Optimization of growth and production of protease by *Penicillium* species using submerged fermentation

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Abstract- Enzymes play a vital role in the industry with a wide range of application. Agricutural waste is the maximum waste been produced in India. This Project aims at using various agricultural wastes for the production of Protease. To achieve this various research is being conducted. Various Bacterial and Fungal species are being used for the production of Protease. *Penicillium* species was being used in this work for the production of Protease .In this work the various components of the media were studied and were optimized and used for the production of Protease.

Introduction

Proteases are primarily considered as enzymes of digestion and are one of the largest and most diverse families of enzymes known. They are classified under Group III (Hydrolases) and the fourth sub-group according to nomenclature Committee of International Union of Biochemistry and Molecular Biology (IUBMB). Proteases catalyze the addition of water across amide bonds to effect cleavage using a reaction involving nucleophilic attack on the carbonyl carbon of the scissile bond. The exact mechanisms of cleavage and the active site substituents vary widely among different protease [15]. Protease has wide application in the industry. Protease covers 60 % of the market value in the Industry. Proteases are primarily considered as enzymes of digestion and are one of the largest and most diverse families of enzymes known. Protease is found in all forms of organisms regardless of kingdom. Some examples include the plant proteases like papain of papaya and bromalein of pineapple, trypsin, chymotrypsin, renin and pepsin are few of the animal and human digestive proteases [7]. Proteases of bacteria, fungi and viruses are increasingly studied due to its importance and subsequent application in industry and biotechnology [16]. Microbial alkaline proteases dominate the worldwide enzyme market, accounting for a two-thirds share of the detergent industry [18]. Commercial application of microbial proteases is attractive due to the relative ease of large-scale production as compared to proteases from plants and animals. Proteases have application in the industry such as Leather, Detergents, Food Processing, Pharmaceutical industry, Proteases in the study of protein conformation and many more [21].

Materials and Method

Various parameters were studied during the work. Media optimization was the main area of interest.

Fungal species have an ability of using any kind of nutrient source for its growth.

Culture conditions: The culture was obtained from NCIM Pune in lyophilized form and the culture was grown on PDA and sub-cultured for 30 days.

Carbohydrates sources: A microorganism requires a lot amount of carbohydrate source for its production. Two different carbohydrate sources were used for the production of Protease.

1. Primary Carbohydrate Source.
2. Secondary Carbohydrate Source.

The primary carbohydrate sources were actually the chemical sources available at various laboratories. The secondary carbohydrate sources were the agricultural waste, which were used as a major carbohydrate source for the production of Protease.

The primary carbohydrate sources used was as follows:
Dextrose
Sucrose
Lactose

The secondary carbohydrate sources used were as follows:
Wheat Bran
Maize Bran
Rice Bran

These sources were altered with variations and studied accordingly .The concentrations were used according to the Eagles Basal medium.

The components of the Basal Medium (Production medium) were as follows:
Casein
Sodium Chloride
Dipotassium Phosphate
Bromothymol Blue
Dextrose, Sucrose, Lactose
Wheat Bran, Rice Bran, Maize Bran.

Nitrogen Sources: The other major component required for the production of microorganism is the nitrogen source. Organic sources such as Casein, Yeast Extract, Meat Extract and Inorganic Sources such as Ammonium Sulphate, Ammonium Bicarbonate, Ammonium Nitrite were studied in the production Medium.

pH: The pH of a production medium plays a vital role for the production of various products .In this Experiment a range of pH was studied and used for final production .The range of pH used was 6.6- 9.6 .

Temperature: The production medium was kept at various temperatures such as 26,27,28,29,30 on a shaker incubator and studied .

The final production medium was prepared and autoclaved at 15 lbs pressure for 15 mins . The medium was cooled and 2 ml of culture was transferred aseptically in the production medium. The flask was then transferred on a shaker incubator for 7 days. The Production medium was then filtered and used for the enzyme assay.

Enzyme Assay: The Standard protocol of Sigma Quality Control Department was used for the enzyme Assay. The pH of the assay was altered and checked. The best pH was being used for the enzyme assay.
100 mM Sodium Tetraborate
0.6% Casein in 10 ml. sodium tetraborate and 90 ml. distilled water
110 mM Trichloroacetic solution (50 ml.)
Trichloroacetic acid
Sodium acetate
Acetic acid

Table no 1:

Reagent	**Test**	**Blank**
Casein solution	3 ml.	3 ml.
Enzyme solution	0.5 ml.	-
Trichloroacetic acid solution	3.2 ml.	3.2 ml.
Enzyme solution	-	0.5 ml.

Dialysis Protocol: Various percentage of Ammonium Sulfhate was being for the precipitation of the enzyme sample.
35%
50%
75%
These concentrations were added in the enzyme samples and incubated at 4^0C for 24 hrs. The samples were removed and precipitated and used for the dialysis. The Dialysis bags were kept for further studies in a beaker consisting of 0.05 Tris-HCl buffers.

Ion Exchange Chromatography: The basic principle behind the use of the column preparation was to treat the enzyme sample with various concentration of salt solutions .This process actually helped with the removal of various bound and unbound proteins were removed. For the Ion Exchange Chromatography DEAE Cellulose was used for column Preparation.
The protocol for the column preparation is as follows:
2.5 gm of DEAE cellulose was prepared in 12 ml of 0.05 M Tris HCl and the beads were allowed to swell for 30 minutes.
The bottom of the syringe was packed with glass wool.
The matrix was poured into syringe column to the level of 7.5 cm and allowed to settle.
The pH of column was maintained at 8.0.
Column was always filled with buffer to avoid from drying the matrix .The column was used again for the separation of the proteins.

Observation and Results:
Carbohydrate Source: The best Carbohydrate Found in Combinations was used seeing the best results in the graph.

Table 2: Sample: Dextrose

Samples	Reading at 275 nm
Wheat Bran	0.5127
Maize Bran	0.6361
Rice Bran	0.6032
Control	0.2341

Table 3: Sample: Sucrose

Samples	Reading at 275 nm
Wheat Bran	0.7875
Maize Bran	1.5681
Rice Bran	1.3142
Control	0.0770

Table 4: Sample: Lactose

Samples	Reading at 275 nm
Wheat Bran	0.8134
Maize Bran	1.2341
Rice Bran	0.6832
Control	0.2561

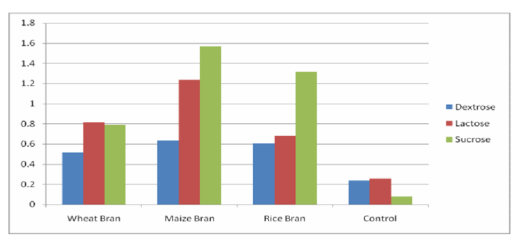

Fig: 1 Various Carbohydrate Sources

The graph here showed the best results with Maize bran and Sucrose in combination which was used for the Production of Protease.

Nitrogen Sources:
The best results were seen with Casein compared to Yeast extract and Malt extract. The

Graphical representation shows us the best Organic Nitrogen Source used for the production of Protease.

Table 5: Organic Nitrogen Sources

Organic Nitrogen Source	Readings at 275 nm
Casein	1.1075
Malt Extract	0.7843
Yeast Extract	0.6512

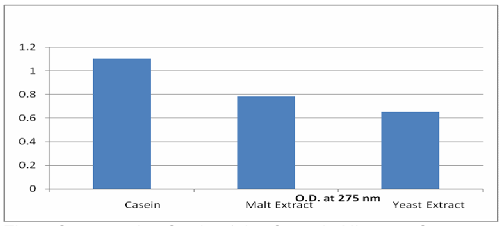

Fig 2: Comparative Study of the Organic Nitrogen Sources

Similarly when the Inorganic Nitrogen Sources were used to enhance the production of the enzyme it was observed and seen that the Inorganic Nitrogen Sources actually hampered the growth of the organisms. The comparison with the controlled production medium showed the difference.

Table 6: Inorganic Nitrogen Sources

Inorganic Nitrogen Source	Readings at 275 nm
Ammonium carbonate	0.3262
Ammonium sulphate	0.3507
Ammonium nitrite	0.3109
Production medium	1.1210

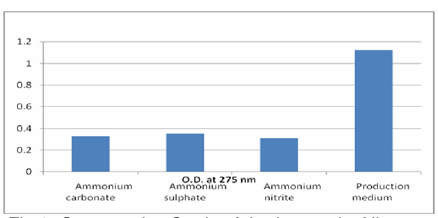

Fig 3: Comparative Study of the Inorganic Nitrogen Sources

pH:
The pH range used in this experiment was altered between 6.6 -9.6 and the best pH was studied and used for the production of protease.The readings clearly indicated that the best pH was 6.6 for the production of protease.

Table 7: pH Optimization

pH used	OD at 275 nm
6.6	1.6006
7.6	0.7896
8.6	0.6202
9.6	0.6581

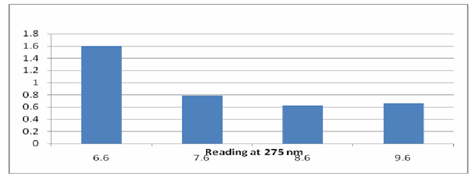

Fig 4: The various pH being studied and Results seen

Optimum Temperature:
The Temperature played a vital role and the best temperature used for the production of protease was seen at 27 and 30 respectively.
Table 8:

Temperature	OD at 275 nm
25	0.5491
26	0.8793
27	1.1134
28	0.8909
29	0.9801
30	1.1304

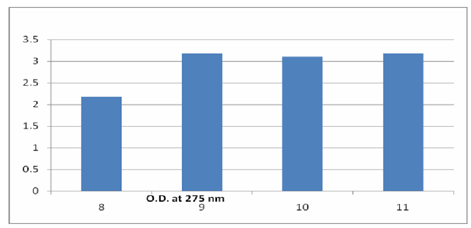

Fig 5: Temperature Optimization for the Production of Protease

Enzyme Assay:
The pH for the enzyme was best seen at 9 as compared to the other two pH respectively .The graphical representation shows the same result.
Table 9:

pH of assay	O.D. at 275 nm
8	2.1775
9	3.1786
10	3.0991
11	3.1786

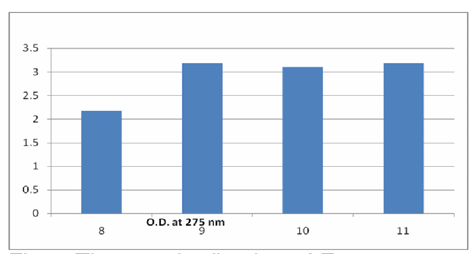

Fig 6: The standardization of Enzyme assay

Dialysis Results:
The supernatant samples obtained were added with 5 grams of ammonium sulfate and were kept at 4^0C for overnight. The samples which were removed after 24 hrs were checked whether these precipitate solutions formed. When the precipitate was formed the samples were centrifuged at 5000 rpm for 5 mins, and the

pellets were used and preserved with 0.05 M Tris – HCl.

Ion Exchange Results:
The samples of the partially purified pellets and the ammonium sulfate fractanionation samples were transferred on the column one by one. The column was then treated with four different solutions as follows:
Tris – HCL (0.05 M)
0.1 M NaCl + Tris – HCl
0.2 M NaCl + Tris – HCl
0.5 M NaCl + Tris – HCl
Table 10:

Samples	Tris–HCl(0.05M)	0.1MNaCl + Tris-HCl	0.2MNaCl + Tris-HCl	0.5MNaCl + Tris-HCl
1	0.0011	0.0030	0.0653	0.0098
2	-0.0045	-0.0065	0.0229	0.0831
3	-0.0020	-0.0059	0.0070	0.0275
4	-0.0059	-0.0093	0.0056	0.0181
5	-0.0090	-0.0116	0.0011	0.0115
6	-0.0186	-0.0072	-0.0010	0.0098
7	-0.0239	-0.0123	0.0018	0.0062
8	-0.0247	-0.0093	-0.0001	0.0140
9	-0.0277	-0.0126	-0.0024	0.0178
10	-0.0282	-0.0049	-0.0077	0.0209

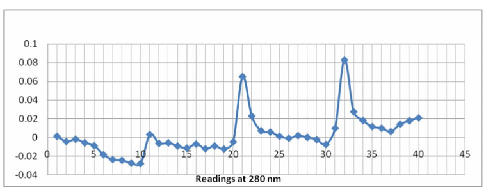

Fig 7 : Readings of Ion Exchange Chromatography

SDS PAGE:
The Samples with the highest O.D. readings were used for inoculating on the SDS –PAGE. The Molecular weight of the Protein was found to be 33KDa .The samples used for the inoculation on the PAGE are mentioned below:
0.1381
0.1030
0.1074
0.1980
0.0452
0.0732

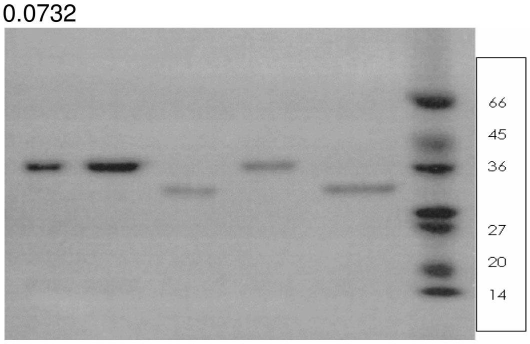

Fig 8 : SDS –PAGE

Protein Estimation :
The readings of protein samples at 280 and 260 are described as below.

Table 11:

Sample no .	O.D. Reading at 280 nm.	O.D. Reading at 260 nm.
1	0.1381	0.0834
2	0.0452	0.0472
3	0.0732	0.0607
4	0.0332	0.0198
5	0.1030	0.2341
6	0.1074	0.1826
7	0.1980	0.2968

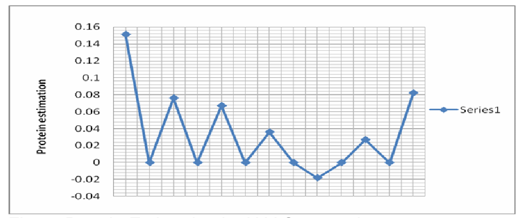

Fig 9 : Protein Estimation by U.V.Spectrophotometer

Discussion
With the markets increasing demand for the protease enzyme the supply has to be increased immensely. With the reports clearly indicating that the market value about 6.7 billion US $ in the recent years and their would be a tremendous increase in the production , in the next few years .Microbial protease can be clearly stated as the most important enzyme in various industries such as detergents , dyes , leather industry with many medicinal applications as well. Although the protease production is done by various sources such as plants animals fruits the protease from the microbial source is considered as the most efficient. Shake flask studies: Optimizations of cultural condition for growth were studied for 7 days, along with the optimization of production parameters. *Penicillium* Species showed maximum growth at pH 6.6 as compared its growth at other various respective pH range varying from pH 6.6 to pH 9.6. During growth studies, the maximum biomass or cell mass was obtained in between 4th day to 6th day. The production of Protease from *Penicillium* species is greatly influenced by initial culture pH. The optimum pH for production of Protease by *Penicillium* species was 6.6. The other parameter, which played a vital role was the Temperature. Though a particular range of temperature was studied the best results were seen at 30^{0}C. Earlier studies have stated that the optimum temperature for the production of Protease from *Penicillium* species was found out to be 27^{o}C [2]. The main source studied was the carbon sources i.e., the primary source and the secondary source where the best results were seen Sucrose and Maize Bran. Though the other source which could be used in place of Maize Bran was Rice Bran which was the second with the best results. The next parameter studied was

the use of Nitrogen Sources for the production was organic Nitrogen sources and Inorganic Nitrogen sources where various materials were used which could actually help in increasing the growth. The best Organic Nitrogen source was Casein whereas the case was completely different in the use of Inorganic Nitrogen source, Ammonium sulphate, Ammonium carbonate, Ammonium nitrite could increase neither the growth rate nor the productivity but they actually hampered the growth of the organism. So in the entire Experiment the Inorganic Nitrogen sources were never used .The total soluble extracellular protein content of the fermentation medium was estimated using the spectrophotometer method of protein estimation. It is important to note that, the total soluble extracellular protein content estimated using spectrophotometer method in this study, essentially, signifies the quantification of all the proteins present in the sample inclusive of our protein of interest. With respect to the different pH ranges Temperature, Carbon Sources and Nitrogen Sources being assayed for production, the highest amount of total extracellular soluble protein content was found to be at pH 6.6, and was calculated to be 0.151 mg/ml.

References

[1] Hameed A.C., Natt M.A. and Evans C.S. (1996) *World J. Microbiol. Biotech*. 12,289-291.

[2] Ito S., Kobayashi T., Ara K., Ozaki K., Kawai S., Hatada Y. (1998) *Extremophiles*, 2,185-190.

[3] Mohsen Fathi Najafi, Dileep Deobagkar, Deepti Deobagkar. (2005) *Electronic J. of Biotechnology*, 8, 2.

[4] Ishikawa H., Ishimi K., Sugiura M., Sowa A. and Fujiwara N. (1993) *Journal of Fermentation and Bioengineering*, 76, 4, 300-305.

[5] Gupta R., Beg Q.K., Khan S., Chauhan B. (2002) *Applied Microbiology and Biotechnology*, 60,381-395.

[6] Hossain M.S., Iqbal M.S. and Hoq M.M. (2004) *Bangladesh J. Microbiol*, 21,86-89.

[7] Bradford M. (1976) *Analytical Biochemistry* 72, 248-254.

[8] Stanbury P.F, Whitaker A., Hall J.S. *Principles of Fermentation Technology, 2'nd edition, Pergamon press*, 14, 167-272.

[9] Aneja K.R. *Experiments in Microbiolog, plant pathology, tissue culture and Mushroom production technology, 3'rd edition, New Age International Publishers, New Delhi*, 357-358.

[10] Kannan N., *Handbook of Laboratory Culture Media, Reagents, Stains and Buffer, Panima Publishing Corporation New Delhi/Bangalore,* 126-127.

[11] Rao M. B., Tanksale A. M., Ghatge M .S. and Deshpande V. V. (1998) *Microbiol. Mol Biol. Rev*. 62, 597-635.

[12] Friedrich J., Gradisar H., Mandin D. and Chaumont J. P. (1999) *Lett. Appl. Microbiol* 28,127-130

[13] Boyer P. D. (1971) *The enzymes, 3rd ed. Academic Press, Inc., New York, N.Y.*

[14] Hoffman T (1974) Adv. Chem. Ser. 136, 146– 185.

[15] International Union of Biochemistry. (1992) Enzyme nomenclature. Aca-demic Press, Inc., Orlando, Fl

[16] Barett A. J. (1994) *Methods Enzymol*. 244, 1– 15.

[17] Menon A. S. and Goldberg A. L. (1987) *J. Biol. Chem*. 262, 14929– 14934.

[18] Argos P. (1987) *J. Mol. Biol.* 193, 385–396.

[19] Rawlings N. D. and Barrett A. J. (1993) *Biochem. J.* 290, 205– 218.

[20] Cerny G. (1978) *Eur. J. Appl. Microbiol. Bio-technol*, 5, 113– 122.

[21] Barrett A.J., Rawlings N.D. and Wessner J. F. (eds). (1998) *Handbook of proteolytic enzymes, Academic Press*.

