Screening of bacteria producing amylase and its immobilization: a selective approach – By Debasish Mondal

Article Summary (In short - What is your article about – Just 2 or 3 lines)	*Bacillus* sp produce amylase, so a selective approach was made to ease the screening of such stains from soil. Attempt was made to enhance the product by whole cell immobilization.
Category:	Microbiology

Your full article (between 500 to 5000 words) - - Do check for grammatical errors or spelling mistakes

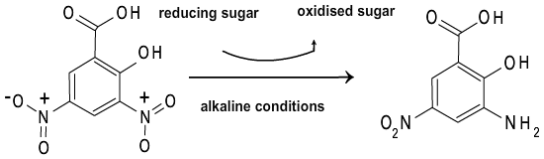
Screening of bacteria producing amylase and its immobilization: a selective approach –

Experiment-1: Preparation of standard curve of Maltose

Theory: Maltose is a disaccharide made up of two subunits of glucose monomers. Maltose is a reducing sugar.

Constructing a standard curve graph for maltose helps us to estimate concentration of reducing sugars present in an unknown sample and for determining the activity of amylase enzyme in forthcoming experiments. The standard curve for maltose is usually constructed using 3, 5-Dinitro salicylic acid (DNS) as the reagent.

Maltose reduces the pale yellow coloured alkaline 3, 5-Dinitro salicylic acid (DNS) to the orange- red coloured, 3 amino, 5 nitro Preparation of reagent:



This intensity change in colour is measured using a colorimeter as the absorbance at 540nm wavelength. A series of solutions containing varying concentrations of maltose are prepared in test tubes and a known quantity of DNS is added to each. These test tubes are then heated on a water bath for few minutes and their optical densities are measured using a colorimeter. A graph is then plotted with amount of maltose on X axis and the observed optical density at Y axis. The plot thus obtained is called a standard maltose curve.

Preparation of reagents:

DNS reagent: 0.25 gm. of DNS is dissolve in 5ml distilled water which already contains 0.4gm of NaOH. To this is added a solution of Sodium- potassium tartrate (7.5gm in 12.5 ml distilled water). The final volume is made to 25 ml adding about 7.5ml distilled water.

2N NaOH: prepared dissolving 1.2gm of NaOH in 30 ml of distilled water.

Maltose working solution: 100mg of maltose is dissolve in 100 ml water in a volumetric flask.

To make a final conc of 1000µg

Materials required: Glassware's (tubes and pipettes)

Spectrophotometer and water bath

Procedure:

1) Pipette out 0.1ml, 0.2ml ……. to 1ml in separate tubes. Keeping a blank tube.
2) Using distilled water bring the final volume to 1ml.

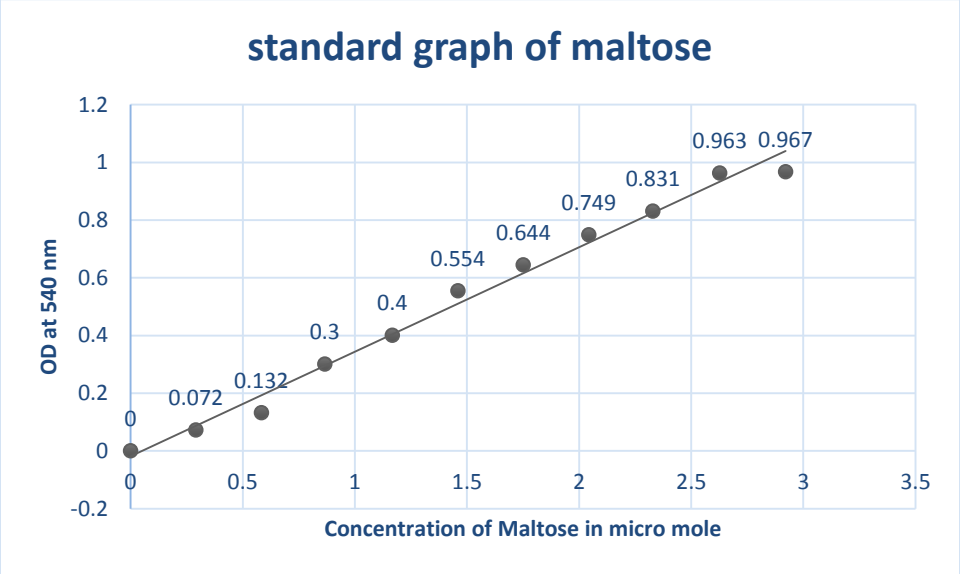
Test tube no	Conc of maltose In µgm	Conc of maltose in µ mole	Volume of Maltose solution	Volume of distilled water added	Volume of DNS added	Heating in boiling water bath for 10 minutes and then cool the tubes	Add 2N NaOH	Take OD at 540 nm
Control	0µg	0	0ml	1.0 ml	1ml		2.5ml	0.00
T1	100 µg	.292	0.1ml	0.9 ml	1ml		2.5ml	0.072
T2	200 µg	.584	0.2ml	0.8 ml	1ml		2.5ml	0.132
T3	300 µg	.867	0.3ml	0.7 ml	1ml		2.5ml	0.300
T4	400 µg	1.168	0.4ml	0.6 ml	1ml		2.5ml	0.400
T5	500 µg	1.46	0.5ml	0.5ml	1ml		2.5ml	0.554
T6	600 µg	1.752	0.6 ml	0.4ml	1ml		2.5ml	0.644
T7	700 µg	2.044	0.7 ml	0.3ml	1ml		2.5ml	0.749
T8	800 µg	2.33	0.8 ml	0.2ml	1ml		2.5ml	0.831
T9	900 µg	2.628	0.9 ml	0.1ml	1ml		2.5ml	0.963
T10	1000µg	2.921	1.0 ml	0ml	1ml		2.5ml	0.967

3) Add 1ml DNS reagent to each tube and cover the test tube with aluminum foil.
4) Heat the contents in boiling water bath for 10 minutes and then cool.
5) Add 2.5 ml of 2N NaOH to each tube and then measure the Optical density at 540 nm.
6) Record the data and plot the graph with amount of maltose in µ mole on X axis and OD along Y axis.

Observation:

Molecular weight of maltose is 342.3gm mole

Table:1



Expt : 2 Isolation of Amylase producing microorganisms from soil

Principle

Amylase is an enzyme that degrade starch, a polysaccharide into shorter polysaccharide namely dextrin, and ultimately into maltose.

Isolation of amylase producing *Bacillus* sp from soil is done using prior heat treatment of the soil sample

to select only the endospores .A basal medium supplemented with starch, which serves as the only carbon source for the microorganism.

The detection of the hydrolytic activity following the growth period is made by performing the starch test using iodine which produces a blue black color in the case of non-producer and a clear zone in case of producer.

Requirements

Soil samples,

Sterile distilled water,

Sterile petri dish, pipettes and tubes

Starch agar (Peptone.5.0gm, Beef extract3.0gm, Starch.2.0 gm., Agar15gm, DW1lit)

Iodine solution.

Spirit lamp, inoculating loop.

Procedure

Preparation of media

Starch agar media were prepared, autoclaved, poured in petri plates and were allowed to solidify.

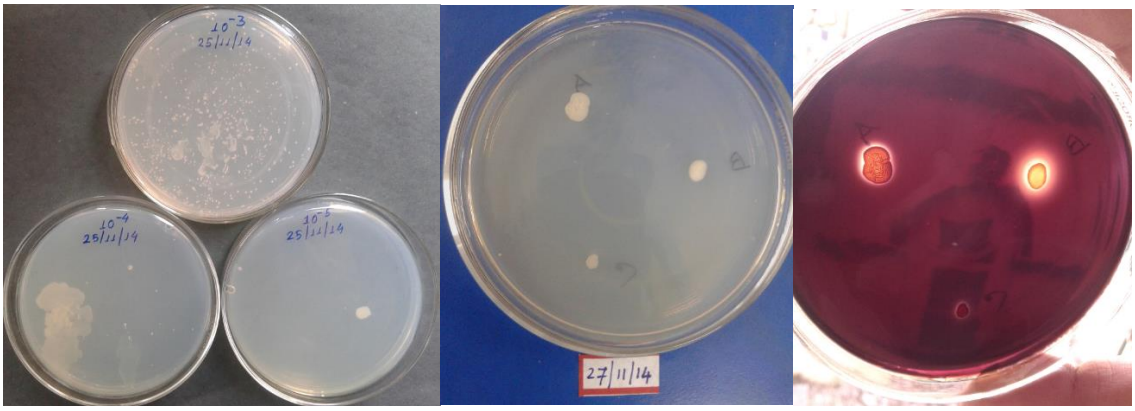
Serial dilution of soil sample and plating

1) 1gm sample (dried soil) each was taken in a conical flask. It was dissolved in 10 ml sterile water separately using magnetic stirrer

2) The suspension was boiled for 20 minutes to kill the vegetative cells. Only the spores will survive.

3) The soil sample was serially diluted to 10^{-5} dilution, and then 0.1 ml of inoculum from 10^{-3} , 10^{-4} and 10^{-5} dilutions were spreaded on the starch agar plate.

4) The petri dishes were incubated at 37^{0}C for 48 hrs.



Plates showing the isolates and their amylase activity in diameter.

Observation

Colonies were found growing in the starch agar plates. Such colonies were subculture in fresh starch agar slants and numbered. After sub culturing the plates were flooded with iodine solution and kept for few minutes.

Clear halo were observed around amylase producing colonies.

The amylase producing colonies were marked and were freshly inoculated in the center of the fresh Starch agar plates and the diameter of the halo were measured after 48hr of incubation and flooding with iodine solution. The diameter of the halo gave a quantitative estimation of the amylase produced.

Results

Results are tabulated as below

Table:2

Sample	Treatment	Dilutions plated	No of colonies	Isolate no producing amylase and respective diameter of the halo	
Soil sample	20 minutes in Boiling water bath	10^{-3}	TNTC	A	16mm
		10^{-4}	8	B	18mm
		10^{-5}	1	C	6mm

Note: TNTC (two numerous to count)

Result: Isolate no A, and B were found to be best amylase producer from halo diameter measurement after iodine treatment.

Precautions

Subculture or replica plating should be made before flooding the plates with the iodine.

Experiment:3

Immobilization of cell preparation, fermentation and amylase estimation

Aim To study Amylase producing microorganisms isolated from previous experiment along with a reference *B. subtilis* culture were entrapped in alginate gel and production of extracellular alpha amylase and compare it with that of free cells.

Principle

Immobilization can be defined as the process whereby the movement of enzymes, cells, organelles etc. in space is completely or severely restricted. Entrapment is chiefly used for the immobilization of cells and commonly polyacrylamide, collagen, cellulose acetate, calcium alginate or carrageenan is used as the matrix.

Alginate a polysaccharide (containing B-D-mannopyranosyl uronate & -L- glucopyranosyl uronate in regular (1-4)linked sequences in the presence of divalent cations, especially calcium forms gel. Since gel formation can take place under mild condition, entrapment in this matrix is very suitable for immobilization of viable cells.

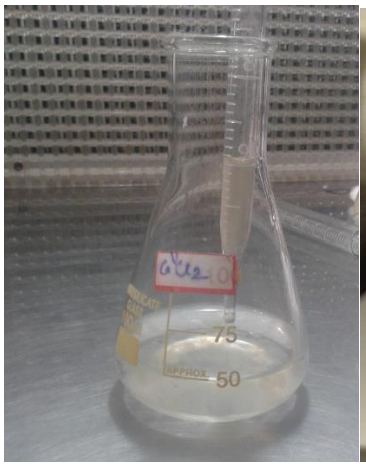
Amylase producing microorganisms isolated from previous experiment along with a reference *Bacillus subtilis* culture were entrapped in alginate gel and production of extracellular alpha amylase were studied and compared with that of free cells.

Requirements:

- LB medium containing 1%w/v Tryptone, 0.5%w/v yeast extract and 0.5% w/v NaCl.
- Fermentation medium contain 0.01%w/v NaCl, 0.02%w/v $(NH_4)_2 SO_4$, 0.05%w/v $MgSO_4$, 0.0075%w/v $CaCl_2$ and 2%w/v Tryptone.
- 4%w/v Na- alginate.(this becomes 2% when diluted with culture)
- 3.5%w/v $CaCl_2$
- 0.9%w/v NaCl solution.
- Phosphate buffer: 0.1 M Na- Phosphate buffer of pH6.5.

68.5ml of 0.2M each ofNaH_2PO4 and 31.5ml of 0.2M Na_2HPO4 and 100ml water was mixed to obtain 0.1M Na-Phosphate buffer pH6.5.

- Substrate: 0.5% Starch in Na- Phosphate buffer.
- DNS reagent: 0.25 gm. of DNS is dissolve in 5ml distilled water which already contains 0.4gm of NaOH. To this is added a solution of Sodium- potassium tartrate (7.5gm in 12.5 ml distilled water). The final volume is made to 25 ml adding about 7.5ml distilled water.
- Conical flasks, pipettes etc.



Procedure

Inoculum preparation

The isolated strains and the reference strain were cultivated at 37^0C on a rotary shaker for 24 hrs. in LB medium containing 1%w/v Tryptone, 0.5%w/v yeast extract and 0.5% w/v NaCl.

Immobilization

5ml of the cell suspension (0.5-0.6OD) were mixed with 5ml of 4% sterile Na- alginate and the mixture were extruded drop wise through a pipette into 25ml 0f 3.5% $CaCl_2$, the beads thus formed were kept for 30 minutes to allow hardening.

Beads were washed in 0.9% saline to remove excess of Ca^{2+}.

Fermentation

Fermentation media 100 ml distributed in conical flasks (250ml capacity) were sterilized. And immobilized cells prepared in the above manner were added in two of flasks (two with isolated strains separately and the other with the reference strain) .For free cell culture the other 100ml medium were inoculated with same no of cells (5ml of the broth of reference and the other with 5ml of isolated strain.)

Fermentation were carried out at 37^0C for 48 hrs in a shaker at 180 rpm

Determination of alpha Amylase activity

Amylase activity were determined after separating the free cells or the beads by centrifugation at low speed 5000rpm.

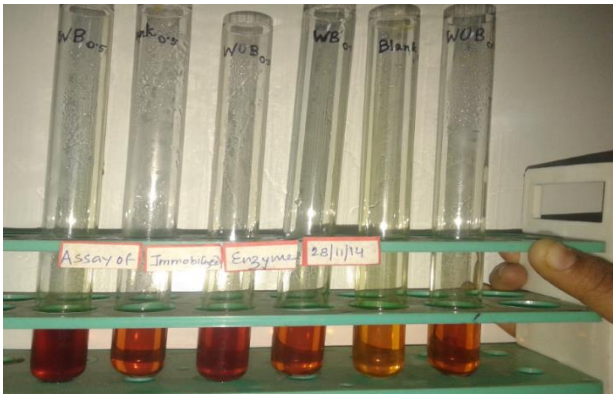
The cell free extract were treated as source of enzyme in the reaction mixture and the reagents were added according to following table shown.

Table:3

Sample type	Tube No	Substrate Starch in buffer	Enzyme soup	Incubation For 15 minutes	2N NaoH	Enzyme soup	DNS	Heat all tubes in boiling water bath for 10 minutes and then cool the tubes and take OD at 540 nm
Blank	T_0	1ml	0 ml		2ml	0.5ml	1ml	
Free cells isolate no A	T1	1ml	0.5ml		2ml	0ml	1ml	
Immobilized cells isolate no A	T2	1ml	0.5ml		2ml	0ml	1ml	
Free cells isolate no B	T3	1ml	0.5ml		2ml	0ml	1ml	
Immobilized cells isolate no B	T4	1ml	0.5ml		2ml	0ml	1ml	
Reference Free cells *B.subtilis*	T5	1ml	0.5ml		2ml	0ml	1ml	
Reference Immobilized cells *B.subtilis*	T6	1ml	0.5ml		2ml	0ml	1ml	

2N NaOH was added after incubation period to stop the reaction.

After addition of DNS the tubes were boiled for 10 minutes .Absorbance were measured against the blank at 540 nm.



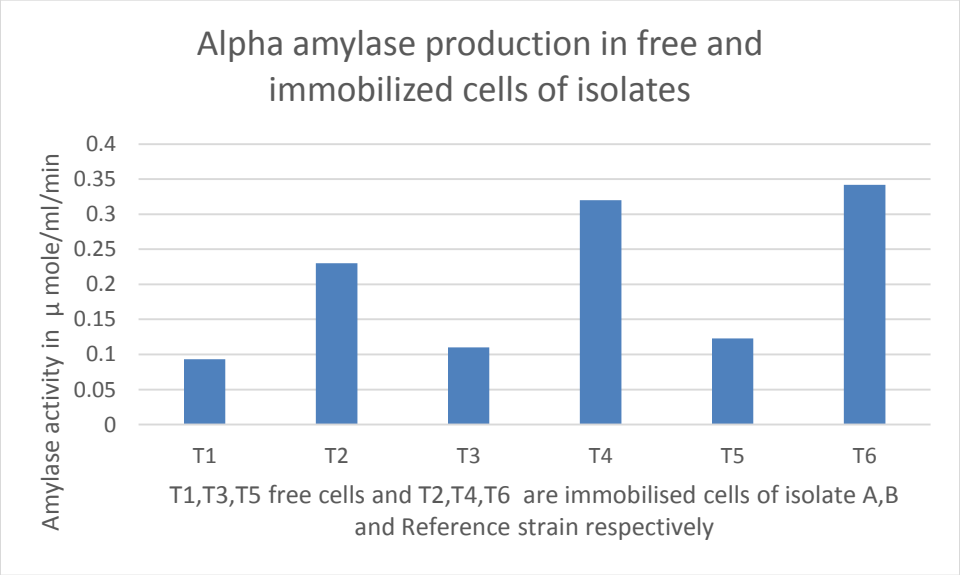
Observations: Table :4

Tube No	OD at 540 nm	Product formed in micro mole /0.5ml enzyme soup	Product formed in micro mole /1ml enzyme soup	Product formed µmole/ml/minute
T_0	0.00	0	0	0

T1	0.257	0.695	1.395 µmole/ml	0.093 µmole/ml/minute
T2	0.637	1.725	3.45 µmole/ml	0.23 µmole/ml/minute
T3	0.304	0.825	1.65 µmole/ml	0.11 µmole/ml/minute
T4	0.901	2.4	4.8 µmole/ml	0.32 µmole/ml/minute
T5	0.341	0.9225	1.845µmole/ml	0.123 µmole/ml/minute
T6	0.941	2.565µmole	5.13 µmole/ml	0.342 µmole/ml/minute

Observations & Results

Observations were tabulated in Table B and histogram was drawn to compare production of amylase by various isolates and reference strain (free and immobilized cells).



Histogram comparing the isolates production of enzyme in free and immobilized state and their comparison with the reference strain.

Results and Interpretation

In reference as well as in the isolated strain the entrapped calcium alginate capsulated cells or immobilized cells were found to produce more amylase than that of the free cells. Moreover the isolated strain B was found to be the best producer among the isolates but it is still less than the compared reference strain in amylase activity. It is possible that the reference strain may be improved upon for its amylase activity by optimizing its various growth parameters and even by physical or chemical mutagenic treatment.

Precautions

1.Care is to be taken while making Na- alginate suspension by slowly heating and then it was autoclaved.

2.Pipette distance and flow should be adjusted to form good size uniform beads.

3.At least 30 minutes curing time in $CaCl_2$ is necessary.

References (if any)

1. Konosoula Z.,Liakopulou Kyriakides M. Thermostable α-amylase production by *Bacillus subtilis*

entrapped in calcium alginate gel capsules. Enzyme and Microbial Technology **39** (2006)690-696.

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