Optimization of process parameters for production of protease by Aspergillus oryzae from Kitchen waste

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MAY– 09

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CERTIFICATE

This is to certify that the work entitled, "**Optimization of process parameters for production of protease by Aspergillus oryzae from Kitchen waste** " submitted by Puneet Agarwal (051564) and Anubhav Agrwal (051552) in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

25/05/09

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ACKNOWLEDGEMENT

We acknowledge the presence of Almighty GOD for his kind benedictions and blessing and his unseen hand over our head to make us feel that HE is always with us.

We are highly indebted to Dr R.S. Chauhan , Honorable HOD, Department of Biotechnology, Jaypee University Of Information Technology Waknaghat for giving us support to carry out work in Lab.

With a deep sense of gratitude we express our heartiest veneration and gratitude to Runni Mukherjee for her benevolent and thoughtful guidance , keen interest, patience and immense help during the course of our work.

A special thanks to all the Scholars for giving us great advice and help from time to time during our project work.

Our eternal gratitude goes to our gracious and affectionate parents and friends for their never ending support.

Place: JUIT, Waknaghat

Puneet Agarwal

Anubhav Agarawal

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Chapter 1

ABSTRACT

1.1 Introduction

Protease:

A protease is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain. Proteases occur naturally in all organisms. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly-regulated cascades (e.g., the blood-clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase-activating cascade. [1] .

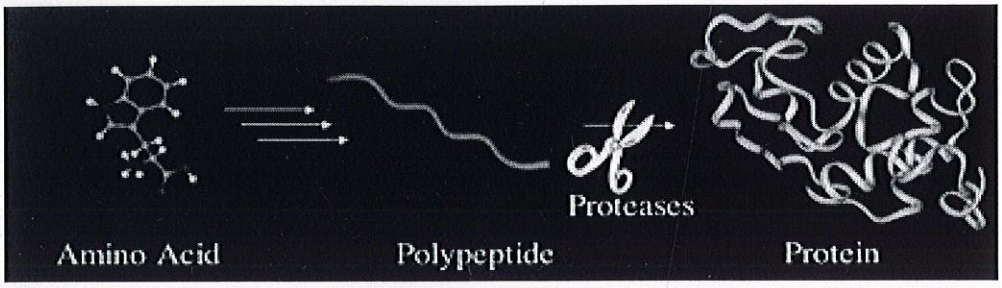


Fig.1.1 Protein synthesis

Proteases play pivotal regulatory roles in conception, birth, digestion, growth, maturation, ageing, and death of all organisms. Proteases regulate most physiological processes by controlling the activation, synthesis and turnover of proteins. Proteases are also essential in viruses, bacteria and parasites for their replication and the spread of infectious diseases, in all insects, organisms and animals for effective transmission of disease, and in human and animal hosts for the mediation and sustenance of diseases. [1]

In medicine, proteases represent important potential targets for medical intervention because of their important regulatory roles in life. It is now known that single amino acid mutations in over 50 human proteases result in hereditary/genetic diseases. [3]

In our environment, proteases are key regulators of the life of insects and other agricultural pests, key regulators of growth and health of farm animals, and principal regulators of plants and marine food sources. [3]

Substrates commonly used for production of industrial proteases are rice bran, maize etc. Mainly fungi are used due to higher production efficiency. Use of different waste materials for production of enzymes is the latest interest of research as they are abundant & low cost. Suitability of various waste materials as carbon source is being found out. Kitchen waste can be a good carbon source as it is one of the most abundant wastes apart from being of no cost. [2]

Chapter 2

2.1 OBJECTIVE

- Selection of strain
- Selection of a low cost carbon source
- Protease production by solid state fermentation
- Quantification of enzymatic activity in crude form
- Optimization of various process parameters
    i. pH
    ii. Time
    iii. Concentration of carbon source

Chapter 3

LITERATURE REVIEW

3.1 Introduction

The properties of proteases [2] produced by *Aspergillus oryzae* under various conditions differ in many respects. The influence of different methods upon the production of extracellular proteases by *Aspergillus oryzae* and to present some of the properties of the enzyme. Proteases, also known as proteinases or proteolytic enzymes, are a large group of enzymes. Proteases belong to the class of enzymes known as hydrolyses, which catalyse the reaction of hydrolysis of various bonds with the participation of a water molecule. Proteases are involved in digesting long protein chains into short fragments, splitting the peptide bonds that link amino acid residues. Proteases, being themselves proteins, are known to be cleaved by other protease molecules, sometimes of the same variety. This may be an important method of regulation of peptidase activity. Advances in analytical technique have demonstrated that protease conduct highly specific and selective modification of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secreatory protein across the membrane.[7]

TABLE 1. *Influence of fermentation methods upon the formation of proteinase by Aspergillus oryzae*

Method	Proteinase activity				Collagenolytic activity[c]	
	Gelatin digestion[a]		Caseinolysis[b]			
	pH 6.8	pH 10.5	pH 6.5	pH 10.5	pH 6.8	pH 10.5
Submerged culture[d]	nil	nil	nil	nil	nil	nil
Static liquid culture[d]	nil	nil	nil	nil	nil	nil
Solid substratum (wheat bran)	3.17	0.51	0.319	0.377	nil	nil

A comparative study of this proteinase [3] with other common proteinases reveals that the proteinase from our strain differs in many respects . It does not possess any collagenolytic activity and thus differs from the proteinase isolated from *Aspergillus oryzae* . Advances in analytical

technique have demonstrated that protease conduct highly specific and selective modification of proteins such as activation of zymogenic forms of enzymes by limited proteolysis, blood clotting and lysis of fibrin clots, and processing and transport of secreatory protein across the membrane.

The current estimated value worldwide sales of industrial enzymes are $1 billion .of the industrial enzymes 75% are hydrolytic. There are over 500 human proteases, accounting for 2% of human genes (DNA sequences that code for amino acids), and similar numbers of proteases occur in every plant, insect, marine organism and in all infectious organisms that cause disease. Protease represents one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide of sales of enzyme.[1]

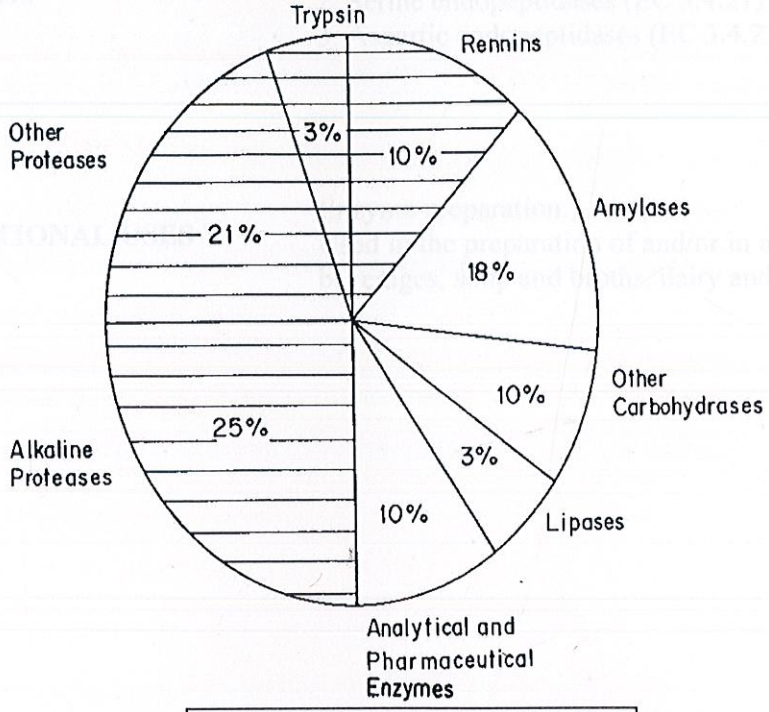


Fig 3.1 Distribution of enzymes sales

Proteases are proteolytic (protein-digesting) enzymes that are mainly classified on the basis of their pH optimum as acidic, neutral, and alkaline proteases. These biocatalysts find wide applications in many industries such as textile, laundry, healthcare etc. Neutral proteases are mainly used in food processing such as baking, brewing, and also in the healthcare sector. One of the more recent applications of these proteases exploit their eco-friendly nature and hence their suitability to act as food-processing aids, wherein these enzymes can be used for the extraction of plant oils thus largely replacing hazardous organic solvents such as hexane which has been traditionally used for such processes. [2].

3.2 PROTEASE from *ASPERGILLUS ORYZAE*: [3]

SOURCES	Produced by the controlled fermentation of non-toxicogenic and non-pathogenic strains of *Aspergillus oryzae* and isolated from the growth medium.
Systematic names and numbers	1. Aminopeptidases (EC 3.4.11) 2. Serine endopeptidases (EC 3.4.21) 3. Aspartic endopeptidases (EC 3.4.23)
FUNCTIONAL USES	Enzyme preparation. Used in the preparation of and/or in meat and fish products, beverages, soup and broths, dairy and bakery products[2]

3.3 Rationale behind potato peels as carbon source

- Easily available & cheaper than other raw materials being used for protease production.
- Provides an alternative for waste treatment.
- No costly pretreatment is required.
- High starch content.
- Suitable for solid state fermentation.

3.4 Response surface methodology:

- In traditional optimization method each parameter is considered to be insensitive to the other process variables involved. In many processes where operating variables interact and influence each other's effect in the response, it is essential that optimization should account for these interactions.
- It is an empirical statistical technique for multiple regression analysis by using quantitative data to solve multivariate data to solve multivariate equation simultaneously.

3.3.1 Steps used in Response surface methodology are:

- Setting up a series of experiments that will yield adequate and reliable measurements of the response of interest.
- Determining a mathematical model that best fits the data collected from the design chosen , by conducting appropriate test of hypothesis concerning the model's parameter.
- Determining the optimal setting of the experimental factor that produces the maximum value of the response.

Chapter 4

MATERIALS AND METHODS

4.1 MATERIALS

4.1.1 Materials used:

- Potato peels as carbon source
- Aspergillus oryzae (MTCC no. 6993) procured from IMTECH, Chandigarh
- Czapek-Dox media without glucose as carbon source.

4.1.2 Composition of Czapek-Dox agar :[1]

Glucose-	5%
$NaNO_3$-	0.2%
KCl-	0.05%
$MgSO_4$,7H2O-	0.05%
FeSO4,7H2O-	0.001%
KH2SO4-	0.1%
Agar-	3%
pH-	5.0

- *Aspergillus oryzae* (MTCC no. 6993) was maintained and stored on Czapek-Dox agar slants at 30^0C and used for the production of spores.

4.1.3 Instruments used

- Digital pH Meter
- UV- Visible Spectrophotometer
- Incubator
- General purpose Balance
- Centrifuge
- Vertical autoclave
- Laminar Flow Cabinet
- Orbital Shaker
- Magnetic Stirrer & Hot Plate

4.1.4 Conditions for production of protease:

- pH – 5.0 – 7.0
- Temperature - 30°C
- Shaker speed – 150 rpm
- Time – 1-6 days

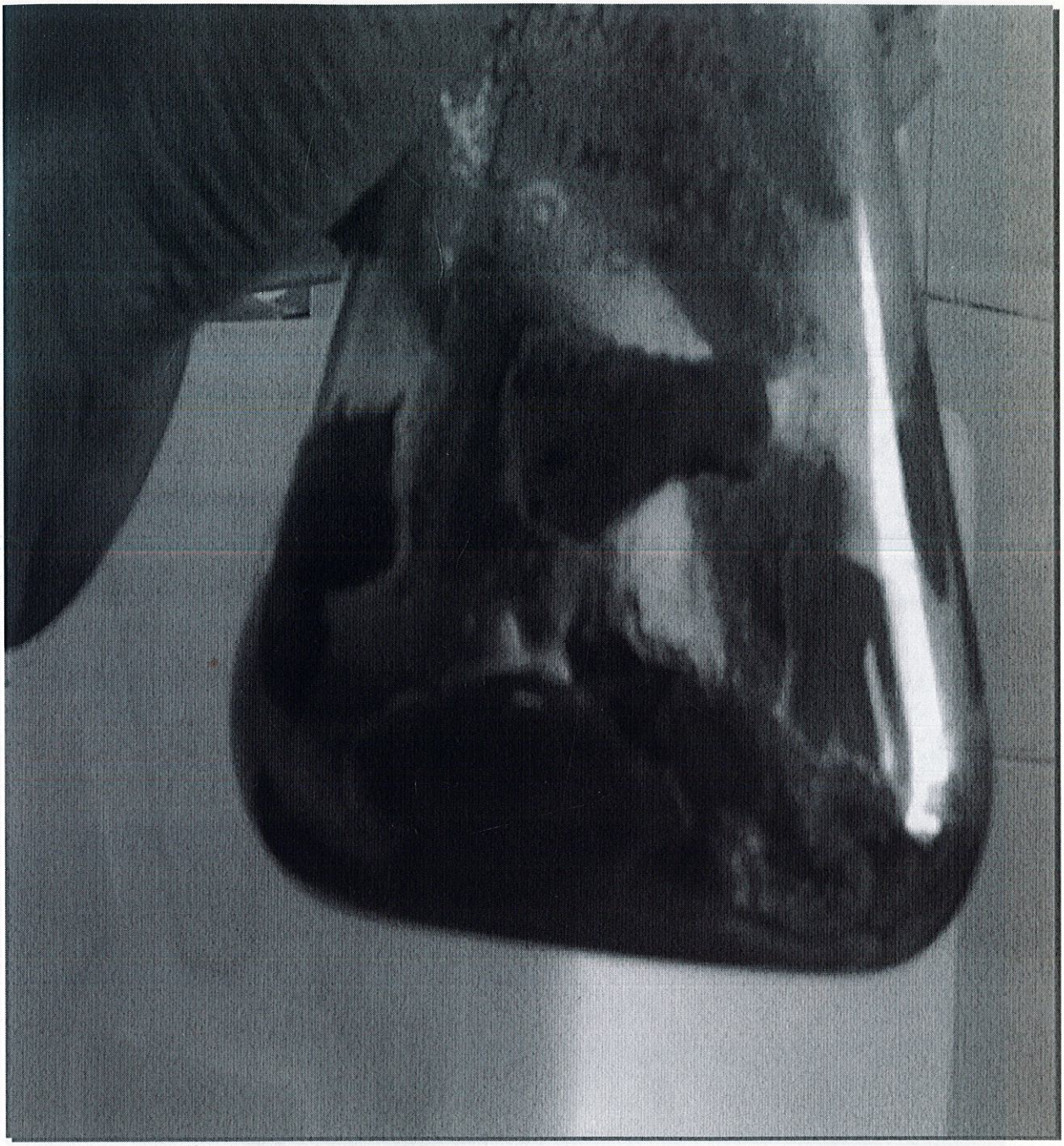


Fig 5.4.2 solid media

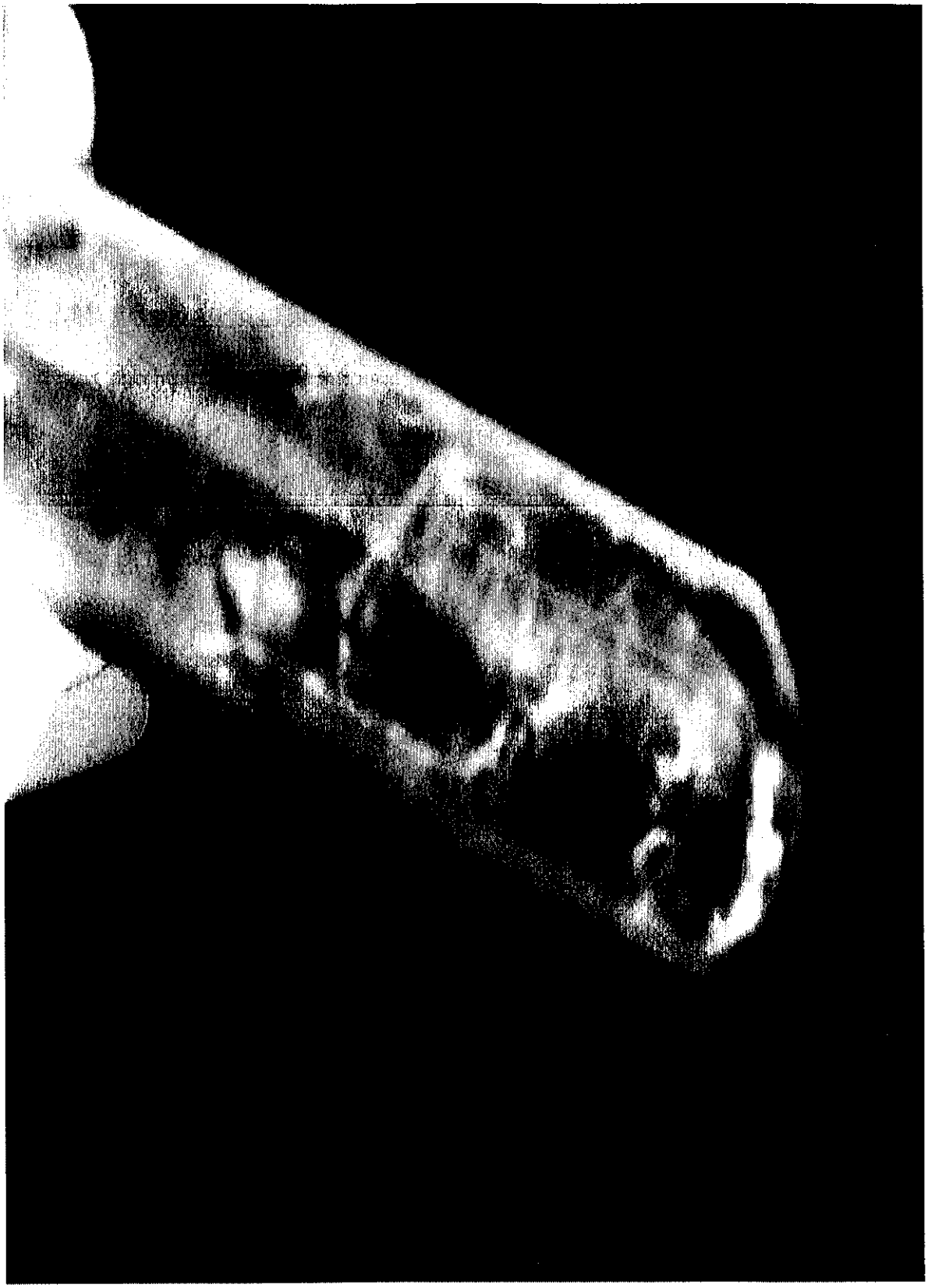


Fig 4.3.3 slant culture

4.2. Methodology

4.2.1 Standard curve

A standard curve (OD vs. concentration) was plotted using BSA. This curve helps in determining the amount of enzyme produced.

4.2.1.1 Preparation of standard curve

Concentrations of reagents used:

1. Tyrosin (stock) 0.0199g/100ml
2. Na_2Co_3 2.65g/50ml
3. F-C reagent (N=1) 5ml

4.2.1.2 Stock dilutions

samples(µl)	Blank	A	B	C	D	E
tyrosin	0	15	30	75	150	300
water	750	735	720	675	600	375
Na_2Co_3	1950	1950	1950	1950	1950	1950
F-C reagent	375	375	375	375	375	375

4.2.2 Optimization of amount of potato peels

4.2.2.1 Fermentation :

1. The fermentation process was carried out in two 250 mL conical flasks. Each flasks are filled with different amount of solid substrate and 15 mL sterile water supplemented with 1% w/w $NaNO_3$.(pH=5)
2. Spores were used as inoculum.
3. Incubation at 30°C for 6 days.

4.2.3.2 Protease Extraction Procedure

After 6 days incubation…..

- Centrifuged at 10,000 rpm for 30min. at 4°c.
- Supernatant after centrifugation was kept at 4°c until used further.
- BSA (bovine albumin serum) stock of conc. 1mg/ml was prepared
- Dilutions were made followed by addition of supernatant (enzyme)

4.2.4 Optimization procedure

4.2.4.1 pH optimization

Experiment was conducted by taking seven fungal cultures and varying the pH values from 5.0 to 7.0. Amount of enzyme was obtained for each pH value after every 24 hours. Temperature was maintained at 30°C. Media used was same as mentioned earlier. The output in the form of optical density was given as input in minitab software to generate a probability graph that will give optimized pH.

4.2.4.2 Time optimization

Experiment was conducted by taking seven fungal cultures and varying the number of hours of growth, keeping other parameters constant as above .Optical density was measured after every 24 hours. Media was same as mentioned earlier. The output in the form of optical density was given as input in minitab software to generate a graph that will give optimized time for growth.

4.2.4.3 Carbon content optimization

Experiment was conducted by taking seven fungal cultures and varying the amount od carbon content keeping other parameters constant as above .Optical density was measured after every 24 hours. We used 4 set of values: 15g/15ml, 30g/15ml, 45g/15ml, and 60g/15ml. The output in the form of optical density was given as input in minitab software to generate a graph that will give optimized amount for growth.

Chapter 5

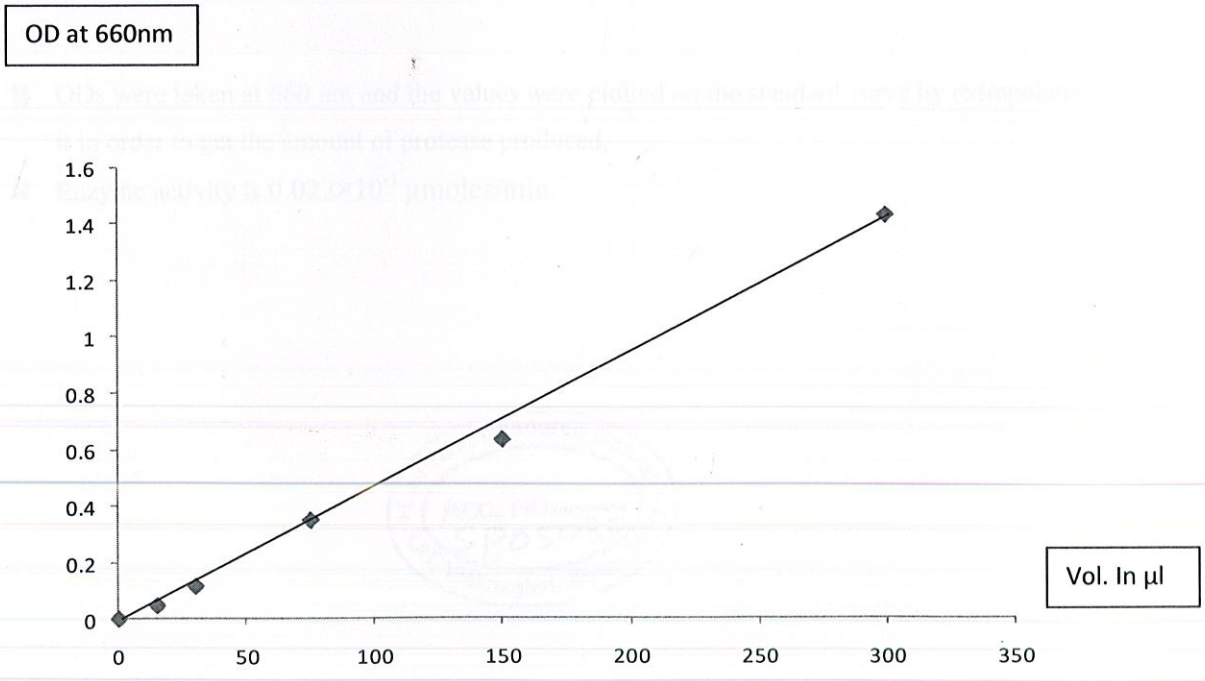
5.1 RESULTS AND DISCUSSION

5.1.1 Calculation of standard

OD at 660nm

samples	OD_{660}
A	0.047
B	0.116
C	0.350
D	0.635
E	1.431

5.1.1.2 Standard Curve Plotting



5.1.2 Carbon Source Optimization

15g/15ml	30g/15ml	45g/15ml	60g/15ml
No significant enzyme activity	No significant enzyme activity	Very low activity	Satisfactory activity

BSA(ml)	Water(ml)	Crude Enzyme(ml)	OD_{660}
0.8	3.2	1	0.105
1.2	1.8	0.5	0.110

Thus potato peels were added to the production media in concentration of 60g/15mL.

5.1.3 Production Results

- ODs were taken at 660 nm and the values were plotted on the standard curve by extrapolating it in order to get the amount of protease produced.
- Enzyme activity is 0.022×10^{-1} µmoles/min.

5.1.4 Optimisation results

The substrate concentration was doubled in order to increase the enzyme production. Final amount of potato peels was 120 gm per 30 ml of water.

	pH	5	5.5	6	6.5	7
Time(days)						
1		0.011	0.025	0.038	0.042	0.035
2		0.043	0.042	0.046	0.061	0.052
3		0.068	0.069	0.065	0.089	0.041
4		0.058	0.072	0.082	0.072	0.032
5		0.021	0.054	0.067	0.055	0.029
6		0.011	0.021	0.031	0.042	

OD AT 660nm

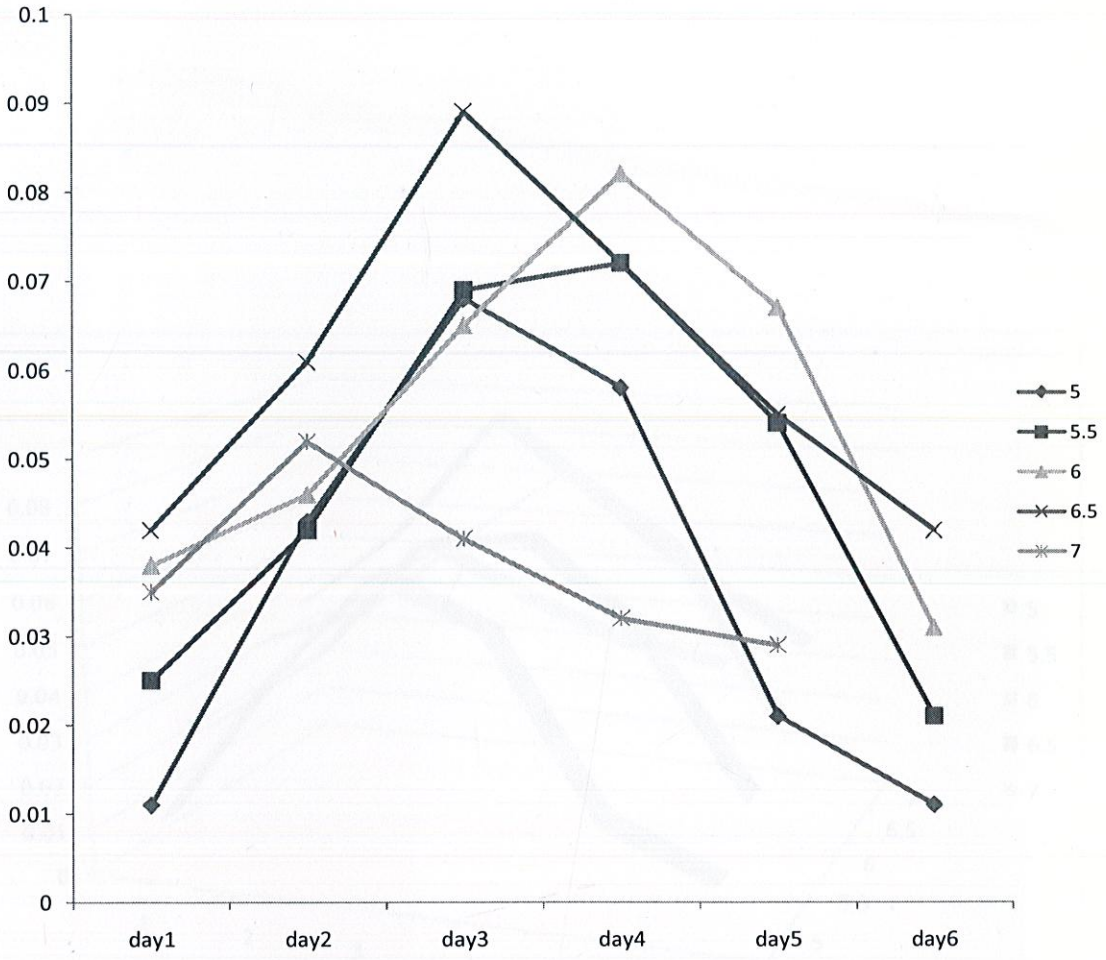
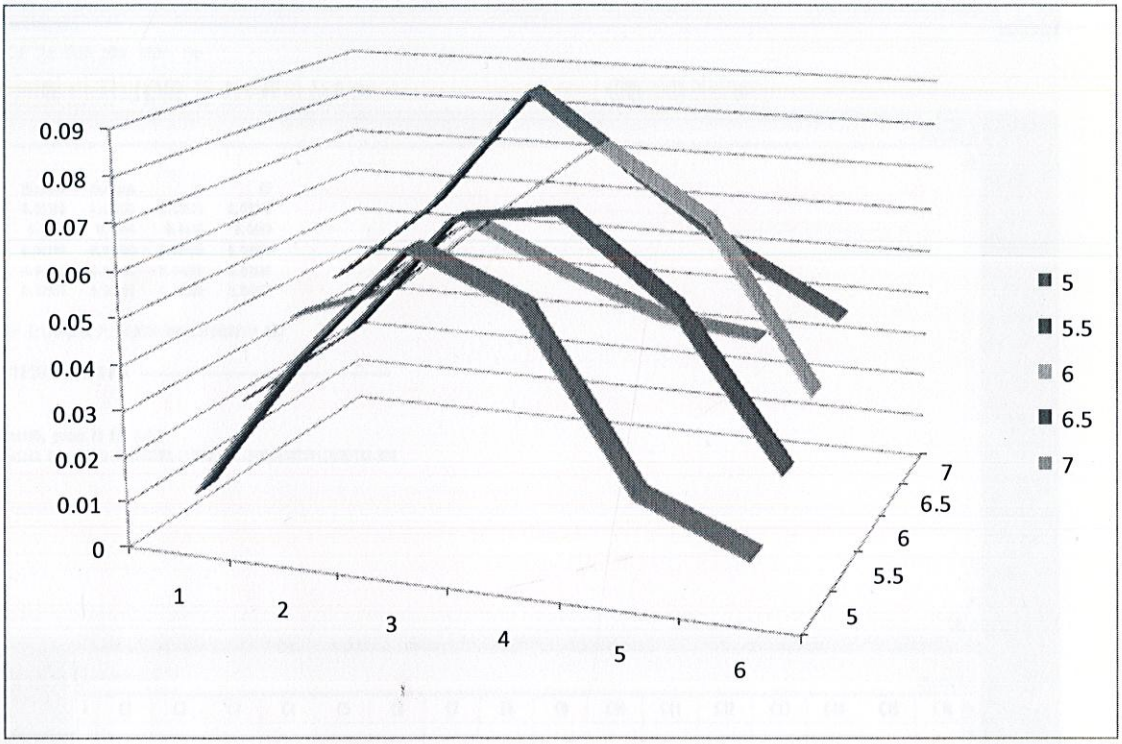


Figure 5.1: Time vs OD at different pH values

It was observed that the amount of protease showed a gradual increase till 3rd day of incubation and then decreased after 72 hours. Thus 72 hours of incubation is the optimum time for production of protease from potato peels using *Aspergillus oryzae.* As we can see from the graph that on day1 OD of pH=5 is very low but increases up to day 3 but after that it started decreasing. If we observe the OD of pH=5.5, we see that it started exponentially up to day 4 after that it started decreasing. Similarly OD of PH=6 shows increment up to day 4 and after that decrement in OD.As we have discuss the optimized result at PH=6.5 on day3 and after that start decreasing .At PH=7 OD is not satisfactory as it started decreasing after day 2.

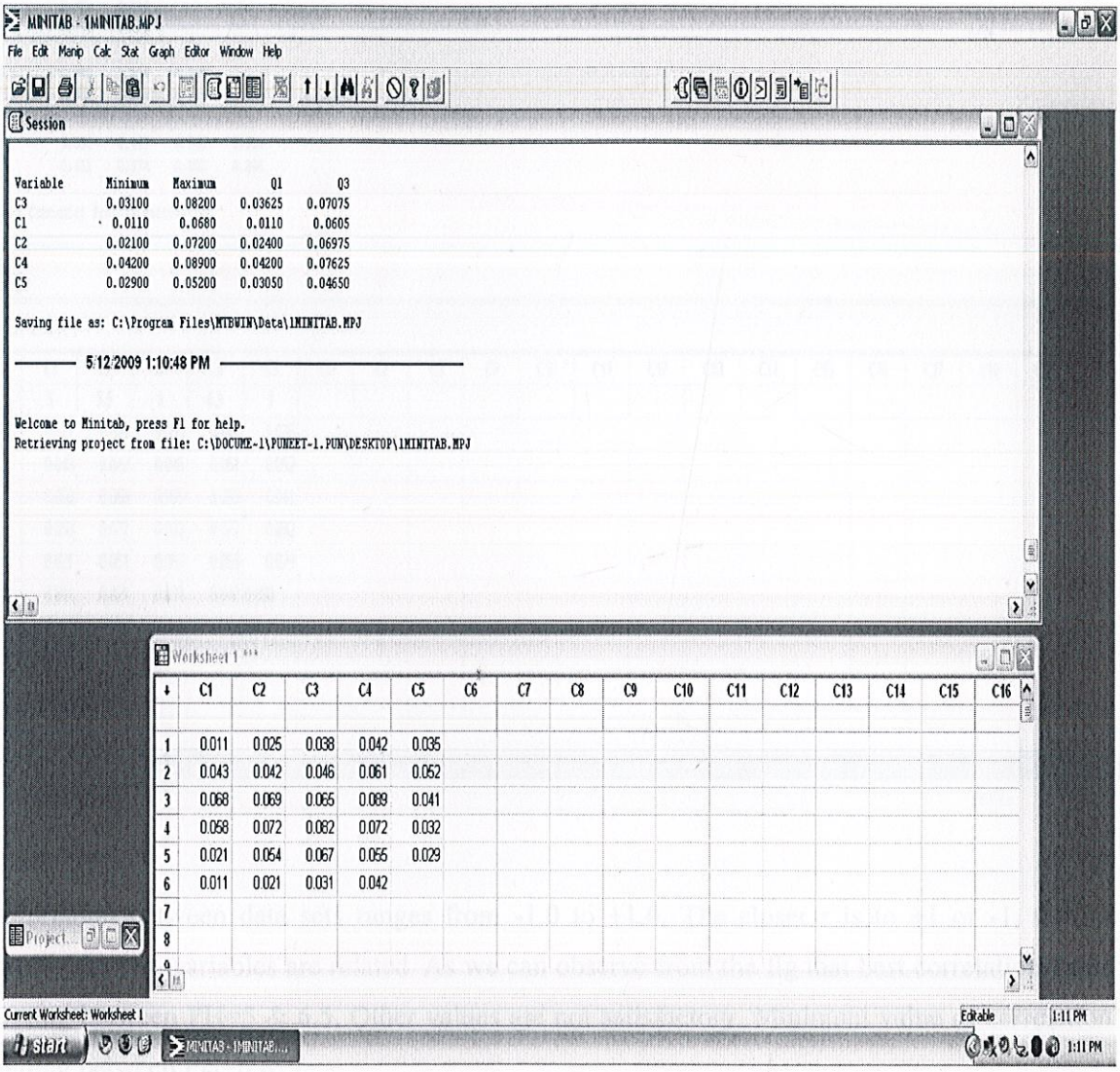


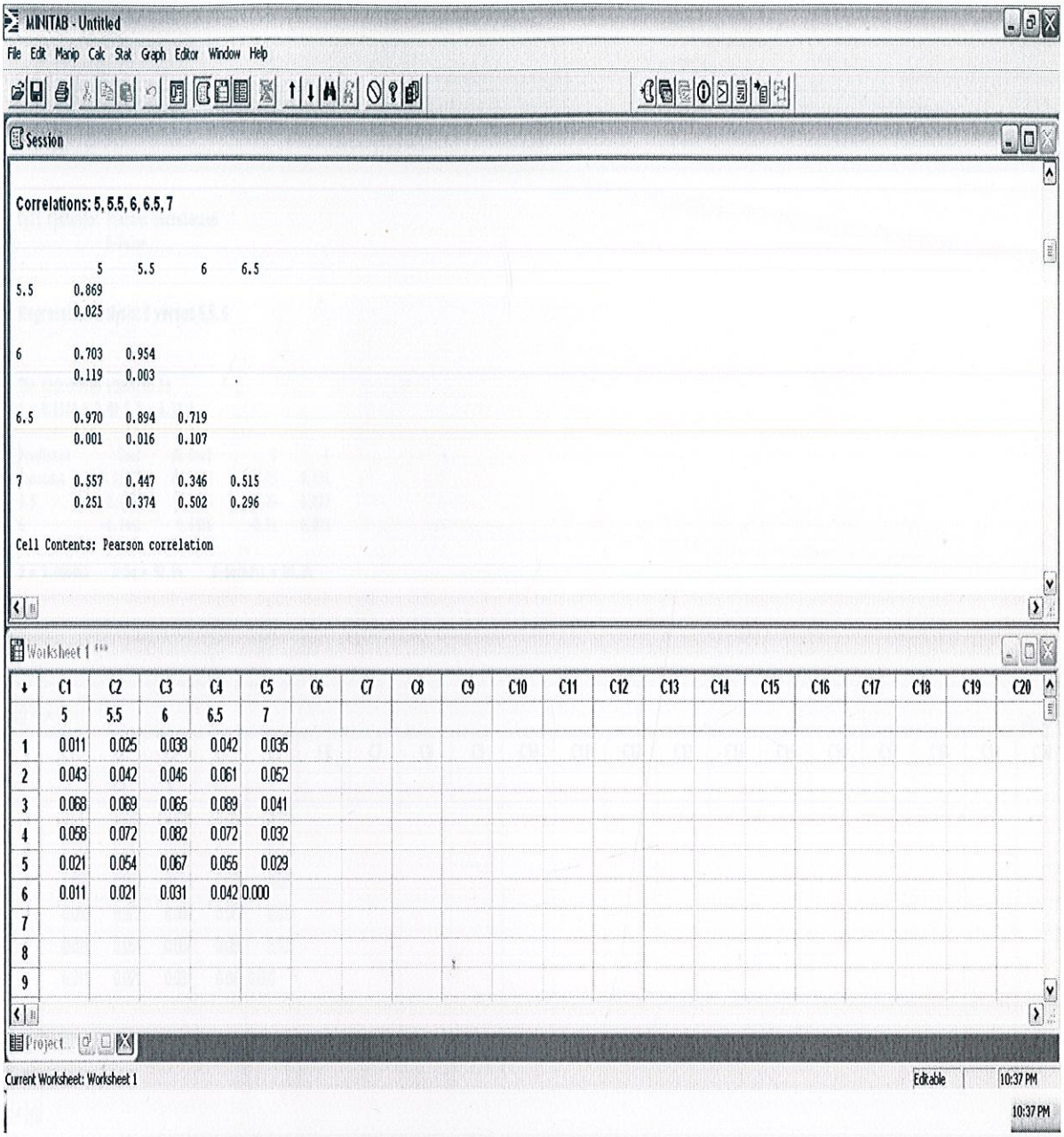
3D graph of optimized result

This figure is showing the 3D analysis of above linear graph. As we can observe from the graph that maximum covered area is at PH = 6.5, also it has highest peak on day 3.

5.1.5 Data analysis using MINITAB

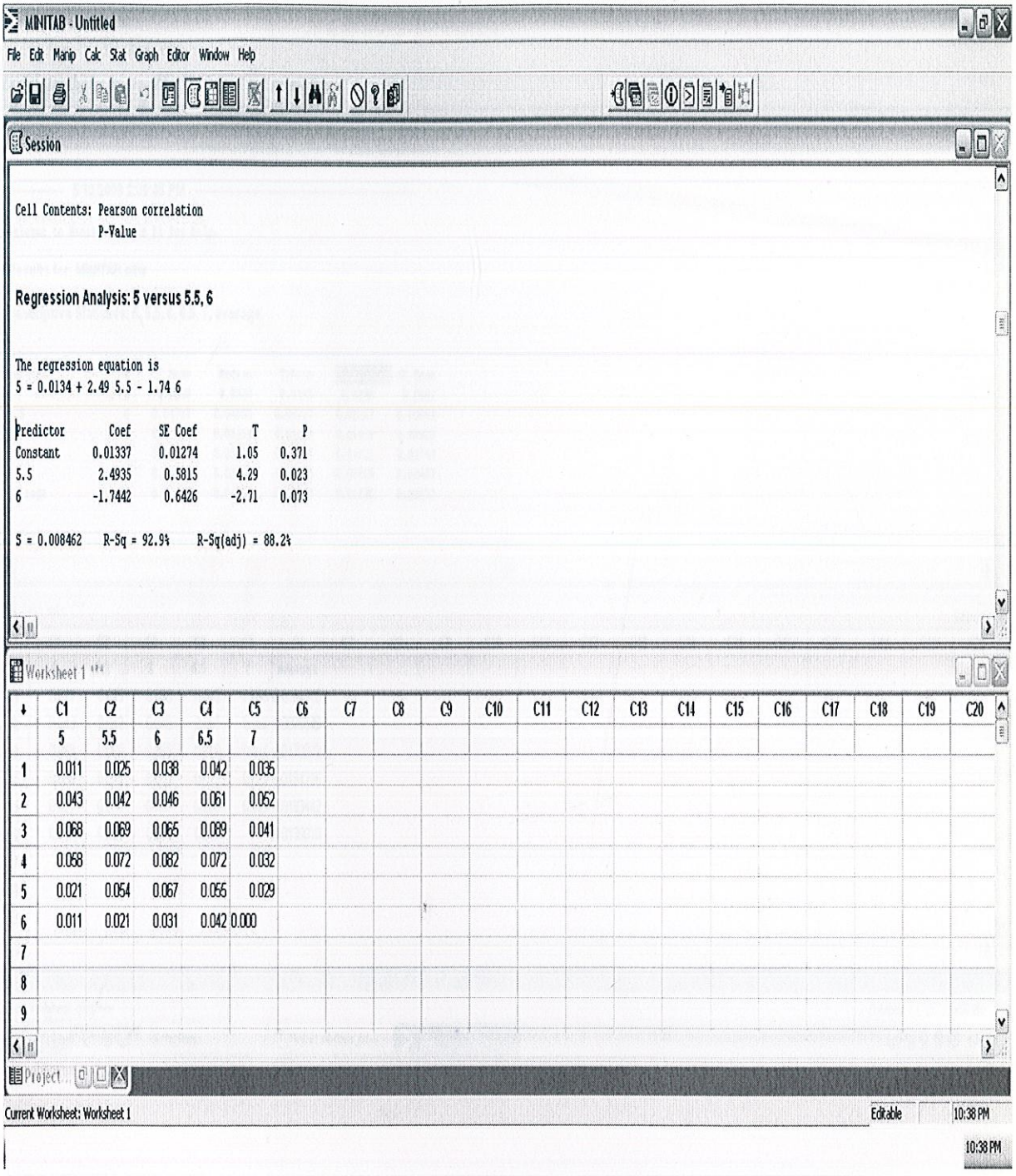
Data analysis for optimization of parameters using MINITAB.





	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20
	5	5.5	6	6.5	7															
1	0.011	0.025	0.038	0.042	0.035															
2	0.043	0.042	0.046	0.061	0.052															
3	0.068	0.069	0.065	0.089	0.041															
4	0.058	0.072	0.082	0.072	0.032															
5	0.021	0.054	0.067	0.055	0.029															
6	0.011	0.021	0.031	0.042	0.000															
7																				
8																				
9																				

Correlation between data sets ranges from -1.0 to +1.0. The closer r is to +1 or -1, the more closely the two variables are related. As we can observe from the fig that best correlation value is coming between PH=5 & 6.5. Other values are not satisfactory. Minimum value of correlation is coming between PH=6 & 7.



	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	C20
	5	5.5	6	6.5	7															
1	0.011	0.025	0.038	0.042	0.035															
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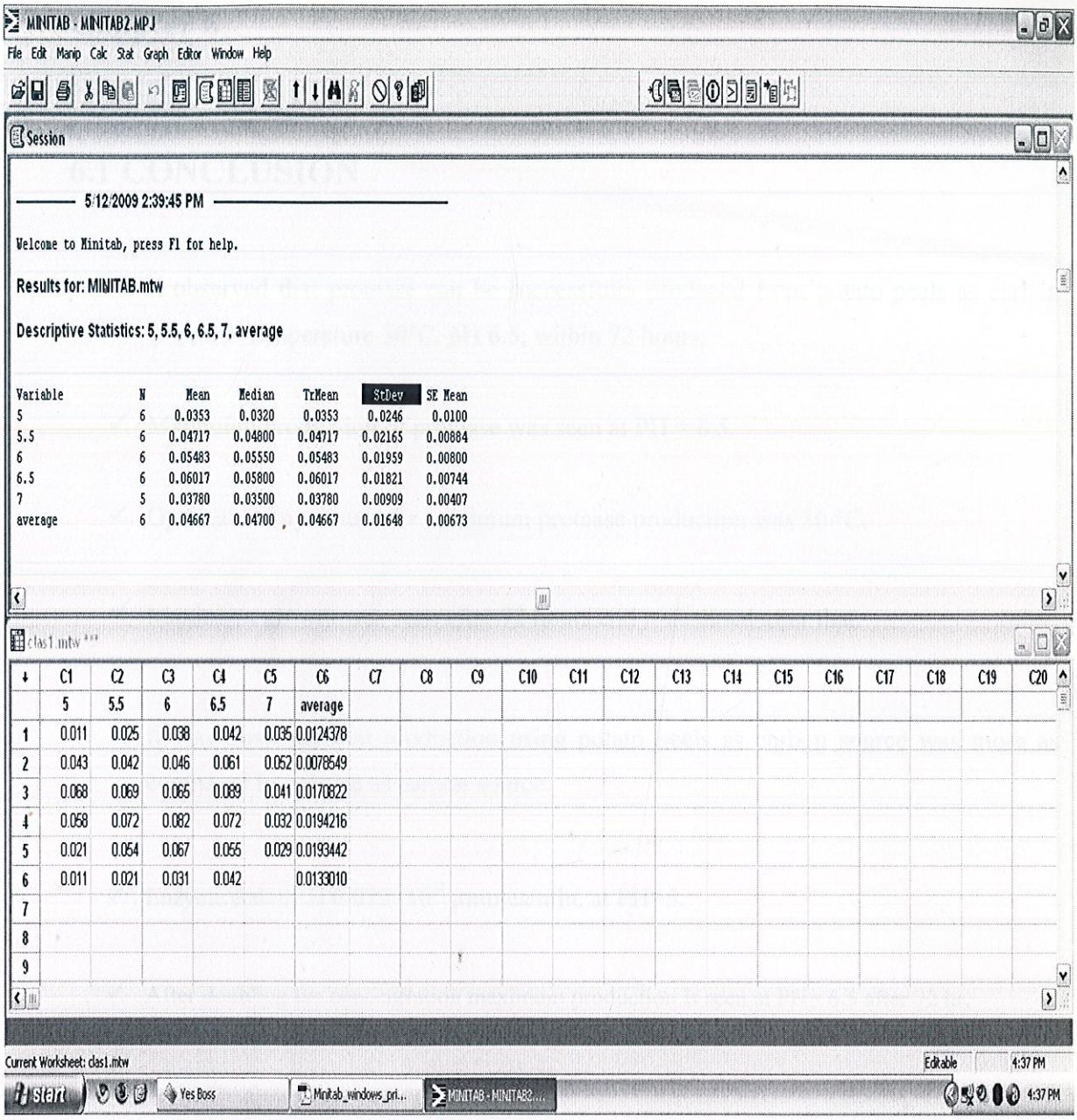
6	0.011	0.021	0.031	0.042	0.000															
7																				
8																				
9																				

Regression analysis refers to techniques for the modeling and analysis of numerical data consisting of values of a variable. As we can see from the above fig. that best fit coming between PH=5, 5.5 & 6 is 92.9% & average of the variable is 0.008462.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Variable N Mean Median TrMean StDev SE Mean
5 6 0.0353 0.0320 0.0353 0.0246 0.0100
5.5 6 0.04717 0.04800 0.04717 0.02165 0.00884
6 6 0.05483 0.05550 0.05483 0.01959 0.00800
6.5 6 0.06017 0.05800 0.06017 0.01821 0.00744
7 5 0.03780 0.03500 0.03780 0.00909 0.00407
average 6 0.04667 0.04700 0.04667 0.01648 0.00673
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C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 C13 C14 C15 C16 C17 C18 C19 C20
5 5.5 6 6.5 7 average
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3 0.068 0.069 0.065 0.089 0.041 0.0170822
4 0.058 0.072 0.082 0.072 0.032 0.0194216
5 0.021 0.054 0.067 0.055 0.029 0.0193442
6 0.011 0.021 0.031 0.042 0.0133010
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start
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Chapter 6

6.1 CONCLUSION

- ✓ It observed that protease can be successfully produced from potato peels as carbon source at temperature 30°C, pH 6.5, within 72 hours.
- ✓ Maximum production of protease was seen at PH = 6.5.
- ✓ Optimum temperature for maximum protease production was 30 °C.
- ✓ Maximum growth was seen after 72 hours and it declined after that.
- ✓ It was observed that production using potato peels as carbon source was more as compared to sucrose as carbon source.
- ✓ Enzyme activity is 0.022×10^{-1} µmoles/min. at PH=5.
- ✓ After doubling the concentration maximum production is seen at PH= 6.5 after 72 hr.

Chapter 7

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