus Niger is highly acid resistant is used as a digestive acid at pH-5.
- The α–amylase from Bacillus lichenoformis is used in all starch industries and detergent industries and to produce starch sizing pastes for use in paper coatings.

II. Objective of the Study

Enzymes are Proteins which catalyze variety of reaction in the Biological systems. There are many methods used to produce the enzymes among that the biological methods are widely used. In this type of biological method of production, solid state fermentation is applied for the production. In all the types of fermentation processes, the cultures has been prepared using yeast extract and peptone etc. These are added to the culture in terms of nutrients as a carbon and nitrogen sources for the microorganism. The cost of these chemicals are much expensive. So the alternative method has been proposed for the preparation of culture medium using some low cost agricultural byproducts such as defatted cotton seed, defatted soybean, mustard seed etc. The fermentation has to carryout using these type of low cost medium to check the productivity and enzyme activity.

III. Experimental Setup

a) Biostat E fermentor

The fermentation was carried out in a B. BRAUN CO, Biostat E fermentor. It is a compact and comprehensive fermentation system on a laboratory scale, which can be used in microbiological and biotechnological research and development. Biostat E fermentors are designed for use in discontinuous fermentation (Batch operations) as well as in continuous

process. The measurement and control system used in compatible with computers. The Biostat E is protected against unauthorized use with a main key. All modules of the measurement and control section are separately switched on. Therefore they can be installed or removed independently from the control in spite of the central mains switch. Additional modules can be inserted without interruption or disturbance of operations.

The lower front panel of the basic device is provided with installation ports for at least 4 dosing pumps of the four, three are peristaltic pumps for the supply of acid, alkali and antifoam agent, the fourth is prepared to install precision dosing pumps.

The arrangements of the various technical appliances in the basic devices are:

- Thermostat system which containing heating and cooling water circuit for tempering as well as for sterilization.
- Gas supply system including exhaust equipment.
- Motor and drive system for the stirrer shaft drive.

The recorder, of 6 channels dot printer records the following measurement values in the basic devices.

- Temperature
- Speed
- pH Value & Antifoam consumption

The culture vessel is mounted on the console laterally fixed at the fermentor where there are the corresponding borings for the feet of the culture. Simultaneously the connection to the stirrer drive is guaranteed. For starting operating the device the filling state of the fermentor thermostat is to be checked. The set point temperature is adjusted at the corresponding digital switch of the module. A good mixing of the culture vessel is a prerequisite. For that a stirrer system is provided which is driven by a controlled DC motor. The stirrer speech can be directly adjusted by the digital switch of the speed controlled module. The adjustable speed range is 50 – 1500 minutes^{-1}.

The pH – value in the culture medium can be electro chemically determined via a combined – glass electrode. The pH set point desired can be adjusted with the digital switch of the pH controller.

b) Dimensions of the fermentor

Total volume of the fermentor	:	6 lit.
Working volume	:	5 lit.
Max working temperature	:	138^{o} C
Max working pressure	:	124^{o} C
Diameter of the fermentor	:	17.5 cm
Height of the fermentor	:	40 cm
Agitator type	:	6 Blade, Paddle type Agitator

IV. Materials and Methods

a) Microbial strain

Bacillus Lichenoformis, NCIM 2051 Received from National Chemical Laboratory, Pune, India.

b) Chemicals

Beef extract
Peptone
NaCl
$MgSO_4$
KH_2PO_4
$CaCl_2$
Yeast extract
Agar
Corn Starch
Defatted Cotton Seed
Defatted Soya flour
Mustard Seed

c) Medium

i. *Universal medium for bacteria*

Beef extract	:	1.0 %
Sodium Chloride	:	0.5 %
Peptone	:	1.0 %
pH	:	7.0 - 7.2

Sterilize the medium, and adjust the pH at 7.2. Add 2% Agar for making slants.

ii. *Corn starch medium: (Basal Medium)*

Corn starch	:	1 %
Yeast extract	:	0.2 %
Peptone	:	0.5 %
$MgSO_4$	:	0.05 %
KH_2PO_4	:	0.05 %
NaCl	:	0.15 %
$CaCl_2$	:	0.015 %

iii. *Low cost medium*

Corn starch	:	1 %
$MgSO_4$	:	0.05 %
KH_2PO_4	:	0.05 %
NaCl	:	0.15 %
$CaCl_2$	:	0.015 %
Soya bean	:	0.5 %
Mustard Seed	:	2 %
Cotton seed	:	3 %

d) Procedure

Shake flask cultures were operated at constant temperature of 37^{0}C and fixed rpm with 100 ml of medium in a 500 ml Erlenmeyer flask and inoculated with the culture. Fermentation studies were carried out in above described B. Braun Biostat E fermentor with the cultural conditions of 37^{0}C, pH 7, and 300 rpm. Since it is an aerobic fermentation, the aerobic rate was maintained at 1 vvm. For every six hours the sample were collected from the sampling point provided in the top of the culture vessel, and analyzed.

i. *Stock Culture*

Bacillus Lichenoformis NCIM 2051 was maintained in an Agar slant at 4^{0}C.

ii. *Sub Culture Maintenance*

Subculture was prepared using a universal Bacteria medium and it was maintained in an incubator at 37°C.

iii. *Pre inoculum*

Take 100 ml of the Universal medium inoculate this with a stock agar culture in a 500 ml Erlenmeyer flask and kept in a shaker at 300 rpm and 37^{0}C. It is also called as seeding of culture.

iv. *Enzyme activity*

One unit of enzyme activity (DUN) is defined as the quantity of enzyme that causes 1 % reduction of blue color intensity of starch-iodine solution in 1 min. The optical density was first measured at 660 nm using an UV spectrophotometer.

V. Results and Discussion

a) Enzyme Activity determination

Different techniques have been used to measure enzyme activities. There is no general method equally applicable to all enzymes. The enzyme activity may be depends on the time, enzyme concentration, substrate concentration.

Extra cellular amylase activity was determined by measuring the decrease in iodine color reaction showing dextrinization of starch. The reaction contained 1 ml of enzyme (cell free supernatant) and 10ml of 1% starch solution incubated at 40^{0}C for 10 min. The reaction was stopped by adding 10ml of 0.1N HCl. 1 ml of this acidified solution was added to 10ml 0.1N HCl. From this 1ml was added to iodine solution (0.05% iodine in 0.5% KI). The optical density of the blue colored solution was determined of 660 nm one unit of enzyme activity (DUN) is defined as the quantity of enzyme that causes 1% reduction of blue color intensity of starch iodine solution at 40^{0}C in 1 min.

For amylase activity determination requires the standard chart for starch iodine solution. Take six test tubes in that add 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of 1% starch solution respectively and add 1, 0.8, 0.6, 0.4, 0.2 and 0 ml of water reply. Add 10ml of iodine solution (0.05% I_2 and 0.5% KI) in all the six text tubes. The inference is the light blue color formation. The optical density of the blue colored solution was measured at 660 nm in the UV spectrophotometer. The standard graph was drawn by plotting starch concentration Vs absorbance. From the standard graph the enzyme activity was calculated.

b) Production of enzyme

The growth pattern of Bacillus Lichenoformis NCIM 2051 and α–amylase production was observed for three days in basal medium with 1% cornstarch as a carbon source. The formation of α–amylase started from 4 hours. The maximum enzyme production was achieved at 24 hours. The pH of the broth increased from 7 at the beginning to 8.9 at the end of fermentation. The maximum yield was achieved at 35^{0}C.

c) Effect of corn starch concentration

The effect of corn starch concentration was further studied. The α–amylase production was studied, by changing the Corn starch concentration at 0.5%, 1% and 2.5%. It was found that with an increase of starch concentration in the medium beyond 1%, enzyme production did not increase. At higher starch concentration, enzyme production was comparatively lower and the time required to reach the maximum enzyme level was longer.

d) Effect of pH

The bacterium was found to grow at pH 3-11, with growth resulting in an increase of the patient's media's pH. Enzyme production started at 5.0 and ceased at pH 10.0. Maximum enzyme production occurred at pH 6-9. Very little enzyme production in the medium at initial pH of 3 - 4. At higher pH values (10-11), growth was quite high, but the amount of enzyme production was very low.

e) Effect of temperature

The strain was found to grow and produce enzyme at temperatures from 25 to 50^{0}C. Maximum enzyme production was observed at 35^{0}C. Growth and enzyme production both started decreasing drastically above 40^{0}C.

f) α–amylase production in low cost medium

The α–amylase production was further studied by using the low cost medium which containing the carbon and nitrogen sources like corn flour, mustard seeds. Since the cost of yeast extract and peptone in the Basal medium is very high, we can replace the yeast extract and peptone with the above mentioned things. The low cost medium produced 2 times more enzyme than the high cost synthetic medium (yeast extract and peptone). The medium containing 0.5% defatted, 2% mustard seed in the place of yeast extract and peptone, was found to yield high enzyme activity of α–amylase. The experiments were conducted for 6 different batches

with various concentrations, which are given in the below table and graph.

Table 1 : Enzyme production for 1% corn starch concentration

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Enzyme Activity DUN/ml
0	7.0	36.9	104.7	300	0.003
3	7.1	36.8	101.8	300	2.01
6	7.2	37.0	100.2	300	3.37
12	5.8	37.1	22.6	300	4.51
18	7.3	35.7	89.3	300	29.31
24	8.2	35.6	86.8	300	55.84
48	9.0	35.6	96.4	300	55.93
72	8.9	36.2	98.7	300	56.11

Table 2 : Enzyme production for 2.5% corn starch concentration

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Enzyme Activity DUN/ml
0	6.3	37.0	100.8	300	0.0007
3	5.8	36.4	100.1	300	1.37
6	6.1	35.9	92.7	300	2.56
12	6.7	35.7	95.6	300	8.48
18	6.9	35.4	97.9	300	33.49
24	7.1	35.3	98.3	300	40.83
48	7.9	35.1	88.5	300	40.71
72	8.5	34.8	84.3	300	40.74

Table 3 : Enzyme production for 0.5% corn starch concentration

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Enzyme Activity DUN/ml
0	6.1	37.0	120.3	300	0.0007
3	6.1	36.7	110.8	300	1.53
6	6.9	36.6	102.6	300	4.92
12	7.5	35.9	100.9	300	12.19
18	7.8	35.8	98.7	300	39.43
24	7.9	35.6	98.5	300	43.38
48	8.3	35.7	98.1	300	42.31
72	8.8	35.4	83.6	300	42.34

Table 4 : Enzyme production using Basal medium + 0.5% defatted soya flour

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Enzyme Activity DUN/ml
0	7.0	37.0	120.3	300	0.98
3	7.1	38.3	110.4	300	11.91
6	7.3	37.1	93.6	300	27.56
12	8.1	36.3	83.9	300	48.29
18	8.7	36.1	70.8	300	61.49
24	8.9	35.9	64.7	300	81.24
48	8.7	36.3	53.9	300	81.31
72	8.9	37.8	48.7	300	80.9

Table 5 : Enzyme production using Basal medium + 3% defatted cotton seed

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Enzyme Activity DUN/ml
0	7.0	37.0	120.1	300	1.90
3	7.3	38.1	117.3	300	18.53
6	7.9	37.5	93.5	300	46.93
12	8.5	36.3	83.8	300	64.71
18	9.1	35.3	77.9	300	84.18
24	9.7	35.5	64.2	300	91.5
48	9.9	36.1	56.9	300	92.3
72	10.3	38.5	28.5	300	91.9

Table 6 : Enzyme production using Basal medium + 2% mustard seed

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Enzyme Activity DUN/ml
0	7.0	37.0	120.1	300	2.41
3	7.3	38.1	117.3	300	12.49
6	7.9	37.5	93.5	300	32.33
12	8.5	36.3	83.8	300	65.91
18	9.1	35.3	77.9	300	95.41
24	9.7	35.5	64.2	300	121.49
48	9.9	36.1	56.9	300	120.83
72	10.3	38.5	28.5	300	121.10

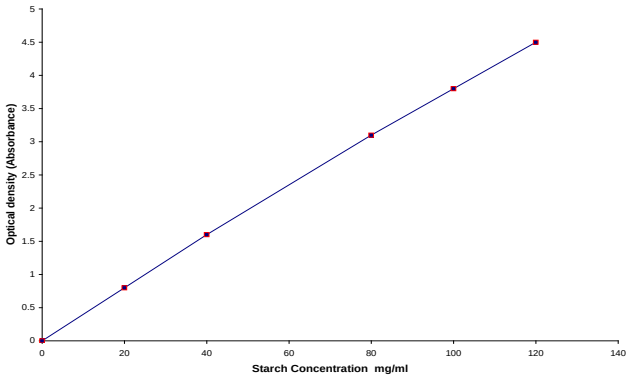

Figure 1 : Standardization graph of starch iodine solution for α–amylase activity

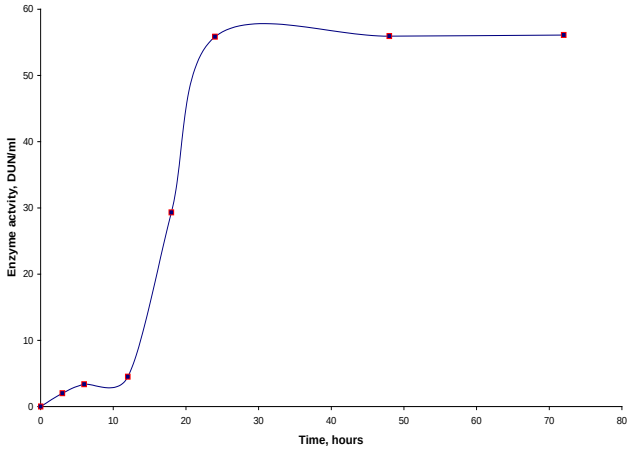

Figure 2 : Enzyme activity for 1% corn starch concentartion

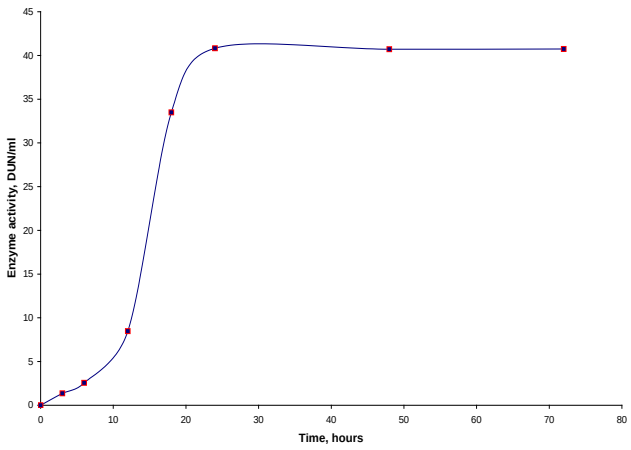

Figure 3 : Enzyme activity for 2.5% corn starch concentartion

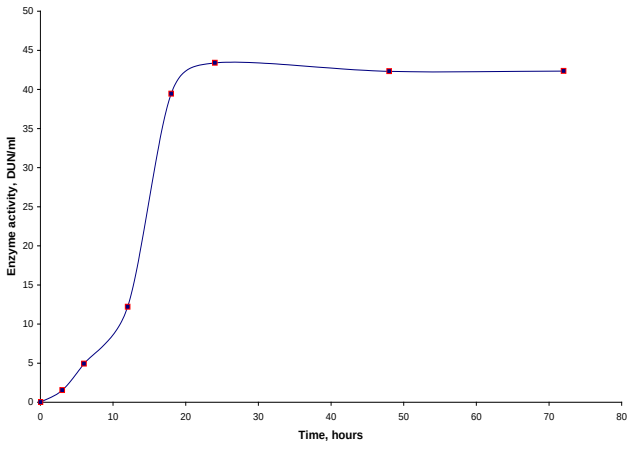

Figure 4 : Enzyme activity for 0.5% corn starch concentartion

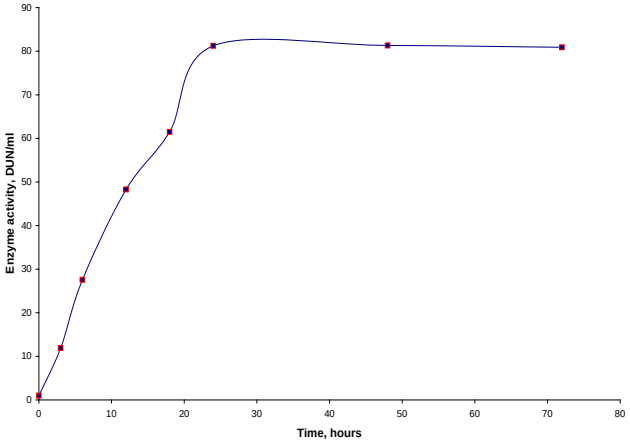

Figure 5 : Enzyme activity for Basal medium with 0.5% defatted Soya flour

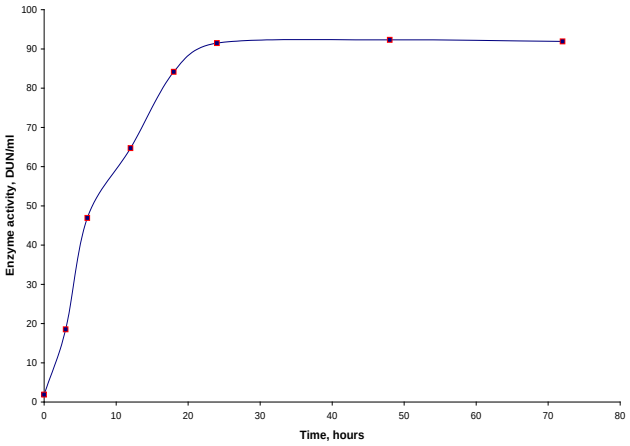

Figure 6 : Enzyme activity for Basal medium with 3% defatted Cotton seed

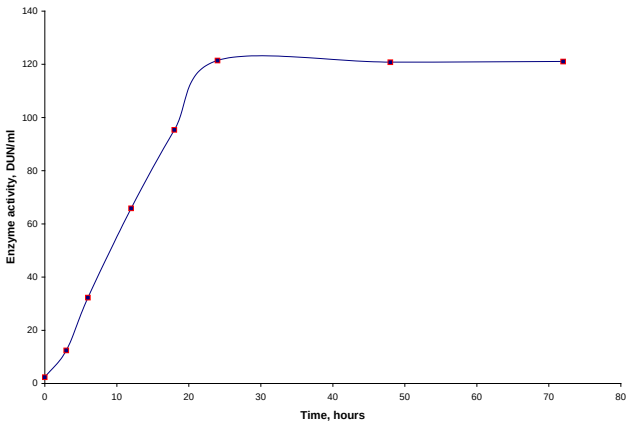

Figure 7 : Enzyme activity for Basal medium with 2% Mustard seed

VI. Conclusion

The Bacterial strain, *Bacillus lichenoformis* NCIM 2051 was obtained from National Chemical Laboratory, Pune, which produced high temperature alkaline α–amylase enzyme. The optimum cultural conditions are found to be 35°C, pH 7 and 300 rpm. The α–amylase produced from this Bacterial strain, *Bacillus lichenoformis* was quite active even at 100°C, however it showed optimum activity at 90°C, and also it exhibited optimum activity in the broad pH range 5.5 – 10, thus α-amylase *of Bacillus lichenoformis* seems to have a very broad pH range. A low cost synthetic medium producing large quantities of α–amylase has been developed from *bacillus lichenoformis* was used for α–amylase production. The α–amylase of this strain showed excellent stability at high temperatures and over a wide pH range. The enzyme activity were determined and optimized. The low cost medium which contains, Defatted soya flour, Defatted cottonseed, and Mustard seed, produces around three times more enzyme than the high cost synthetic medium using yeast extract and peptone in the B. Braun Biostat E fermentor. So it is further suggested to change the cheapest different nitrogen sources components in this low cost medium like corn steep liquor etc.

References Références Referencias

1. A.P. Gandhi and L. Kjaergaard, "Effect of CO_2 on the formation of α -amylase by *Bacillus subtillis* growing in continuous and batch cultures, Biotechnology & Bioengineering, Vol.17, pp. 1109-1118 (1975).
2. Fogarty .W.M, Griffon and A.M. Joyce, "Enzymes of *Bacillus* species process" Biochemistry, Vol.9, pp. 11-24 (1974).
3. H.J. Rehm and G. Read, Biotechnology, Volume 7a, Enzyme technology, VCH publishers (1987).
4. J. Jayaraman, "Laboratory manual in Biochemistry", (1981).
5. James E. Bailey and David F.Ollis, "Biochemical engineering fundamentals", McGraw.Hill international editions, second edition, (1986).
6. Martha H.M.Oseley and Leonard Keay, "Purification and characterization of the α -amylase of *Bacillus subtillis* NRRL B3411, Biotechnology & Bioengineering, Vol.12, pp. 251-271 (1970).
7. Peter F. Stanbury and Allen Whitkar, "Principles of Fermentation technology", Pergamon press, (1984).
8. Pratima Bajpai and Promod Bajpai, "High temperature alkaline α-amylase From *Bacillus lichenoformis*", Biotechnology & Bioengineering, ol.33 pp. 72-78 (1989).
9. Pratima Bajpai and Umender Sharma, "Production of α -amylase in a low cost medium by *Bacillus lichenoformis",* Journal of Fermentation & Bioengineering, Vol.67, No 6, pp.422-423 (1989).
10. Seung-Hyeon moon and Satish J .Parulekar, "A parametric study of x-amylase production in Batch, Fed batch and continuous suspension culture of *Bacillus firmus*", Biotechnology & Bioengineering, Vol.41, pp. 43-54 (1993).
11. Yong Hee Lower et al, "Production of alkaline protease by *Bacillus lichenoformis* in an aqueous two phase system, Journal of fermentation and Bioengineering, Vol.69, pp. 89-92 (1990).

This page is intentionally left blank