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

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

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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

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.

membrane. Their catalytic properties are regulates by conformational changes in their three dimensional structure accomplished by allosteric cofactor molecules.

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 Iproduce 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° C
Max working pressure	:	124° 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 40C.

ii. *Sub Culture Maintenance*

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

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 370C. It is also called as seeding of culture.

iv. *Biomass*

Biomass was estimated by the method of dry weight for every sample. It was expressed in terms of 1 dry weight/lit.

v. Results and Discussion

a) Biomass estimation

The Biomass for the sample, which got from the fermentation broth was determined by the dry weight method. Take some known amount of liquid from the fermentation of liquid from the fermentation broth in the centrifuge test tube, and kept in a centrifuge for 20 minutes at 5000 rpm. The supernatant liquid was collected and kept for α– amylase activity determination. The cells settled in the bottom of the centrifuge tube was transferred to a funnel contains the gravimetric filter paper (ash less), and washed thoroughly with distilled water. Transfer this gravimetric filter paper in to the known weight silica crucible, and incinerate for 30 min. Cool the contents and measures the weight from this calculate the cell concentration.

Sample calculation

Weight of empty crucible	:	15.8603 gms.
Volume of fermentation liquid taken:		11.4 ml
Weight of crucible with cells	:	15.8895 gms.
Cell mass/volume of liquid taken	:	
	:	15.8895 – 15.8603
	:	0.0292 gms
For 1 litre, cell concentration weight.	:	2.57 gm dry

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 350C.

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 500C. Maximum enzyme production was observed at 350C. Growth and enzyme production both started decreasing drastically above 400C.

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 the maximum amount 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	Cell concentration gm dry wt per lit
0	7.0	36.9	104.7	300	0.052
3	7.1	36.8	101.8	300	0.236
6	7.2	37.0	100.2	300	0.480
12	5.8	37.1	22.6	300	1.215
18	7.3	35.7	89.3	300	2.882
24	8.2	35.6	86.8	300	2.843
48	9.0	35.6	96.4	300	2.745
72	8.9	36.2	98.7	300	2.461

Table 2 : Enzyme production for 2.5% corn starch concentration

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Cell concentration gm dry wt per lit
0	6.3	37.0	100.8	300	0.035
3	5.8	36.4	100.1	300	0.21
6	6.1	35.9	92.7	300	0.53
12	6.7	35.7	95.6	300	0.78
18	6.9	35.4	97.9	300	1.52
24	7.1	35.3	98.3	300	2.61
48	7.9	35.1	88.5	300	2.48
72	8.5	34.8	84.3	300	2.57

Table 3 : Enzyme production for 0.5% corn starch concentration

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Cell concentration gm dry wt per lit
0	6.1	37.0	120.3	300	0.026
3	6.1	36.7	110.8	300	0.21
6	6.9	36.6	102.6	300	0.58
12	7.5	35.9	100.9	300	0.97
18	7.8	35.8	98.7	300	1.38
24	7.9	35.6	98.5	300	1.85
48	8.3	35.7	98.1	300	1.61
72	8.8	35.4	83.6	300	1.43

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

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Cell concentration gm dry wt per lit
0	7.1	37.0	98.3	300	0.042
3	7.8	36.8	90.7	300	1.19
6	8.3	36.1	83.6	300	2.25
12	8.5	35.7	85.9	300	3.37
18	9.2	35.4	70.2	300	3.93
24	9.8	35.3	56.3	300	4.89
48	9.7	35.4	79.3	300	4.91
72	9.8	35.3	73.2	300	4.87

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

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Cell concentration gm dry wt per lit
0	7.0	37.0	120.3	300	0.02
3	7.1	38.3	110.4	300	1.25
6	7.3	37.1	93.6	300	2.31
12	8.1	36.3	83.9	300	3.80
18	8.7	36.1	70.8	300	4.93
24	8.9	35.9	64.7	300	5.29
48	8.7	36.3	53.9	300	5.3
72	8.9	37.8	48.7	300	5.32

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

Time hours	pH	Temp ^0C	$\%PO_2$	rpm	Cell concentration gm dry wt per lit
0	7.0	37.0	120.1	300	0.01
3	7.3	38.1	117.3	300	1.23
6	7.9	37.5	93.5	300	2.56
12	8.5	36.3	83.8	300	3.19
18	9.1	35.3	77.9	300	4.84
24	9.7	35.5	64.2	300	6.71
48	9.9	36.1	56.9	300	6.68
72	10.3	38.5	28.5	300	6.70

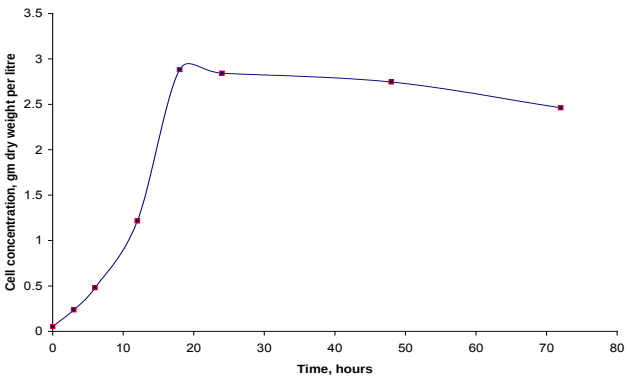

Figure 1 : Biomass cell concentration for 1% corn starch

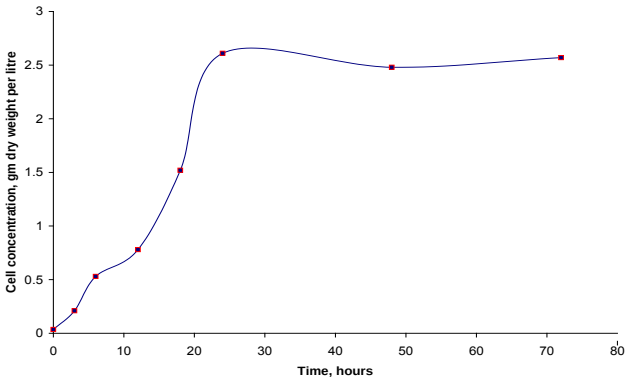

Figure 2 : Biomass cell concentration for 2.5% corn starch

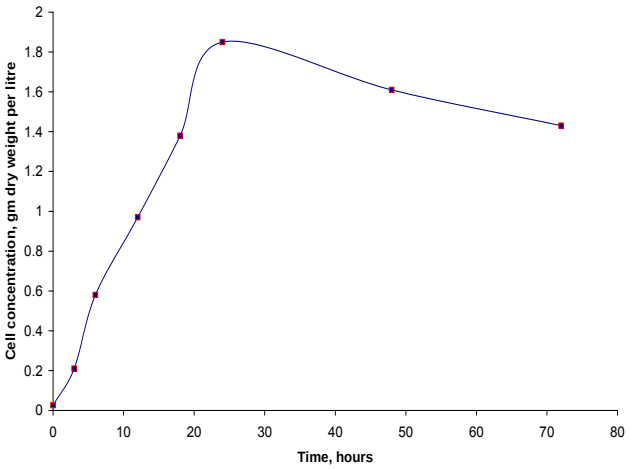

Figure 3 : Biomass cell concentration for 0.5% corn starch

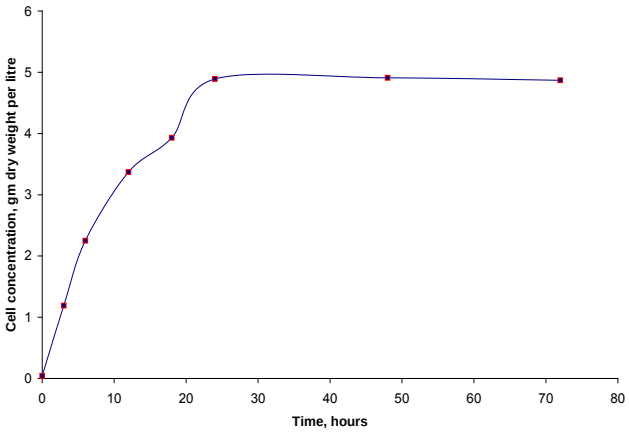

Figure 4 : Biomass cell concentration for Basal medium with 0.5% defatted soya flour

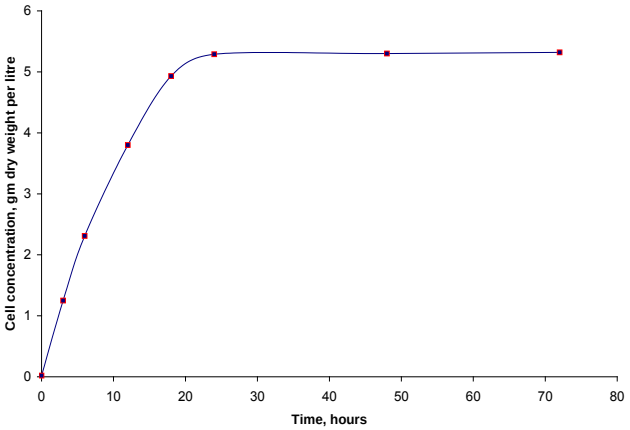

Figure 5 : Biomass cell concentration for Basal medium with 3% defatted cotton seed

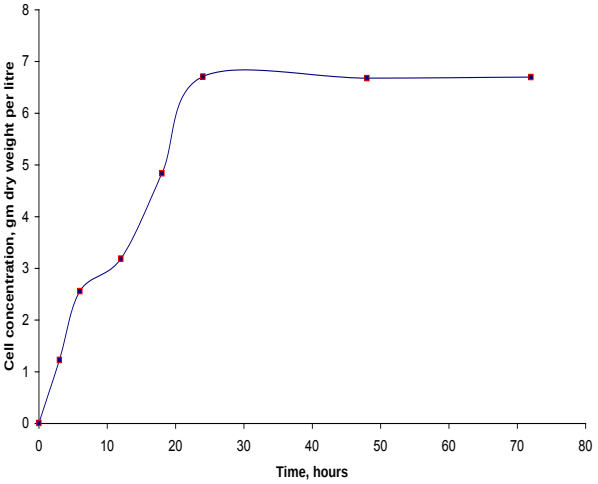

Figure 6 : Biomass cell concentration for Basal medium with 2% mustard seed

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 35oC, pH 7 and 300 rpm. The α–amylase produced from this Bacterial strain, *Bacillus lichenoformis* was quite active even at 1000C, however it showed optimum activity at 90oC, 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 cell mass concentration and the enzyme activity were determined and optimized. The low cost medium which contains, Defatted soya flour, Defatted cottonseed, and Mustard seed, produces two

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.

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