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Microbial Production of Protease: Isolation and Optimization

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Abstract: ***The microbial production of protease is the need of rapidly growing industrial field. So due to its wide utility protease production becomes the centre of attraction for commercial market. The present study emphasized on microbial production and optimization of protease enzyme. The potential protease production depends on effective strain selection, for this purpose we took different samples of stagnant water from different locations. Then we isolated the single colony of protease producing microorganism from it, through repeated sub- culturing. The presence of protease enzyme in the microbial culture was checked by skimmed milk assay followed by protease assay. Then the effective strain is selected for the optimization. The protease enzyme was optimized in various carbon sources such as glucose, starch, molasses, and glycerol for potential protease production.***

Keywords: Alkaline protease, isolation, optimization, characterization, future scope of proteases.

1. Introduction

Protease refers to the group of enzyme whose catalytic function is to hydrolyze peptide bonds of proteins. Proteases are the degradative enzymes, which catalyze the total hydrolysis of protein. Advance in analytical techniques have demonstrated that protease conduct highly specific and selective modification of protein such as activation of zymogenic form of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secretory protein across the membrane. The current estimated value of worldwide sales of industrial enzyme is $1 billion. Of the industrial enzyme, 75% are hydrolytic. Protease represents one of the three largest groups of the industrial enzyme and account of about 60% of the total worldwide sale of enzyme. Protease executes a large variety of functions, extending from cellular to organ and organism level, to produce cascade system such as hemostasis and inflammation. They are responsible for the complex processes involved in the normal physiology of the cell as well as in abnormal pathophysiological conditions. Their involvement in the life cycle of disease causing organism has led them to become a potential target for developing therapeutic agents against fatal diseases such as cancer and AIDS. Protease has a long history of application in food and detergent industries. Their application in leather industry for dehairing and bating of hides to substitute currently used toxic chemicals is a relatively new development and has conferred added biotechnological importance. The vast variety of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempt to exploit their physiological and biotechnological applications.

2. Materials and Methodology

a) Methodology

1) Collection of sample
2) Inoculum preparation
3) Repeated sub culturing
4) Single colony isolation of protease producing strain
5) Biochemical characterization of protease enzyme
6) Optimization of various assay conditions

b) Collection of sample

For the large scale production of protease it is essential to collect the potential microbial strain from various suitable sources. The main source of protease producing microbial strains includes: - stagnant water from sewages or from the drain near a place where butcher work has been done. Three samples were taken:

1) PI from "Abu Ka Naala", Kankar khera, Meerut Cantt.
2) P2 sample is Sewage Water from Municipal drain, Shraddhapuri colony, Meerut Cantt.
3) P3 from a drain near the Butchery, Lal Kurti, Meerut City.

c) Inoculum preparation

The three samples were filtered through Watmann's filter paper and 10 ml of filtrate from each sample was taken. The 10 ml of filtrate were divided into two parts of 5 ml each and then they were transferred into Erlenmeyer flask (250 ml) containing 50 ml of sterile Inoculum media for the growth of protease producing microbial strain.

d) Media composition

The Inoculum media consists of the following nutrients

Constituents	*Quantity (gm/100ml)*
Starch	1
Casein	0.5
KNO3	0.2
NaCL	0.2
K2HPO4	0.2
MgSO4.7H20	0.005
FeSO4.7H20	0.001
CaCO3	0.002
DH2O	100

3. Method

The inoculum media which is 100 ml in composition was then divided into two parts of 50 ml each. The prepared media was steam sterilized at a pressure of 15 psi, 121 ° C for 15 minutes. The sample was then inoculated after the

cooling of media for 15-20 minutes and then was kept on a rotary shaker at 180 rpm at 55° C for about 24 hrs. After 24 hrs the growth of microbial strain is checked and the strain which was obtained was then cultured on the Agar media having the following composition:-

Constituents	*Quantity (gm/100ml)*
Beef extract	0.1
Yeast extract	0.2
Peptone	0.5
NaCI	0.5
Agar	1.5
DH2O	100

The Petri dish containing the cultured strain was then put into the incubator for about 48 hrs at 37° C. After 48 hrs the growth was checked and strains so obtained was then further sub cultured to get desired strain from the mixed colony of the different microbial strains for the production of protease enzyme.

3.1 Repeated Sub culturing

Repeated sub culturing is performed to get the desired strain out of the mixed colonies of the microbes for the production of the protease enzyme. The sub culturing is performed in the media used for the culturing of the sample i.e. Agar medium. The sub culturing is performed carefully under aseptic condition in laminar flow to avoid any type of contamination.

3.2 Single colony isolation

The serial dilution method is performed for single colony isolation from mixed colonies of microbes to get the desired strain which is used in the maximum production of protease enzyme. The serial dilution method was performed as follows:-

Firstly 10 test tubes were selected in which we added 9 ml of distilled water. Then in the first tube we added 1 ml of microbial culture and mix it thoroughly. From the first tube we transferred 1 ml of the culture mixture to the second test tube and so on till the last test tube. In this way we got the series of concentrations as 10^{-1} to 10^{-10}.

After that we selected 10^{-10} microbial concentration to be used for the further sub culturing of the strain. After 48 hrs of growth in Petri dish put into an incubator we got the isolated colonies of microbes. From these colonies we picked the single colony for further sub culturing.

3.3 Biochemical characterization

The isolated single colony was further sub cultured in a medium containing a film or layer of Skimmed milk to check the protease activity. This was kept for about 72 hrs in an incubator at 37° C. The activity was checked after 72 hrs, it was found that the layer of Skimmed milk was degraded by the enzyme produced by the microbial colony. This confirmed the enzyme produced by the microbes was protease. Now further assay is done to check the desired protease level.

3.4 Assay of Enzyme Activity

Casein (1.0%) was dissolved in buffer (pH 8.0) by heating at 70° C. 2 ml of casein solution was mixed with 0.5 ml of culture supernatant and incubated at 45° C in water bath for 50 min. The reaction was terminated by adding an equal volume of 10 % TCA followed by 10 min holding time. Suspension was filtered through Watmann's filter paper. To 1 ml of filtrate 5 ml of 0.5 $Na2CO_3$ solutions and 0.5 ml of 3-fold diluted folin reagent were added and mixed thoroughly. The colour development after 30 min of incubation at 30° C was measured as O.D at 660nm with blank as reference.

One unit (EU) of enzyme activity was defined as the amount of enzyme required to liberate 1 p.tg of tyrosine / min under assay conditions.

Sample	Measured O.D
P1	1.301
P2	1.2491
P3	1.3798

The maximum protease activity was found in the sample P3, so sample P3 was selected for optimization of various conditions for maximum yield of the enzyme protease.

3.5 Optimization of various assay conditions

The optimization of sample P3 was done using various assay conditions to check the maximum yield of protease enzyme in different sources. The different assay conditions included:-

1) *Various Carbon Sources
2) Various Nitrogen Sources
3) Different Temperature Range
4) Different pH Conditions

*We have mainly concentrated on the various carbon sources only due to limited time.

3.6 Optimization using various Carbon sources

The various carbon sources included Glucose, Molasses, Starch and Glycerol.

Method: The common inoculum media is taken in four Erlenmeyer flask (250ml) having altered carbon sources. The common media composition containing altered carbon sources is as follows: -

Constituents	*Quantity (gm/100ml)*
*Carbon Source	1
Starch	1
Casein	0.5
KNO3	0.2
NaCL	0.2
K2HPO4	0.2
MgSO4.7H20	0.005
FeSO4.7H20	0.001
CaCO3	0.002
DH2O	1000

*__Altered carbon sources-__ Glucose, starch, Molasses, and Glycerol.
The four flasks containing the above medium with different carbon sources were inoculated with single isolated colony from the sample P3. The flasks were then incubated for 72 hrs at 37° C on a rotary shaker at 180 rpm for the selection of best carbon source with maximum protease production. After the selected time period the growth in different carbon sources were checked and further enzyme assayed was performed to check the maximum enzyme activity in the four flasks containing the carbon sources.

__Assay of Enzyme Activity__:- Casein (1.0%) was dissolved in buffer (pH 8.0) by heating at 70° C. 2 ml of casein solution was mixed with 0.5 ml of culture supernatant and incubated at 45° C in water bath for 50 min. The reaction was terminated by adding an equal volume of 10 % TCA followed by 10 min holding time. Suspension was filtered through Watmann's filter paper. To 1 ml of filtrate 5 ml of 0.5 Na_2CO_3 solutions and 0.5 ml of 3-fold diluted folin reagent were added and mixed thoroughly. The colour development after 30 min of incubation at 30° C was measured as O.D at 660nm with blank as reference.

One unit (EU) of enzyme activity was defined as the amount of enzyme required to liberate 1 µg of tyrosine/min under assay conditions.

Carbon sources	Measured O.D
*Glucose	1.5808
Starch	1.3214
Molasses	1.2786
Glycerol	1.1827

* From the following data it was concluded that the best carbon source is **Glucose** having maximum protease production.

4. Result and Discussion

From the previous data obtained we can now calculate the **#Enzyme Activity (EA**) using the following formula:

EA = $(OD_{samp} - OD_{con})$*DF/V_EIT

Where, EA =Enzyme Activity
OD= OD of Sample
OD= OD of control
DF= Dilution Factor (1.00)
V_E= Volume of enzyme (0.5ml)
IT = Incubation Time (30min)

#Enzyme Activity is measured in **Enzyme Unit** (**EU**). One EU of enzyme activity may be defined as as "**The amount of enzyme required to liberate 1 µg of tyrosine / min under assay conditions".**

__Calculations__

1) From the above formula we calculated the Enzyme Activity of the sample strains PI, P2 and P3 and we got the following result:

Sample strains	Enzyme Activity(EA)
P1	0.087EU
P2	0.084EU
P3	0.092EU

* From the above calculations we plotted a graph of enzyme activity v/s Sample strain.

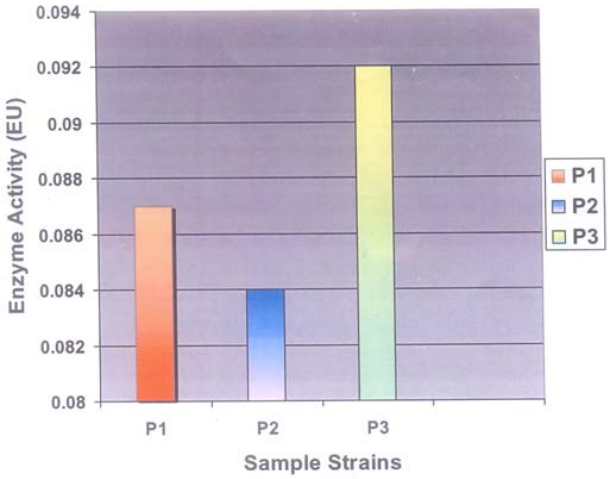

Figure 1: Enzyme activity versus sample strains

- The above plotted graph shows enzyme activity measured in enzyme unit (EU) v/s the different sample strains taken.
- The graph shows the maximum activity of strain P3 which is suited best for the production of protease enzyme.

2) In the similar way we calculated the Enzyme Activity in different Carbon Sources used for the production of protease enzyme and got the following result:

Carbon sources	Enzyme activity
Glucose	0.1054EU
Starch	0.0880 EU
Molasses	0.0852 EU
Glycerol	0.0789 EU

* From the above data we plotted a graph of Enzyme Activity v/s Carbon Sources used.

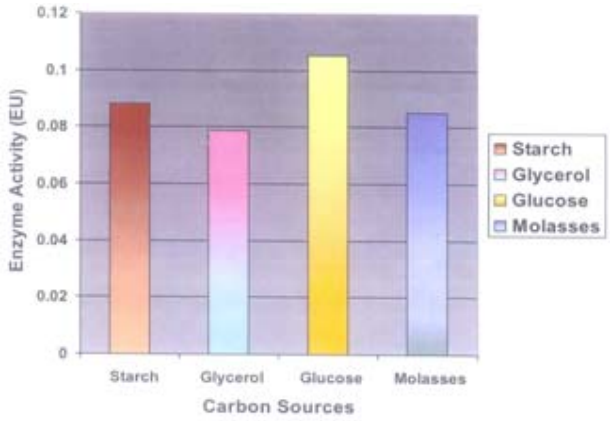

Figure 2: enzyme activity versus different carbon sources

- The above plotted graph shows enzyme activity measured in enzyme unit (EU) v/s the different Carbon sources taken.

- The graph shows the maximum activity of protease enzyme in glucose which is suited for the production of protease enzyme.

5. Conclusion

The maximum protease activity was found in the glucose, suggesting that glucose is the best carbon source for the effective protease production. In future we like to work on some other factor of the related topic.

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