PRODUCTION OF ALKALINE PROTEASE FROM *Bacillus amyloliquefaciens* AND MOLECULAR CHARACTERIZATION

A PROJECT REPORT

Submitted by

GANESH KUMAR.M

SIVA ELANGO.P

VASANTHAN.M

P-1830

in partial fulfillment for the award of the degree

of

BACHELOR OF TECHNOLOGY

in

INDUSTRIAL BIOTECHNOLOGY

ANNA UNIVERSITY: CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report "**PRODUCTION OF ALKALINE PROTEASE FROM *Bacillus amyloliquefaciens* AND MOLECULAR CHARACTERIZATION**" is the bonafide work of "**GANESH KUMAR.M** and **SIVA ELANGO.P**" who carried out project work under my supervision.

SIGNATURE

Dr. P. Rajasekaran
PROFESSOR AND HEAD
Department of Biotechnology,
Kumaraguru College of Technology,
Coimbatore-641006.

SIGNATURE

Mr. T. Sathish Kumar
SUPERVISOR
Lecturer,
Department of Biotechnology,
Kumaraguru College of Technology,
Coimbatore-641006.

ANNA UNIVERSITY: CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report **"PRODUCTION OF ALKALINE PROTEASE FROM *Bacillus amyloliquefaciens* AND MOLECULAR CHARACTERIZATION"** is the bonafide work of **"VASANTHAN.M"** who carried out project work under my supervision.

SIGNATURE

Dr. P. Rajasekaran
PROFESSOR AND HEAD
Department of Biotechnology,
Kumaraguru College of Technology,
Coimbatore-641006.

SIGNATURE

Mr. M. Shanmuga Prakash
SUPERVISOR
Lecturer
Department of Biotechnology,
Kumaraguru College of Technology,
Coimbatore-641006.

Lark Training & Research Institute

No 7, Guberaganapathy
Street, Mathiyazhagan
Nagar, Padi, Chennai-50

Ph: 044 26243912,
Fax: 044 42146141
Mail: ltri@scientist.com

This is to certify that Mr M.Ganesh Kumar, Final B.Tech (Industrial Biotechnology) of Kumaraguru College of Technology, Coimbatore, TN, India has undergone a short term project work on ***"Production Of Alkaline Protease from Bacillus amyloliquefaciens & Molecular Characterization"*** under my supervision from 18th Jan 2007 to 29 th March 2007

Dated: 28-03-2007
Place: Chennai

H.Abbas Ali
Scientific Officer LTRI

LARK TRAINING & RESEARCH INSTITUTE
No. 7, GUBERA GANAPATHI STREET,
MATHIYAZHAGAN NAGAR,
PADI, CHENNAI-600 050.

Lark Training & Research Institute

No 7, Gubera ganapathy
Street, Mathiyazhagan
Nagar, Padi, Chennai-50

Ph: 04426243912,
Fax: 04442146141
Mailltri@scientist.com

This is to certify that Mr. P. Siva Elango, Final B.Tech (Industrial Biotechnology) of Kumaraguru College of Technology, Coimbatore, TN, India has undergone a short term project work on ***"Production Of Alkaline Protease from Bacillus amyloliquefaciens & Molecular Characterization"*** under my overall supervision from 18 th Jan 2007 to 29th March 2007.

Dated: 28-03-2007
Place: Chennai

H.Abbas Ali
Scientific Officer LTRI

LARK TRAINING & RESEARCH INSTITUTE
No. 7, GUBERA GANAPATHY STREET,
MATHIYAZHAGAN NAGAR,
PADI, CHENNAI-600 050.

Lark Training & Research Institute

No 7, Guberaganapathy
Street, Mathiyazhagan
Nagar, Padi, Chennai-50

Ph: 044-26243912,
Fax: 044-42146141
Mailltri@scientist.com

This is to certify that Mr. M. Vasanthan, Final B.Tech (Industrial Biotechnology) of Kumaraguru College of Technology, Coimbatore, TN, India has undergone a short term project work on ***"Production Of Alkaline Protease from Bacillus amyloliquefaciens & Molecular Characterization"*** under my supervision from 18 th Jan 2007 to 29th March 2007.

Dated: 28-03-2007
Place: Chennai

H.Abbas Ali
Scientific Officer LTRI

LARK TRAINING & RESEARCH INSTITUTE
No. 7, GUBERA GANAPATHI STREET,
MATHIYAZHAGAN NAGAR,
PADI, CHENNAI-600050

CERTIFICATE OF EVALUATION

College : KUMARAGURU COLLEGE OF TECHNOLOGY

Branch : INDUSTRIAL BIOTECHNOLOGY

Semester : Eighth Semester

S.No	Name of the Students	Title of the Project	Name of the Supervisors with Designation
01	GANESH KUMAR.M (71203204008)	**"Production of alkaline protease from *Bacillus amyloliquefaciens* and molecular characterization"**	Mr.T.Sathish kumar Lecturer
02	SIVA ELANGO.P (71203204032)		
03	VASANTHAN.M (71203204037)		Mr.M.Shanmuga Prakash Lecturer

The Report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Industrial Biotechnology of Anna University was confirmed to be the report

ACKNOWLEDGEMENT

With our deepest sense of gratitude we extend our heartfelt thanks to **Mr. T. Sathish Kumar** & **Mr. M. Shanmuga Prakash** Lecturers, Department of Biotechnology, Kumaraguru College of Technology, for their relentless support, masterly guidance, creative ideas and patient efforts for successful completion of the project.

Our sincere thanks to **Dr. P. Rajasekaran**, Professor & Head, Department of Biotechnology, Kumaraguru College of Technology. His gracious and ungrudging guidance all through our project work is highly acknowledged with gratitude.

We are happy to thank **Mr. S. Shanmugam**, Senior Lecturer, Department of Biotechnology, Kumaraguru College of Technology for his timely help and encouragement without any hesitation.

We are happy to thank **Dr. N. Saraswathy**, Senior Lecturer, Department of Biotechnology, Kumaraguru College of Technology for her timely help and encouragement without any hesitation.

We wish to extend our thanks to all **Teaching and Non-Teaching staffs** of the **DEPARTMENT OF BIOTECHNOLOGY** for their kind and patient help throughout the project work.

We are happy to thank our **Principal, Dr. Joseph.V.Thanikal and Management**, Kumaraguru College of Technology for providing us all the

We are happy to thank **Mr. H. Abbas Ali**, Scientific officer, Lark Training and Research Institute (LTRI), for his support, guidance and timely helps for the successful completion of this project.

We also thank **The Management, Lark Innovative Fine Teknowledge**, for providing all the facilities and timely helps for the successful completion of this project.

We thank all our friends who physically and emotionally helped us to bring out the work successfully.

Finally, we wish to express our deep sense of gratitude to our beloved parents and family members for their constant encouragement and love, without whose inspiration this study would not have seen the dawn of day.

[Ganesh Kumar.M]

[Siva Elango.P]

[Vasanthan.M]

ABSTRACT

Bacillus amyloliquefaciens is a rod shaped gram positive organism that is most commonly used for the production of amylases. Molasses is the waste produced from cane and beet during sugar extraction. The project is planned to use *Bacillus amyloliquefaciens* to produce protease with molasses as a sole carbon source which is rich in various nutrients favouring the microbial growth. The enzyme production is compared with different immobilization methods and also temperature and pH stabilities of the enzyme and it was found with polyacrylamide immobilization the production of enzyme was high and had an optimum activity at 50°C and at a pH 9. The enzyme production is scaled up using airlift fermenter with polyacrylamide immobilized *Bacillus amyloliquefaciens*. The crude enzyme produced is purified by different methods. The enzyme producing gene is amplified for molecular characterization.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ABBREVIATIONS

APS	Ammonium persulphate
IUB	International Union of Biochemistry
DEAE	Di ethyl amino ethyl
TCA	Tri chloro acetic acid
TEMED	N,N,N'N'- tetra methyl ethylene diamine
PAGE	Polyacryl amide gel electrophoresis
SDS	Sodium dodecyl sulphate
PCR	Polymerase Chain Reaction

LIST OF FIGURES

1.0 INTRODUCTION

Enzymes are biocatalysts that catalyze a specific chemical reaction without affecting the equilibrium of the catalyzed reaction providing the reaction path with lower activation energy. Wherever a substance needs to be transformed into another, nature uses enzymes to speed up the process. According to IUB enzymes have been classified as oxidoreductase, transferase, hydrolases, lyases, isomerase and ligases.

Hydrolases are group of enzymes that carry out hydrolysis i.e., transfer of functional group to water (e.g.) proteases. Proteases catalyze the hydrolysis of the peptide bonds that link amino acids together in a protein. Proteases can be classified based on their activity in different pH as

- Acid proteases
- Neutral proteases
- Alkaline proteases

Alkaline proteases are produced by a wide range of organisms including bacteria, moulds, yeasts and various mammalian tissues (Adinarayana *et al.,* 2002). Globally large proportion of commercially available alkaline proteases was produced by various *Bacillus sp.,* which possess significant proteolytic activity and stability at considerably high pH and temperatures (Kumar *et al.,* 2003). Proteases generally execute diversified functions and have important biotechnological applications in

cassava waste and sugarcane baggasse as a sole carbon source in the medium for the increased production of alkaline proteases by *Bacillus amyloliquefaciens* (Glenn *et al*., 1973).

Similarly molasses the final byproduct obtained in the preparation of sucrose by repeated evaporation and crystallization of juices from sugar cane and from sugar beets has been used as a potent carbon source for alkaline protease production now-a-days (Gupta *et al*., 2002). Molasses is rich in different nutrients that favour microbial growth. The composition and nutrient content of cane molasses is given in the table below

Table 1.1- Composition of molasses

Item	**Concentration (%)**
Total Solids	75.0
Total Sugars	46.0
Crude Protein	3.0
Nitrogen Free Extract	63.0
Total Fat	0.0
Total Fiber	0.0
Ash	8.1
Calcium	0.8
Phosphorus	0.08

Modification of biotechnological processes, using immobilized cells and biocatalysts, has recently gained the attention of many biotechnologists. Application of immobilized enzymes or whole cells is advantageous, because such biocatalysts display better operational stability and higher efficiency of catalysis than the free enzymes or cells and they are reusable. Whole cell immobilization by entrapment is widely used as a simple technique (Adinarayana *et al.*, 2005) for the production of alkaline proteases.

With this background, our study was planned with the following objectives.

1. To optimize the molasses concentration for the production of alkaline protease from *Bacillus amyloliquefaciens*.
2. To characterize (pH & temperature) the crude alkaline protease.
3. To immobilize the *Bacillus amyloliquefaciens* using various gel entrapment techniques for the production of alkaline protease.
4. To characterize (pH & temperature) the alkaline protease of the immobilized *Bacillus amyloliquefaciens*.
5. To scale up the enzyme production using immobilized *Bacillus* cells in airlift fermenter.
6. To purify the alkaline protease produced by *Bacillus amyloliquefaciens*.
7. To identify the alkaline protease gene in *Bacillus amyloliquefaciens*.

2.0 LITERATURE REVIEW

B. amyloliquefaciens was discovered in soil in the year 1943 by a Japanese scientist named Fukumoto, who gave the bacterium its name since it has produced (*faciens*) a liquifying (*lique*) amylase (*amylo*) (Fukumoto, 1943).

B. amyloliquefaciens is Gram-positive, catalase positive, aerobic, rod-shaped and motile in nature. This organism is naturally found in soil samples. As with other members in the family Bacillaceae, it forms a strong endospore when conditions are not favorable and can be dispersed in this form into dust which gets into water supplies for plants and animals. *Bacillus amyloliquefaciens* is further classified as a low G+C organism (Fukumoto, 1943). *B. amyloliquefaciens* is known for its ability to degrade proteins extracellularly (Gill *et al.*, 2002).

The dependence of enzyme production from *Bacillus amyloliquefaciens* during the growth phase in batch culture has been studied previously (Cumming *et al.*, 1984). The results showed the rate of enzyme production has been enhanced by ball milling, ultrasonication, and autolysis. It also suggested that autolysis is a feasible method for disrupting *Bacillus sp* and thereby an enhancement in the enzyme production has been observed.

Bacillus amyloliquefaciens isolated from meju, a Korean soybean fermentation starter was used to produce neutral metalloprotease and an

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. They constitute one of the most important groups of industrial enzymes, as it accounts for at least a quarter of the total global enzyme production (Kumar *et al.,* 2003) Proteases have been widely used in detergents, leather, food and in pharmaceutical industries.

For an enzyme to be used as an detergent additive it should be stable and active in the presence of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators, fillers, fabric softeners and various other formulation aids. Alkaline proteases can withstand high pH & possess good thermo stability. Alkaline protease treatments can modify the surface of wool and silk fibers to provide new and unique finishes. Alkaline proteases have been used in the hide dehairing process, where dehairing is carried out at pH values between 8 and 10 (Mohsen *et al.,* 2005).

Previous reports are there for the isolation of heat stable alkaline protease and lactamase from *Bacillus species* obtained in a local hot spring. The activity of protease and lactamase was stable up to 60°C in isolated condition, and at immobilized condition in the sodium alginate gel the enzyme showed high protease activity than free enzyme (Chandi *et al.*, 1998).

The extracellular production of protease by *Bacillus sp* isolated from

bran as its sole carbon source. Optimum temperature has been identified as 55°C and the pH as 9.0 with a 4% inoculum in the media.

Precipitation of enzymes is a useful method of concentration and is ideal as an initial step in their purification and it can be used on a large scale. Salting out of proteins, particularly by use of ammonium sulphate, is one of the best known and used methods of purifying and concentrating enzymes, particularly at the laboratory scale. Increase in the ionic strength of the solution cause a reduction in the repulsive effect of like charges between identical molecules of a protein. When these forces are sufficiently reduced, the protein will precipitate. Ammonium sulphate is convenient and effective because of its high solubility, cheapness, lack of toxicity to most enzymes and its stabilizing effect on some enzymes (Yeng *et al*., 2000).

Ion exchange chromatography separates molecules based on differences between the overall charges of proteins. It is usually used for protein purification but may be used for purification of oligonucleotides, peptides, or other charged molecules. The protein of interest must have a charge opposite that of the functional group attached to the resin in order to bind. Elution is achieved by increasing the ionic strength to break up the ionic interaction, or by changing the pH of the protein (Scawen et al., 1980).

DEAE cellulose is an anion exchanger applied for the chromatography of proteins. Serum components, enzymes, peptides,

mortem aging were detected by this technique. Such changes observed were the disappearance of one fraction and appearance of new components, while other fractions diminished (Rampton *et al.*, 1965).

Immobilization of whole cells has been defined as the physical confinement or localization of intact cells to a certain defined region of space with preservation of some (Adinarayana *et al.*, 2003), or most, catalytic activity. The increased stability under extreme conditions of pH and temperature, as well as the re-use and applicability in continuous processing systems that enclose immobilized cells instead of soluble enzymes make the cells a preferred, versatile tool in both food industry and medicine. There are several different approaches to the classification of immobilized biocatalysts, but the most frequently employed classification is based upon the method of immobilization selected for a specific application (Banik *et al.*, 2004).

Microbial products are usually produced either by free or immobilized cells. The use of immobilized cells as industrial catalysts can be advantageous compared to batch fermentation process. Whole cell immobilization has been a better choice over enzyme immobilization. Whole cell immobilization by entrapment is a widely used as a simple technique. It was reported that the viability of microbial cells was maintained over a period of 18 months under entrapped conditions (Adinarayana et al., 2005).

using molasses as a sole carbon source, purifying the enzyme and molecular characterization.

3.0 MATERIALS AND METHODS

3.1 BROTH PREPARATION

Commercially available strain of *Bacillus amyloliquefaciens* was obtained from IMTECH Chandigarh, India and was used for the broth preparation.

MATERIALS

Peptone	-	5g/l
NaCl	-	5g/l
Beef extract	-	3g/l

METHOD

1. The broth is made upto 150ml with the above mentioned substances in a 250ml conical flask.
2. The pH was adjusted to 7.2 and autoclaved at 121°C for 15 minutes.
3. The flask is allowed to cool to room temperature and then was inoculated with the Bacillus strain and incubated at 36°C for 12 hrs which is then used for protease production.

3.2 MEDIA OPTIMIZATION WITH MOLASSES

The media used for protease production should be optimized with

METHOD

1. The protease producing media was prepared using the above composition varying the carbon source and made upto 150ml in conical flask.
2. The pH was adjusted to 9.0 and autoclaved at 121°C for 15 minutes.
3. To the media 15ml of 12 hrs broth culture was inoculated under aseptic conditions and mixed well.
4. The media was then incubated in orbital shaker at 150rpm.
5. Samples were collected for every six hours and protease assay was performed.

3.3 PROTEASE ASSAY

Protease activity was assayed spectrophotometrically according to the method proposed by Kunitz using casein as a substrate (Kunitz, 1947). The activity of enzyme was expressed in (Units/ml) and the absorbance was measured at 280nm. One unit of protease activity was defined as the amount of the enzyme needed to liberate 1 µg / ml of tyrosine per minute, under assay condition.

PRINCIPLE

The enzyme alkaline protease cleaves the substrate casein under assay

MATERIALS

(See appendix 6.2)

METHOD

1. 1.5ml of the culture was taken from the shake flask after every six hours.
2. The sample was then centrifuged, and the supernatant was used as the enzyme for assay.
3. To 50µl of the supernatant 450µl of 1% casein in (50mM; pH 8.0) Tris HCl was added and incubated at 55°C for 20 minutes.
4. After incubation 750µl of TCA solution was added and kept at room temperature for 30 minutes.
5. The sample was then centrifuged for 15000rpm, 15 minutes and the supernatant was collected and absorbance was measured at 280nm using an UV spectrophotometer and activity in U/ml was calculated using the formula below.

$$\text{Activity} = \frac{(O.D)_{280} * \text{cuvette volume (3 ml)} * \text{dilution factor}}{18.4 * \text{sample volume (50 µl)}}$$

Where,

MATERIALS

(See appendix 6.2)

METHOD

1. 50µl of the enzyme was taken in 6 different test tubes each and 450µl of 1% casein was added to each tube.
2. Each tube was then incubated at different temperatures 40°C, 50°C, 60°C, 70°C, 80°C, 90°C for 20 minutes.
3. After incubation 750µl of TCA solution was added to all the tubes and kept at room temperature for 30 minutes.
4. The sample was then centrifuged for 15000rpm, 15 minutes and the supernatant was collected and absorbance was measured at 280nm using an UV spectrophotometer.

3.3.2 EFFECT OF pH ON CRUDE ALKALINE PROTEASE ACTIVITY

The stability of the enzyme at different pH was found out by carrying out the assay using the substrate with different pH.

MATERIALS

(See appendix 6.2).

3. After incubation 750µl of TCA solution was added to all the tubes and kept at room temperature for 30 minutes.
4. The sample was then centrifuged for 15000rpm, 15 minutes and the supernatant was collected and absorbance was measured at 280nm using an UV spectrophotometer.

3.4 IMMOBILIZATION

Microbial immobilization involves the entrapment of microbial cells with an insoluble matrix so it can be maintained in proper reactor for its reuse under stabilized conditions. The various entrapment techniques used for immobilization are

- Sodium alginate
- Polyacrylamide
- Gelatin

3.4.1 IMMOBILIZATION OF *Bacillus amyloliquefaciens* USING SODIUM ALGINATE

MATERIALS

Sodium alginate (3%) (See appendix 6.3)

Calcium chloride (0.2 M)

Sterile syringe

2. To 15ml of the sodium alginate slurry, 15 ml of the cell suspension was mixed to get a uniform mixture.
3. The slurry was taken into a sterile syringe and added dropwise into 0.2M Calcium chloride.
4. The beads were preserved in 0.9% Sodium chloride before use.
5. 150 ml of the production medium was prepared and inoculated with sodium alginate beads.

3.4.2 IMMOBILIZATION OF *Bacillus amyloliquefaciens* USING POLYACRYLAMIDE

This immobilization is based on condensation copolymerization of a water-soluble, functionalized prepolymer into a polymer. Gel containing the cells can be used directly in suspension

MATERIALS

Phosphate buffer (Sterile) (pH-8.0)

Acrylamide – 2.85 g

Ammonium persulphate – 10 mg

TEMED (NNN^1N^1 tetra methyl ethylene diamine) – 1 ml

Sterile distilled water

Petri plates

3. The slurry was poured into sterile petriplates and allowed to solidify.
4. After solidification, the polyacrylamide gel was cut into small pieces and transferred into 0.2M phosphate buffer for washing.
5. The gel was then stored in refrigerator in sterile distilled water.

3.4.3 IMMOBILIZATION OF *Bacillus amyloliquefaciens* USING GELATIN

MATERIALS

Gelatin (sterile) (20%) (See appendix 6.4)

Gluteraldehyde (5%) (See appendix 6.4)

Sterile distilled water

Petri plates

METHOD

1. To 20% of sterile gelatin, the cell suspension of *Bacillus amyloliquefaciens* was added and then poured into petri plate.
2. The gel was overlayed with 5% gluteraldehyde for hardening.
3. The resulting blocks was cut into approximately 4 mm small size cubes and washed with sterile distilled water. The immobilized *Bacillus amyloliquefaciens* was then used for the production of alkaline protease in shake flask.

MATERIALS

(See appendix 6.2)

METHOD

1. 50µl of the enzyme extract from 3 types of immobilized cells were taken in 6 different test tubes each and 450µl of 1% casein was added to each tube.
2. Each tube was then incubated at different temperatures 40°C, 50°C, 60°C, 70°C, 80°C, 90°C for 20 minutes.
3. After incubation 750µl of TCA solution was added to all the tubes and kept at room temperature for 30 minutes.
4. The samples were then centrifuged for 15000rpm, 15 minutes and the supernatant was collected and absorbance was measured at 280nm using an UV spectrophotometer.

3.4.5 EFFECT OF pH OF ALKALINE PROTEASE ACTIVITY USING DIFFERENT IMMOBILIZATIONS OF *Bacillus amyloliquefaciens*

The stability of the enzyme at different pH was found out by carrying out the assay using the substrate with different pH.

METHOD

1. 50µl of the enzymes from 3 types of immobilized cells were taken in 6 different test tubes each and 450µl of 1% casein in (50mM; pH 6 - 10) Tris HCl was added to the tubes.
2. Each tube was then incubated at 55°C for 20 minutes.
3. After incubation 750µl of TCA solution was added to all the tubes and kept at room temperature for 30 minutes.
4. The samples were then centrifuged for 15000rpm, 15 minutes and the supernatant was collected and absorbance was measured at 280nm using an UV spectrophotometer.

3.5 SCALE UP PRODUCTION OF ALKALINE PROTEASE USING POLYACRYLAMIDE IMMOBILIZED *Bacillus amyloliquefaciens* IN AIRLIFT FERMENTER

The scale up was carried out in an airlift fermenter using polyacrylamide immobilized *Bacillus amyloliquefaciens*. The volume of the fermenter was 4 litres and its working volume was 2.5litres.

MATERIALS

Production medium – 2 litres (See appendix 6.1)

Polyacrylamide immobilized cells

2. The medium was then inoculated with 10% of 18 hour broth culture under aseptic condition and the temperature was maintained at 37 °C with pH 9.0.
3. An increase in temperature was controlled by running water through the U tube provided in the fermenter jar.
4. The pH, temperature and dissolved oxygen probes and also the foam sensor was inserted into the fermenter and the fermentation process was started.
5. The pH was controlled with the use of 0.5N NaOH as the base and 0.5 N H_2SO_4 as the acid.
6. The foam produced in the fermenter was controlled by the addition of sunflower oil as antifoaming agent.
7. 2 lpm of air was supplied to the media.
8. The sample was collected on each hour through the sampling point and was assayed to find the enzyme activity.

3.6 PURIFICATION OF ALKALINE PROTEASE FROM *Bacillus amyloliquefaciens*

The fermentation process was stopped at a condition when the alkaline protease enzyme showed the maximum activity. This was followed by the purification process.

MATERIALS

Activated charcoal

Phosphate buffer (pH - 8.0) (see appendix 6.5)

Glass wool

Column

METHOD

1. 5g of activated charcoal was added to 10 ml phosphate buffer.
2. The mixture was then transferred to a column packed with glass wool.
3. After the charcoal got packed with the glass wool in the column, 15ml of crude sample was added to the column.
4. A colourless pure sample was collected separately at the bottom of the column.

3.6.2 AMMONIUM SULPHATE PRECIPITATION

Precipitation of enzymes is a useful method of concentrating it and is an ideal initial step in their purification. It can be used on a large scale and is less affected by the presence of interfering materials. It is a simple and effective means of fractionating proteins. The equation used to calculate the amounts of ammonium sulphate is

$$X = [50.6 \times (S_2 - S_1)] / (1 - 0.3 \times S_2)$$

Where, X = the amount of ammonium sulphate to add in grams per 100 ml

this amount increases the volume substantially; so the concentration of ammonium sulphate in a saturated solution at 0 c is 56.6g/100ml.

MATERIALS

Phosphate buffer (pH 8.0) (See appendix 6.5)

METHOD

1. 80% of ammonium sulphate was taken as a standard, for 75 ml of protein sample obtained after the treatment of activated charcoal (53.94g) of ammonium sulphate was added.
2. The solution was kept in a magnetic stirrer under refrigeration condition for overnight.
3. The sample was taken and centrifuged to obtain pellet.
4. Discard the supernatant and the pellet was dissolved in phosphate buffer pH 8.0.
5. The protease assay was carried out and the enzyme activity was calculated.

3.6.3 DESALTING OF ALKALINE PROTEASE IN DESALTING COLUMN

Desalting of protein was carried out to remove the salt content present

MATERIALS

Desalting column

Phosphate buffer (pH 8.0)

NaCl (25 mM) (See appendix 6.6)

METHOD

1. The column and the buffers were brought to the room temperature.
2. 15ml of buffer solution was added to equilibrate the column. Care has to be taken to avoid air bubbles.
3. Following the addition of buffer, 1ml of sample was added gently and drained off completely.
4. After the addition of sample 1ml of distilled water was added slowly and collected to wash the column. This was repeated for 5 to 6 times.
5. The sample was eluted with 1ml of 25mM NaCl solution.
6. The enzyme activity was then assayed.
7. The elution of sample was stopped when the activity decreases.

3.6.4 PURIFICATION OF ALKALINE PROTEASE BY DEAE CELLULOSE ANION EXCHANGER

Cellulose helps to retain the protein on the addition of sample to it. The column was equilibrated with low concentration buffer such as 100mM Tris chloride. Sodium chloride of different concentration from 0.2M to 1M

METHOD

1. 2 g of DEAE cellulose was weighed and transferred to 12 ml water.
2. The resin was washed three to four times with water and then centrifuged at 1000-2000 rpm for 5 minutes.
3. The pellet was washed twice with (100mM; pH -7.5) Tris buffer and using the same cellulose Tris solution was prepared.
4. Initially the column was packed with certain amount of glass wool.
5. Following the addition of glass wool cellulose Tris solution was added.
6. The set up was kept as such for few minutes until cellulose settles at the bottom and Tris solution was drained off.
7. 15 ml of phosphate buffer was added to equilibrate the column.
8. The sample collected after the desalting was added slowly to the column and kept for few minutes then drained off.
9. Different concentration of NaCl (0.2 M – 1M) was added through the column one by one and the sample was collected and the enzyme assay was carried out.

3.6.5 CHARACTERISATION OF MOLECULAR WEIGHT OF ALKALINE PROTEASE USING SDS PAGE

SDS PAGE is commonly used to separate protein depending upon their

MATERIALS REQUIRED

(See appendix 6.8)

Electrophoresis power supply

PROCEDURE

1. The glass plates were assembled with spacers between it the assembly was kept in a vertical position in gel casting stand.
2. The separating gel mixture was slowly poured into the gap between the glass plates leaving sufficient space for pouring stacking gel mix.
3. Leave the set up for 10 – 20 minutes for polymerization.
4. After polymerization, add a small quantity of water over the solidified gel to remove the unpolymerized acrylamide.
5. The stacking gel mixture was poured slowly over the surface of polymerized separating gel.
6. Slowly insert a comb over stacking gel without the formation of air bubbles.
7. After polymerization, the spacer was removed carefully and the gel was mounted on the electrophoresis removing the bottom spacer and the comb was removed carefully for the development of well.
8. The top chamber and the bottom chamber of the tank were filled with tank buffer.

12. The gel was removed and subjected for silver staining, to view the protein band.

3.6.5.1 SILVER STAINING

Silver staining was carried out with MEDOX BIO silver staining kit. Silver staining is more sensitive than the common Coomassive G-250 and hence it allows easy detection of proteins. Trichloro acetic acid present in the fixing solution removes the interfering ions and detergents from the gel and prevents the movement of protein out of the gel. Sensitivity of stain was increased by glutaraldehyde present in the cross linking solution .Excess stain was removed and the gel was rehydrated by washing with distilled water. Better image of the band was obtained on the addition of silver nitrate. Finally, the silver ion was reduced to metallic silver at the protein band by the developing solution and the bands appear golden or brown colour.

MATERIALS

MEDOX BIO Silver staining kit.

METHOD

1. After the electrophoresis the gel was removed from the cassette and

5. Cross linking solution was then discarded and the gel was then immersed in double distilled water with gentle shaking for 30 minutes.
6. Water was discarded and 25 ml of reducing solution was added and the gel was kept at room temperature for 20 minutes.
7. The reducing agent was discarded and 25 ml of silver nitrate was added and kept for 10 minutes.
8. The gel was then washed with double distilled water for 2 minutes.
9. Water was discarded and 25 ml of developing solution (with 25 L /50 ml of Formaldehyde) was added and kept for 10 minutes with gentle shaking.
10. The former step was repeated once again.
11. The reaction would stop on the appearance of golden or brown colour to the band. The developing solution was discarded and 25 ml of water was added to the gel.
12. The gel was the observed under white illuminator for the presence of bands.

3.7 ESTIMATION OF PROTEIN BY FOLIN-LOWRY'S METHOD

Protein estimation was carried by Lowry's method (1951). It forms a copper protein complex in the alkaline medium. This complex then reduces a phosphomolybdic phosphotungstate reagent to yield intense blue colour

METHOD

1. Take 0.1 ml sample and 0.9 ml of distilled water in a test tube in duplicate.
2. Add 5 ml solution C in all the tubes and leave for 10 minutes at room temperature.
3. Add 0.5 ml solution D and mix immediately and vigorously.
4. After 10 minutes record the absorbance against blank at 600 nm.
5. Prepare calibration curve by plotting optical density against the standard tyrosine solution.
6. Calculate the protein concentration mg/ml by reading the absorbance of sample on standard curve.

3.8 ISOLATION OF DNA

MATERIALS REQUIRED

(See appendix 6.10)

METHOD

1. The bacterial culture was centrifuged at 7500 rpm.
2. The pellet was then suspended in 180 µL of lysozyme solution.
3. The sample was incubated at 37°C for 30 minutes.
4. Following the incubation 20 µl of proteinase K and 200 µl of buffer

7. 200 µl of 100% ethanol was added to the sample and was mixed by pulse vortexing for 15 seconds.
8. After vortexing, it is then centrifuged for few seconds to remove the drops from the inside lid. The sample, buffer and the ethanol has to be mixed thoroughly to yield a homogenous solution.
9. Carefully pour the mixture to the QIA amp Spin Column and centrifuge at 800 rpm for 1 minute.
10. Spin column was opened carefully and 500 µl of Buffer AW1 was added.
11. The spin column was placed in a collection tube and the tube containing the filtrate was discarded. It was centrifuged at 8000 rpm for 1 minute.
12. The collection tube containing the filtrate was discarded and was replaced by a fresh collection tube.
13. 500 µl of buffer AW2 was added to the spin column and centrifuged at 14000 rpm for 3 minutes.
14. After centrifugation, the collection tube with filtrate was discarded and replaced by a fresh one.
15. 200 µl of buffer AE was added and incubated at room temperature for 5 minutes (increases the yield) and then centrifuged at 8000 rpm for 1 minute.

denaturation, annealing, and extension. The above three process continues for 30 cycles. The primers are added to the target DNA that they go and bind to opposite strands of the target DNA. The synthesis takes place in the 5' – 3' direction. The kit used for PCR was provided by "FERMENTAS", and the 2 X PCR master mixes provided contains the dNTPs, Taq DNA polymerase.

MATERIALS

BANGALORE GENEI PCR KIT

(See appendix 6.11)

METHOD

The following components were added in a PCR vial for both forward and reverse reaction.

POSITIVE REACTION

2X PCR master mix	-	12.5 µl
Forward primer	-	5 µl
Reverse primer	-	5 µl
Template DNA	-	5 µl
Nuclease free water	-	22.5 µl

NEGATIVE REACTION

Table 3.2 PCR

REACTION	Initial Denaturation	Denaturation	Annealing	Extention	Final Extention
TEMPERATURE	94°C	94°C	58.6°C	72°C	72°C
TIME PERIOD	3 Minutes	30 Seconds	30Seconds	1minutes	3minutes
		30 cycles			

The reaction was followed as

1. After PCR 10 µl of sample was mixed with 5µl of gel loading dye.
2. 15µl of sample was loaded in 1.8% agarose gel and electrophoresis was carried out.

Table 3.3 Primer data

	Forward Primer Data	Reverse Primer Data
Sequence	AAGAGTGAGAGGCAA	GATTGAACATGCGGAGGA
GC Content	47	50
Position	445	1645
Degeneracy	0	0
3' GC	50	50
3' Degeneracy	0	0

4.0 RESULTS AND DISCUSSION

4.1 MEDIA OPTIMIZATION WITH MOLASSES

Molasses was used as the sole carbon source. The concentration of molasses was varied and the optimum concentration of molasses in which the enzyme production was high was found out.

Table – 4.1 Alkaline protease activity using 1.0% molasses

Time(hrs)	Activity(U/ml)
1	1.62
7	1.79
13	1.98
19	2.24
25	2.03
31	1.78
36	1.53

Table – 4.2 Alkaline protease activity using 2.0% molasses

Time(hrs)	Activity(U/ml)
1	1.80
7	2.03
13	2.26

Table – 4.3 Alkaline protease activity using 3.0% molasses

Time(hrs)	Activity(U/ml)
1	1.94
7	2.16
13	2.38
19	2.88
25	2.43
31	2.18
36	2.02

Table – 4.4 Alkaline protease activity using 4.0% molasses

Time(hrs)	Activity(U/ml)
1	2.11
7	2.37
13	2.77
19	3.01
25	2.80
31	2.56
36	2.43

Table – 4.5 Alkaline protease activity using 5.0% molasses

Time(hrs)	Activity(U/ml)
1	2.34
7	2.67

Table – 4.6 Alkaline protease activity using 6.0% molasses

Time(hrs)	Activity(U/ml)
1	2.22
7	2.42
13	2.81
19	3.25
25	3.00
31	2.75
36	2.61

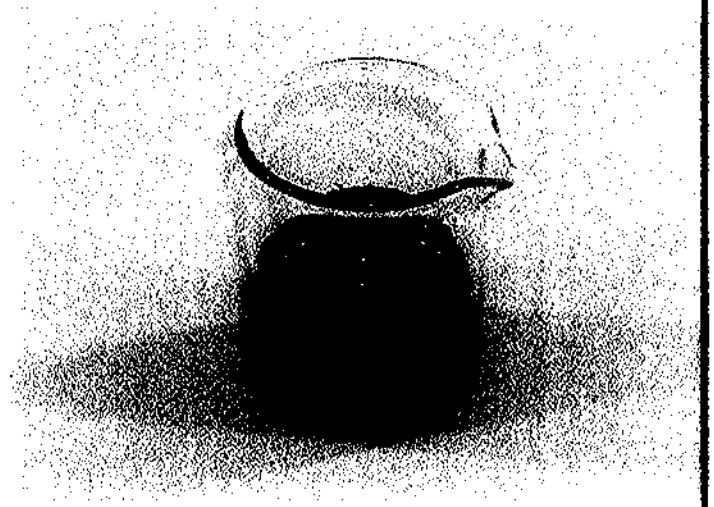
Table – 4.7 Alkaline protease activity using 7.0% molasses

Time(hrs)	Activity(U/ml)
1	2.18
7	2.38
13	2.68
19	3.04
25	2.75
31	2.59
36	2.32

Table – 4.8 Alkaline protease activity using 8.0% molasses

Time(hrs)	Activity(U/ml)
1	2.12
7	2.28

Figure – 4.1 (Molasses)



The table - 4.9 shows the maximum activity of alkaline protease at different molasses concentration during 19th hour of the bacterial growth. The activity increases as the molasses concentration get increased and reaches the maximum at 5% molasses concentration. The activity decreases as the molasses concentration increases further due to substrate inhibition

Table – 4.9 Optimization of molasses for alkaline protease production

Molasses concentration	Maximum activity(U/ml)
1.0%	2.24
2.0%	2.59
3.0%	2.88
4.0%	3.01
5.0%	3.72

4.2 EFFECT OF TEMPERATURE ON CRUDE ALKALINE PROTEASE ACTIVITY

The thermostability of the crude alkaline protease was found out by performing the assay at different temperatures ranging from 40°C to 90°C.

Table – 4.10 Effect of temperature on crude alkaline protease

Temperature (°C)	Activity (U/ml)
40	3.34
50	3.91
60	3.65
70	3.23
80	3.15
90	3.03

Table – 4.10 shows the activity of the crude alkaline protease at different temperatures. Generally, the optimum activity of alkaline protease ranges between 25°C to 70°C (Noguetra *et al.*, 2006). The alkaline protease produced shows the maximum activity at 50°C, which makes it a potent industrial enzyme.

4.3 EFFECT OF pH ON CRUDE ALKALINE PROTEASE ACTIVITY

Table – 4.11 Effect of pH on crude alkaline protease

pH	Activity (U/ml)
6.0	3.29
7.0	3.58
8.0	3.78
9.0	3.93
10.0	3.45

Table – 4.11 shows the activity of the crude alkaline protease at different pH. Generally, the optimum activity of alkaline protease ranges between 6.5 to 10 (Morihara, 1963). The alkaline protease produced shows the maximum activity at 50°C, which makes it a potent industrial enzyme.

4.4 IMMOBILIZATION

Immobilization of the Bacillus cells was carried out using three different types gel entrapment techniques.

Table–4.12 Enzyme activity from sodium alginate immobilized *Bacillus amyloliquefaciens*

Time(hrs)	Activity(U/ml)
1	2.79
7	3.25

Tables–4.13 Enzyme activity from polyacrylamide immobilized *Bacillus amyloliquefaciens*

Time(hrs)	Activity(U/ml)
1	2.98
7	3.33
13	3.89
19	4.46
25	4.04
31	3.96
36	3.72

Table–4.14 Enzyme activity from gelatin immobilized cells

Time(hrs)	Activity(U/ml)
1	2.82
7	3.28
13	3.84
19	4.30
25	3.82
31	3.79
36	3.37

Table – 4.15 Optimal enzyme activity with different types of immobilization of *Bacillus amyloliquefaciens*

Table – 4.15 shows the maximum activity of alkaline protease produced from *Bacillus amyloliquefaciens* under different immobilized conditions. A gradual increase in alkaline protease production was noticed from 6 hours onwards to 19 hours. On further incubation decline in alkaline protease activuty was observed. The alkaline protease activity with immobilized cells in polyacrylamide was found higher followed by alginate. Low level of alkaline protease production was observed with gelatin.

4.5 EFFECT OF TEMPERATURE ON ACTIVITY OF ALKALINE PROTEASE FROM IMMOBILIZED *Bacillus amyloliquefaciens*

The thermostability of the alkaline protease from immobilized cells was found out by performing the assay at different temperatures ranging from 40°C to 90°C.

Table – 4.16 Effect of temperature of alkaline protease from immobilized *Bacillus amyloliquefaciens*

Temperature(°C)	Sodium alginate activity (U/ml)	Polacrylamide activity (U/ml)	Gelatin activity (U/ml)
40	3.75	4.38	4.09
50	4.13	4.50	4.32
60	3.87	4.37	3.98
70	3.72	3.94	3.76

Table – 4.16 shows the activity of the alkaline protease at different temperatures. Generally, the optimum activity of alkaline protease ranges between 25°C to 70°C (Nogueira *et al.*, 2006). The alkaline protease produced by immobilized cells shows the maximum activity at 50°C, which makes it a potent industrial enzyme.

4.6 EFFECT OF pH ON ACTIVITY OF ALKALINE PROTEASE FROM IMMOBILIZED *Bacillus amyloliquefaciens*

The stability of the alkaline protease from immobilized cells was found out by performing the assay at different pH ranging from 6 to 10.

Table – 4.17 Effect of pH alkaline protease from immobilized *Bacillus amyloliquefaciens*

pH	Sodium alginate Activity (U/ml)	Polacrylamide Activity (U/ml)	Gelatin Activity (U/ml)
6	3.63	4.02	3.94
7	4.01	4.39	4.20
8	4.23	4.67	4.51
9	4.60	4.81	4.78
10	4.38	4.61	4.42

4.7 SCALE UP PRODUCTION OF ALKALINE PROTEASE WITH POLYACRYLAMIDE IMMOBILIZED CELLS IN AIRLIFT FERMENTER

The scale up process was carried out with polyacrylamide immobilized Bacillus amyloliquefaciens in airlift fermenter.

Figure-4.2 Scale up enzyme

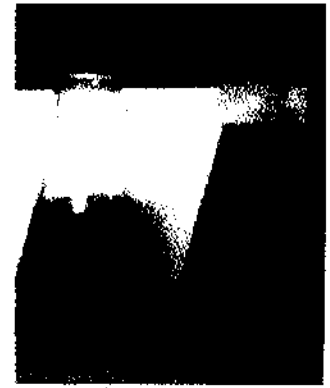


Table – 4.18 Enzyme activity from scale up enzyme production

Time(hrs)	Activity(U/ml)
1	3.29
7	8.64
13	17.97
19	25.32
25	20.47
31	17.55
36	13.69

Table- 4.19 Effect of temperature on enzyme from scale up production

Temperature (°C)	Activity
40	18.88
50	25.74
60	20.00
70	18.07
80	17.52
90	15.88

Table- 4.20 Effect of pH on enzyme from scale up production

pH	Activity
6.0	16.27
7.0	19.96
8.0	23.79
9.0	25.67
10.0	22.64

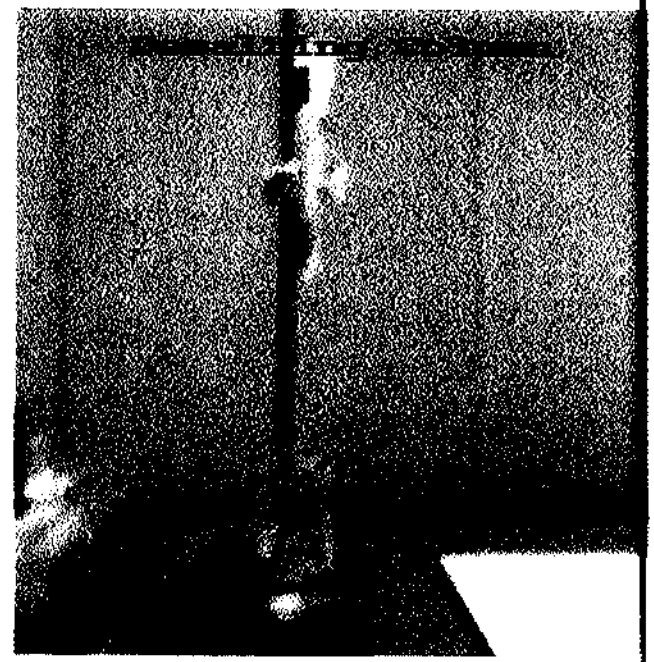
The enzyme was scaled up and the activity was increased to ten folds. The stability of the enzyme was also checked and was found optimum to be

4.8 PURIFICATION OF ALKALINE PROTEASE FROM POLYACRYLAMIDE IMMOBILIZED *Bacillus amyloliquefaciens*

Downstream process involves the purification of enzyme. The various steps involved in the purification process were carried out and the activity of the alkaline protease was found after each step which is given in the table – 4.21.

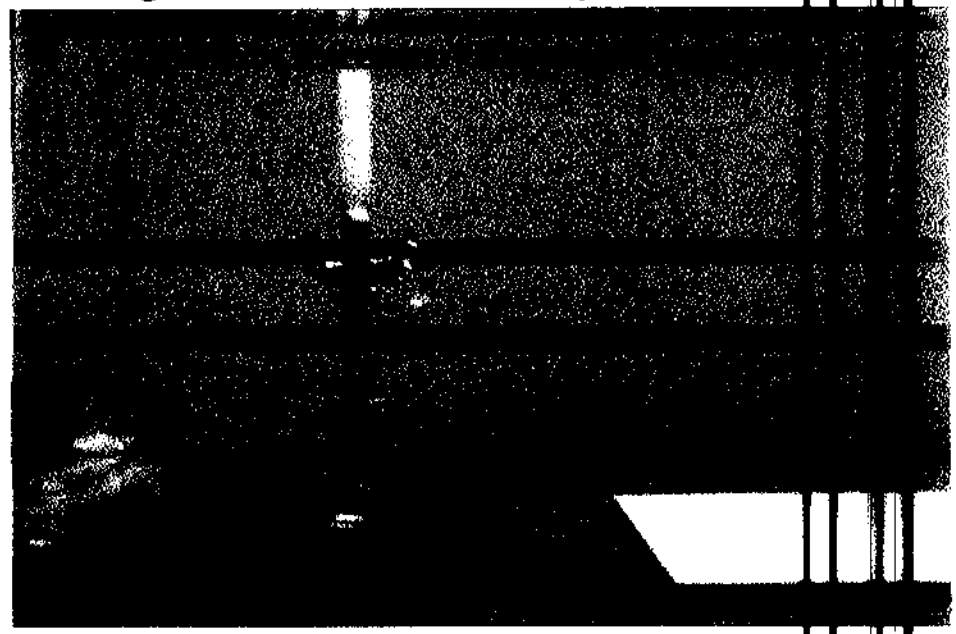
4.8.1 PROTEIN DESALTING WITH DESALTING COLUMN

Figure – 4.3 Purification using desalting column



4.8.2 PURIFICATION OF PROTEIN BY DEAE CELLULOSE ANION EXCHANGER

Figure – 4.4 Purification using DEAE column



4.8.3 MOLECULAR WEIGHT CHARACTERISATION OF ALKALINE PROTEASE USING SDS PAGE

Figure – 4.5 SDS PAGE

The SDS PAGE was carried out with alkaline protease obtained in each step of purification. The molecular weight of alkaline protease was found to be approximately 30000 Da.

The concentration of protein obtained from ammonium sulphate precipitation, protein desalting and DEAE cellulose steps are calculated from the tyrosine calibration curve and the specific activity was calculated by the formula

$$\text{Specific activity (U/mg)} = \frac{\text{Activity (U/ml)}}{\text{Concentration of protein (mg/ml)}}$$

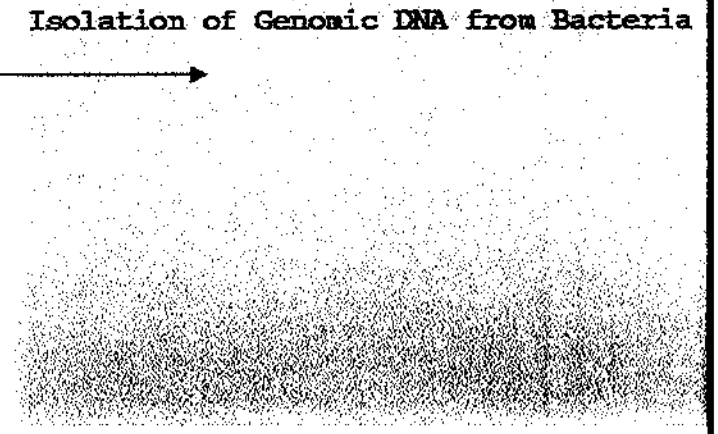
Table – 4.22 Activity of alkaline protease in various purification steps

Steps	Activity (U/ml)	Specific activity (U/mg)
Ammonium Sulphate Precipitation	20.17	70.77
Protein Desalting	17.86	76.32
DEAE Cellulose	14.73	100.86

From table – 4.21, it was clear that the specific activity of alkaline protease increases from one step to another in the purification process.

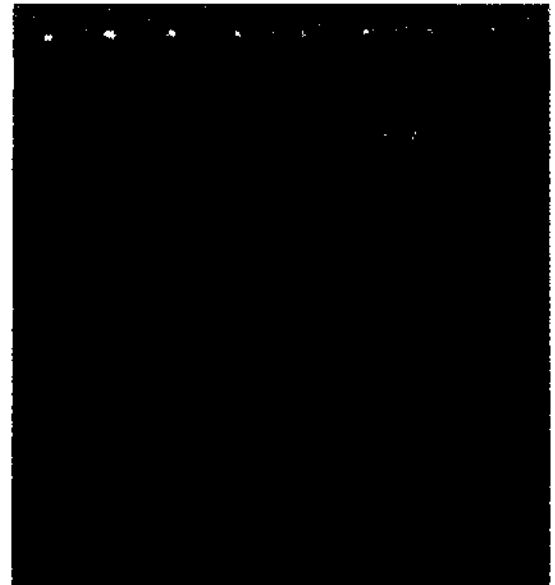
4.9 ISOLATION OF DNA

Figure – 4.6 Isolation of DNA from *Bacillus amyloliquefaciens*



4.10 POLYMERASE CHAIN REACTION

Figure – 4.7 PCR

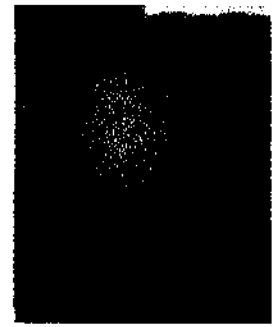


yield products of improved quality and reduce the use of hazardous and polluting chemicals.

4.11.2 DESTAINING OF BLOOD

Figure – 4.10 Cloth with blood stain before applying enzyme

Figure – 4.11 Cloth with blood stain after applying enzyme



In case of removing blood stain from cloth, it was seen that the protease enable to remove blood stain very easily without addition of any detergent. This protease showed high capability for removing proteins and

0.1880

5. SUMMARY AND CONCLUSION

Bacillus amyloliquefaciens was used for the production of alkaline protease with molasses as the sole carbon source. The concentration of molasses was optimized to be 5% in the production medium which favoured the highest enzyme production. The stability of the crude enzyme was checked for various temperature and pH. The activity of the enzyme was found to be optimum at 50°C and at pH 9.0.

The *Bacillus* cells were immobilized with different types of entrapment techniques. The polyacrylamide immobilization of the cells showed higher protease activity than other immobilizations. The temperature for the protease from immobilized cells was optimum at 50C and the pH was optimum at 9.0.

The scaled up production of the enzyme was carried out using polyacrylamide immobilized cells in airlift fermenter with a working volume of 2.5 litres. The enzyme activity was increased to 5 to 10 folds. This was followed by the downstream processing i.e. purification of the enzyme. Ammonium sulphate precipitation was carried out with 80% ammonium sulphate and finally with the DEAE column.

The application of alkaline protease was also checked by using the enzyme in dehairing of skin and destaining of blood. Finally the alkaline

6.0 APPENDICES

6.1 PRODUCTION MEDIUM

6.1.1 MEDIUM COMPOSITION

Carbon source (molasses)	-	(diff. conc)
Nitrogen source (soy bean flour)	-	0.3%
Salt solution	-	5.0%

Adjust the pH to 9.0

6.1.2 SALT SOLUTION

Magnesium Sulphate	-	5.0 g/l
Potassium Dihydrogen Phosphate	-	5.0 g/l
Ferrous Sulphate	-	0.1 g/l

Adjust the pH to 7.0.

EFFECT OF TEMPERATURE ON CRUDE ALKALINE PROTEASE ACTIVITY

6.2 ASSAY MATERIALS

6.2.1 CASEIN SOLUTION

Tris HCl	_	7.8g
Casein	_	10g

Adjust the pH to 8 and make up the volume to 1 liter.

6.2.2 TCA SOLUTION

TCA	_	5%
Sodium acetate		9%

6.3 IMMOBILIZATION OF *Bacillus amyloliquefaciens* USING SODIUM ALGINATE

3% SODIUM ALGINATE

Dissolve 3 grams of sodium alginate in 100 ml of distilled water.

6.4 IMMOBILIZATION OF *Bacillus amyloliquefaciens* USING GELATIN

6.4.1 20% GELATIN

Dissolve 20 gms of gelatin in 100 ml of distilled water and autoclave it at 121°C for 15 minutes.

6.4.2 5% GLUTERALDEHYDE

Make up 5 ml of gluteraldehyde to 100 ml with distilled water.

Acetate

6.5 PHOSPHATE BUFFER 0.1 M (pH – 8)

(1.0M) Potassium Dihydrogen Phosphate	-	61.5ml
(1.0M) Dipotassium Hydrogen Phosphate	-	38.5ml

Dilute the combined 1M stock solutions to 1 liter with distilled water.

6.6 SODIUM CHLORIDE (25mM)

Sodium Chloride	-	1.461g
Distilled Water	-	1 liter

6.8 REAGENTS FOR SDS PAGE

6.8.1 ACRYLAMIDE MONOMER (STOCK SOLUTION 30:0.8)

Acrylamide	-	30g
Bisacrylamide	-	0.8g

Add distilled water to make the final volume to 100ml .Store it in a brown bottle.

6.8.2 SEPERATING GEL MIX

Acrylamide stock solution	-	5 ml
4x separating gel buffer	-	3.75 ml
10% SDS solution	-	0.15 ml
Distilled water	-	6.05 ml
10% APS	-	85 µl
TEMED	-	30 µl

6.8.3 STACKING GEL MIXTURE

Acrylamide stock solution	-	0.665 ml
4x stacking gel buffer	-	1.25 ml
10% SDS	-	0.05 ml
Distilled water	-	3.0 ml
10%APS	-	35 µl
TEMED	-	3.0 µl

6.8.4 SEPERATING GEL BUFFER (4X) PH 8.8

Tris	-	18.15g
Distilled water	-	75ml

Adjust the pH with 0.1 M HCL and make the final volume to 100ml with distilled water.

6.8.5 STACKING GEL BUFFER (4X) pH 6.8

Tris	-	1.5g
Distilled water	-	20ml

Adjust the pH with 1M HCL and make the final volume to 25 ml with distilled water.

6.8.6 SDS 10%

SDS	-	0.1g
Distilled water	-	10ml

6.8.7 AMMONIUM PER SULPHATE (APS) 10% *FRESHLY PREPARED*

APS	-	0.1g
Distilled water	-	1ml

6.8.8 SAMPLE BUFFER (4X) pH 6.8

6.8.9 1X TRIS GLYCINE ELECTROPHORESIS BUFFER

25mM Tris

250 mM glycine (pH 8.3)

0.1% (w/v) SDS

Prepare a 5X stock solution of electrophoresis buffer by dissolving 15.1 g of Tris base and 94g of glycine in 900ml of deionized water. Then add 50 ml of 10% (w/v) stock solution of electrophoresis grade SDS and adjust the volume to 1000ml with water.

6.9 REAGENTS FOR PROTEIN ESTIMATION

6.9.1 TRICHLOROACETIC ACID

Dissolve 10gms of Trichloroacetic acid in 100 ml of distilled water.

6.9.2 SOLUTION A

Sodium carbonate	-	2g
Sodium hydroxide	-	0.1N
Distilled water	-	100ml

6.9.3 SOLUTION B

Sodium potassium tartarate	-	1g
Copper sulphate	-	0.5%

6.9.5 SOLUTION D

Folin and Ciocalteu's phenol reagent: Dilute with distilled water to form 1:2 dilutions.

6.9.6 SODIUM CHLORIDE 1M

Sodium chloride	-	58.44 g
Distilled water	-	1 liter

6.10 ENZYME SOLUTION

Lysozyme	-	20 mg/ml	
Tris HCl	-	20 mM	(pH 8.0)
EDTA	-	2 mM	
Triton	-	1.2%	

6.11 PCR MATERIALS

2X PCR master Mix

Forward Primer

Reverse Primer

Template DNA

Nuclease Free Water

REFERENCES

1. Adinarayana, K. and Ellaiah, P. (2002) 'Response surface optimization of the critical medium components for the production of alkaline protease by a newly isolated *Bacillus sp.*', J Pharm pharmaceut science Vol. 5(3), pp. 272-278.

2. Adinarayana, K., Ellaiah, P. and Siva Prasad, D. (2003) 'Production and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus subtilis PE-11', AAPS PharmSciTech. Vol. 4, pp. 56-60.

3. Adinarayana, K., Bezawada, J. and Poluri, E. (2005) 'Production of Alkaline Protease with Immobilized Cells of *Bacillus subtilis* E-11 in Various Matrices by Entrapment Technique', AAPS PharmSciTech Vol. 6(3), Article 48, pp. 391-397.

4. Banik, R.M. and Prakash, M. (2004) 'Laundry Detergent Compatibility of the Alkaline Proteases from *Bacillus cereus*', Microbial Reasearch, Vol. 159, pp. 113-120.

5. Chandi, C., Rath, V.R. and Subramanyam. (1998) 'Enhanced Protease and β- Lactamase Activity by Immobilization of a Thermophilic *Bacillus sp* Isolated from a Local Hot Spring in Orissa ,India ', Recent Trends In Biotechnology, Vol. 25, pp. 93-99.

6. Cumming, R.H., Tuffnell, J. and Street, G. (1984) 'The Dependence of Protein Release from *Bacillus amyloliquefaciens* On the Growth Phase in Batch Culture', Biotechnology and Bioengineering, Vol. 27,

8. Fukumoto, J. (1943) 'Studies on the production of bacterial amylase. I. Isolation of bacteria secreting potent amylases and their distribution', J. Agr. Chem. Vol. 19, pp. 487-503.

9. Gill, M., Vaughan, T., Fashola-Stone, E., Michael, N.P., Murdoch, H., Sims, M.S., Stubbs, S., Wigley, S. and Minton, N.P. (2002) '*Bacillus amyloliquefaciens* orthologue of *Bacillus subtilis ywrO* encodes a nitroreductase enzyme which activates the prodrug CB 1954', Microbiology Vol. 148, pp. 297-306.

10. Glenn, A.R., Both, G.W., McInnes, J.L., May, B.K. and Elliot, W.H. (1973) 'Dynamic state of the messenger RNA pool specific for extracellular protease in *Bacillus amyloliquefaciens*: its relevance to the mechanism of enzyme secretion', Mol. Biol. Vol. 73, pp. 221-230.

11. Gupta, R., Beg, Q.K., And Lorenz, P. (2002) 'Bacterial alkaline proteases: molecular approaches and industrial applications', Applied Microbiology and Biotechnology Vol. 59, no. 1, pp.15-32.

12. Jose, M., Sanchez-Puelles, J.M., Jose L. and Garcia, E. (1992) 'Immobilization and single-step purification of fusion proteins using DEAE-cellulose', Eur. J. Biochem. Vol. 203, pp. 53- 159.

13. Kumar, C.G., Joo, H.S., Park, G.C., Paik, S.R. and Chang, C.S. (2003) 'Oxidant and SDS-stable alkaline protease from *Bacillus clausii* I-52; production and some properties', Journal of Applied Microbiology Vol. 95, pp. 267-272.

14. Kunitz, M. (1947) 'Crystalline soyabean Trypsin Inhibitor and General Properties', Journal of General Physiology Vol. 30, pp. 291-310.

17. Motoki, K., Keiichi, M., Koji, S. and Tadayuki, I. (1988) 'Highly Thermostable Neutral Protease from *Bacillus steaothermophilus*', Journal of Fermentation Technology Vol. 66(1), pp. 13-17.

18. Naidu, K.S.B. and Devi, K.L. (2005) 'Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran', African Journal of Biotechnology Vol. 4(7), pp. 724-726.

19. Nogueria, E., Beshay, U. and Moreira, A. (2006) 'Characteristics of Alkaline Protease Enzyme Produced by *Teredinobacter turnirae* and its Potential Applications as a Detergent Additive', Deutsche Lebensmittel- Rundschau, Vol. 105, pp. 205-210.

20. Qixian, Z., Norihiro, T., Shigeyoshi, M. and Shigezo U. (1983) 'Increased Production of α-Amylase by *Bacillus amyloliquefaciens* in the presence of Glycine'. Applied and Environmental Microbiology Vol. 46, No. 1, pp. 293-295.

21. Rampton, J. H., Anglemier, A. F., and Montgomery M. W. (1965) 'Fractionation of Bovine Sarcoplasmic Proteins by DEAE-Cellulose Chromatography', Journal of Food Science Vol. 30(4), pp. 636-640.

22. Scawen, M.D., Atkinson, A. and Darbyshire, J. (1980) 'Large Scale Enzyme Purification', Journal of Applied Protein Chemistry Vol. 17, pp. 281-324.

23. Yeng J.K., Shih, I.L., Tzeng, Y.M. and Wang, S.L. (2000) 'Production and purification protease from a *Bacillus* subtilis that can deproteinize crustacean wastes', Enzyme and Microbial Technology Vol. 26, pp. 287-301.