IJNRD.ORG ISSN : 2456-4184
INTERNATIONAL JOURNAL OF NOVEL RESEARCH AND DEVELOPMENT (IJNRD) | IJNRD.ORG
An International Open Access, Peer-reviewed, Refereed Journal

Evaluation of wound healing activity of alkaline protease gel formulation in wistar rats.

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Abstract : STUDIES ON PRODUCTION, CHARACTERIZATION AND APPLICATIONS OF MICROBIAL ALKALINE PROTEASE

For industrial use, only large scale production of alkaline proteases can suffice the requirement. Industrial production of alkaline proteases can be carried out by solid-state and submerged fermentations. Media composition particularly carbon and nitrogen source and process parameters such as temperature, pH, agitation speed , greatly influence the enzyme production. Each micro-organism producing alkaline protease requires a different medium and process conditions. The effect of carbon and nitrogen sources on the enzyme production has been studied extensively. Akhavan Sepahy and Jabalameli studied the effect of various culture conditions on the production of an extracellular protease by *Bacillus* sp. and reported that sucrose and corn steep liquor are the best substrate for enzyme production. Some alkaline proteases also require metal ions in the form of salts in the production media. It has been shown that FeSO4.7H2O and MgSO4.7H2O enhanced the protease production by *Bacillus subtilis* RSKK96 Since cost of fermentation media is considerable, production of alkaline proteases have also been carried out using different agro industrial wastes (green gram husk, chick pea, wheat bran, rice husk, lentil husk, cotton stalk, crushed maize, millet cereal), tannery wastes, shrimp wastes, date wastes etc.. Most of the microorganisms produce alkaline proteases at pH 8-9 and temperature of 32-450C.presents different physiochemical parameters for maximum production of alkaline proteases. Researchers are in a continuous process of optimizing production to achieve maximum yield and economical use of available resources. Traditionally, scientists have adopted “one variable at a time” strategy where each variable is optimized independently. This is very time consuming, expensive and does not reflect true optimum when a large number of variables are involved because of interference by interaction between them. Recently,

a number of statistical methods have been developed such as Taguchi methodology, Plackett–Burman design and response surface methodology (RSM) for optimization to achieve rapid and better understanding of interaction between various variables using a minimum number of experiments .

1.INTRODUCTION

1.1Enzymes

A substance produced by a living organism which acts as catalyst to bring about a specific biochemical reaction. Enzyme-substrate reaction.

1.2 Enzymatic reaction

Enzymes are the catalytic cornerstones of metabolic activities of living being and catalyse most of the reactions in living organisms. Classically enzymes work by providing an alternative path of lower activation energy for reactions and dramatically accelerating its rate. The term enzyme was coined by German physiologist Wilhelm Kuhne and was derived from the Greek word ενζυμον. Various workers like Buchner, Sumner, Northrop, Stanley, Phillips and several others showed that enzymes are specialized proteins. Enzyme focused research is of importance not only to biological community but also to process designers/engineers, chemical engineers and researchers working in other scientific fields. Enzymes being proteins possess properties such as specificity towards the reactions they catalyse and the substrates on which they act upon. Apart from basic approach, enzymes are widely studied from an industrial point of view. Enzymes have been used since the dawn of mankind in cheese manufacturing and indirectly via yeasts and bacteria in food manufacturing. The past few decades of the twentieth century have witnessed spectacular advances and betterment of living standards due to the beneficial integration of enzyme technology with scientific progress and rapid translation of laboratory findings into practical technologies and commercial scale manufacturing processes.

1.3 Enzyme Nomenclature

Enzymes are identified by a common nomenclature system based on the description of what function it performs in the cell and ends with a common phrase. The International Union of Biochemistry and Molecular Biology and the International Union of Pure and Applied Chemistry developed a nomenclature system wherein each enzyme is given an

Enzyme Commission Number called as the EC number. Accordingly the top level classes based on the mechanism of operation of an enzyme are:

1. Oxidoreductases: catalyse oxidation/reduction reactions
2. Transferases: transfer a functional group
3. Hydrolases: catalyse the hydrolysis of bonds with addition of water
4. Lyases: remove groups from their substrates
5. Isomerases: catalyze isomerisation changes within a single molecule
6. Ligases: join two molecules with covalent bonds

From an industrial standpoint, only a limited number of enzymes are commercially available and few of them have found applications in large quantities. More than 75% of industrial enzymes are hydrolases (Rao et al., 1998). More than fifty commercial industrial enzymes are available and their number is being increased steadily.

1.4. Proteases

Proteases are among the oldest and most diverse families of enzymes known and are involved in every aspect of organisms function. They constitute a very large and complex group of hydrolytic enzymes that degrade proteins into small peptides and amino acids. Proteases catalyze the addition of water across amide (and ester) bonds to cleave using a reaction involving nucleophilic attack on the carbonyl carbon of the sessile bond. They differ widely in their properties such as substrate specificity, active site and catalytic mechanism and possess different profiles for mechanical stress, chemical environment, pH and temperature for stability and activity. Because of their broad substrate specificity, proteases have a wide range of applications such as in leather processing, detergent formulations, baking, brewing, meat tenderization, peptide synthesis, cheese manufacture, soy sauce production, protein hydrolysate, pharmaceutical industry, waste treatment, silk industry, organic synthesis, recovery of silver from waste photographic film, as well as analytical tools in basic research and have high commercial value (Godfrey and West, 1996). Among the bulk of industrial enzymes, proteases from plant, animal, and microbe constitute around 60 % of the total worldwide enzyme sales (Kunamneni et al., 2003; Merheb-Dini et al., 2009). Currently, the largest share of the enzyme market has been held by detergent proteases which are active and stable at alkaline pH. They are also important from a physiological point of view, as they are involved in many cellular processes like protein turn over and digestion as well as fungal morphogenesis, spore formation and spore germination. Yet, there is a continued search for proteases having novel properties with known and newer applications. Proteases are enzymes occurring everywhere in nature be it inside or on the surface of living organisms such as plants, animals and microbes These enzymes carry out proteolysis i.e. Break down proteins by hydrolysis of the peptide bond that exists between two amino acids of a polypeptide chain. The proteases available today in the market are derived from microbial sources. This is due to their high productivity, limited cultivation space requirement, easy genetic manipulation, broad biochemical diversity and desirable characteristics that make them suitable for biotechnological applications [1]. The world enzyme market is currently at $5.1 billion and is expected to rise by 6.3% annually by 2013. Since proteases share a major part of the global enzyme market, an upward trend in their demand is

1.5 SOURCES OF ALKALINE PROTEASES

Alkaline proteases are obtained from various microbial sources such as bacteria, fungi and certain yeasts. Of all the microbial sources, bacterial proteases are of particular interest due to their various applications in industries such as detergent, textile, leather, food and feed industry. A major source of

bacterial alkaline proteases is *Bacillus* species, which has been studied extensively. Some fungal species are also known to produce alkaline proteases of industrial use, of which *Aspergillus* species has been extensively studied. A very few studies exist on yeast species. Alkaline proteases from *Aureobasidium pullulans, Yarrowia lipolytica, Issatchenkia orientalis.*

1.6 STUDIES ON PRODUCTION, CHARACTERIZATION AND APPLICATIONS OF MICROBIAL ALKALINE PROTEASE

For industrial use, only large scale production of alkaline proteases can suffice the requirement. Industrial production of alkaline proteases can be carried out by solid-state and submerged fermentations. Media composition particularly carbon and nitrogen source and process parameters such as temperature, pH, agitation speed , greatly influence the enzyme production. Each micro-organism producing alkaline protease requires a different medium and process conditions. The effect of carbon and nitrogen sources on the enzyme production has been studied extensively. Akhavan Sepahy and Jabalameli studied the effect of various culture conditions on the production of an extracellular protease by *Bacillus* sp. and reported that sucrose and corn steep liquor are the best substrate for enzyme production. Some alkaline proteases also require metal ions in the form of salts in the production media. It has been shown that FeSO4.7H2O and MgSO4.7H2O enhanced the protease production by *Bacillus subtilis* RSKK96 Since cost of fermentation media is considerable, production of alkaline proteases have also been carried out using different agro industrial wastes (green gram husk, chick pea, wheat bran, rice husk, lentil husk, cotton stalk, crushed maize, millet cereal), tannery wastes, shrimp wastes, date wastes etc.. Most of the microorganisms produce alkaline proteases at pH 8-9 and temperature of 32-450C.presents different physiochemical parameters for maximum production of alkaline proteases. Researchers are in a continuous process of optimizing production to achieve maximum yield and economical use of available resources. Traditionally, scientists have adopted “one variable at a time” strategy where each variable is optimized independently. This is very time consuming, expensive and does not reflect true optimum when a large number of variables are involved because of interference by interaction between them. Recently, a number of statistical methods have been developed such as Taguchi methodology, Plackett–Burman design and response surface methodology (RSM) for optimization to achieve rapid and better understanding of interaction between various variables using a minimum number of experiments [30].

1.7 CHARACHTERIZATION OF ALKALINEPROTEASES

Alkaline proteases from different sources have been characterized by various workers in order to use them for specific purposes. For example alkaline proteases with broad pH range activity, high thermostability and bleach stable find application in detergent and leather industry. The alkaline protease isolated from *Pseudomonas aeruginosa* is active at a broad pH range of 6–11 and a

temperature range of 25–650C. The studies showed that the purified enzyme retains its activity in surfactants and bleaching agents. These properties indicate its possible use in the detergent industry .

1.8 IMMOBILIZATION OF ALKALINE PROTEASES

Despite of various advantages, the use of enzymes in industrial applications has been limited due to their high cost of production, instability and expensive recovery which restrict the repeated use of the enzyme. This led to technological developments in the field of immobilized enzymes/whole cells systems which offer the possibility of a wider and more economical use of enzymes. Several attempts have been made to increase stability to temperature, pH, and organic recovery and reuse of enzymes by using various techniques of enzyme immobilization. Whole cell immobilization technique has been employed for higher productivity of alkaline proteases by protecting the cells from shear forces, and advantage .and easy recovery of products .Cell free immobilization techniques have also been widely used for the production of proteases. The alkaline proteases are immobilized on solid support matrix by adsorption, covalent binding, ionic binding, cross linking and entrapment. A few examples of the immobilization of whole cells as well as cell free supernatants using various matrices are listed in Table 6.

1.9 MOLECULAR CHARACTERIZATION OF ALKALINE PROTEASES

Attempts have been made to deduce the nucleotide and amino acid sequences of alkaline proteases from various sources. These sequences not only help in deriving the primary structure, but also in identifying various functional regions of the proteases. Studies of DNA and protein sequence homology are important for a variety of purposes and have therefore become routine in computational molecular biology. They serve as a prelude to phylogenetic analysis of proteins and assist in predicting the secondary structure of DNA and proteins, and elucidating the structure function relationship of proteases. Alkaline protease from marine bacterium strain YS-80-122 with 463 amino acid residues.

1.10 USES OF ALKALINE PROTEASES

Alkaline proteases are one of the most important classes of proteases from an industrial point of view, occupying a major share of the total enzyme market. Use of alkaline proteases as active ingredients in detergents is the largest application of this enzyme. They are also widely used in leather industry, medical diagnostics, recovery of silver from X-ray films, silk degumming, food and feed industry etc. Due to their vast applications in the industrial processes, many companies started manufacturing them at commercial level. The table 7 gives the commercial manufacturers of alkaline proteases with their product trade name and different applications.

1.11 Detergent industry

Alkaline proteases have contributed greatly to the development and improvement of modern household and industrial detergents. They are effective at the moderate temperature and pH values that characterize modern laundering conditions in industrial & institutional cleaning. enzymes used in laundry industry are proteases, lipase, cellulases, amaylses etc. Of these, alkaline protease find a major application as

1.12 Leather industry

Soaking, dehairing of hides and skins and bating have traditionally being carried out by using different chemicals which poses a high tannery waste pollution threat. Hence, proteases with a pH optimum around 9–10 are widely used in soaking to facilitate the water uptake of the hide or skin. Alkaline proteases with elastolytic and keratinolytic activity are used for dehairing and bating process to obtain a desired grain, softness and tightness of leather in a short time. Alkaline proteases with keratinolytic activity have been reported for remarkable dehairing properties . A novel protease showing keratinolytic activity from *B. subtilis* has been studied as a potential for replacing sodium sulfide in the dehairing process of leather industry. Verma *et al.* [31] showed the use of protease from *Thermoactinomyces* sp. RM4 for dehairing goat hides.

1.13 Chemical Industry

A high stability in the presence of organic solvents is a feature which is highly desired in applications involving biocatalysis in non-aqueous medium for peptide synthesis. Alkaline proteases from *Aspergillus flavus*, *Bacillus pseudofirmus* SVB1, *Pseudomonas aeruginosa* PseA have shown promising results for potential of peptide synthesis due to their organic solvent stability.

1.14Medical Uses

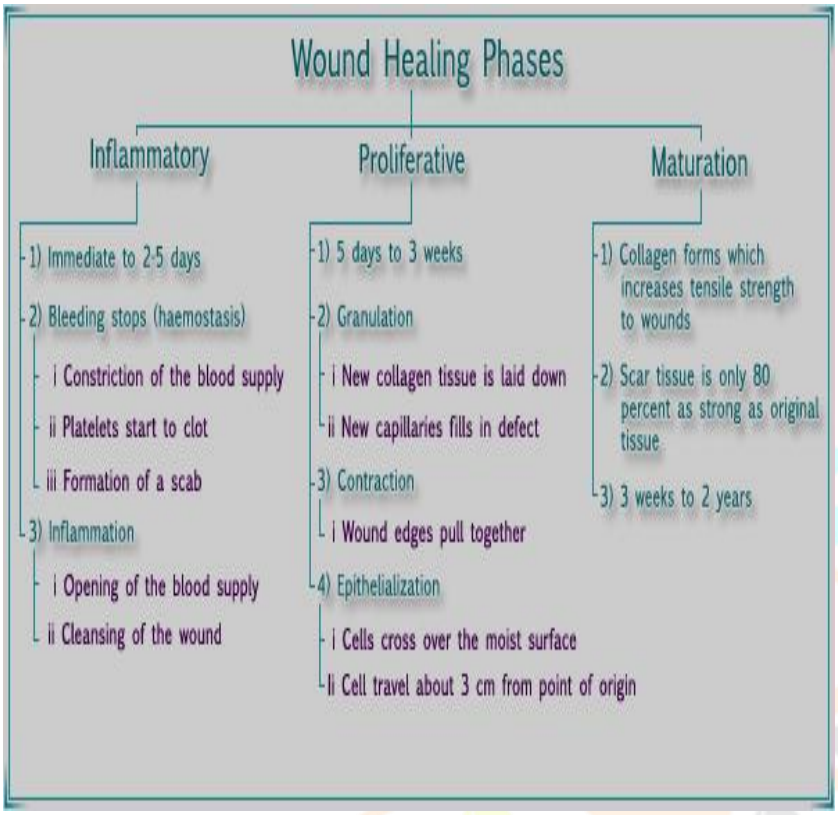
The use of immobilized alkaline protease from *Bacillus subtilis* possessing therapeutic properties has been studied for development of soft gel-based medicinal formulas, ointment compositions, gauze, non-woven tissues and new bandage materials . Oral administration of proteases from *Aspergillus oryzae* has been used as a diagnostic aid to correct certain lytic enzyme deficiency syndromes . Alkaline-fibrinolytic protease have been reported to preferentially

1.15 Wound healing

Is an intricate process where the skin or other body tissue repairs itself after injury? In normal skin, the epidermis (surface layer) and dermis (deeper layer) form a protective barrier against the external environment. When the barrier is broken, an orchestrated cascade of biochemical events is quickly set into motion to repair the damage. (Nguyen, D.T et al., 2009, Rieger, S et al., 2014) This process is divided into predictable phases: blood clotting (hemostasis), inflammation, the growth of new tissue

(proliferation), and the remodelling of tissue (maturation). Sometimes blood clotting is considered to be part of the inflammation stage instead of its own stage. (Stadelmann, WK et al., 1998)

Chart1.16 wound healing phase



1.17 Hemostasis (blood clotting):

Within the first few minutes of injury, platelets in the blood begin to stick to the injured site. This activates the platelets, causing a few things to happen. They change into an amorphous shape, more suitable for clotting, and they release chemical signals to promote clotting. This results in the activation of fibrin, which forms a mesh and acts as "glue" to bind platelets to each other. This makes a clot that serves to plug the break in the blood vessel, slowing/preventing further bleeding. (Rasche, H et al., 2001, Versteeg, H. H et al., 2013)

1.18 Inflammation:

During this phase, damaged and dead cells are cleared out, along with bacteria and other pathogens or debris. This happens through the process of phagocytosis, where white blood cells "eat" debris by engulfing it. Platelet-derived growth factors are released into the wound that cause the migration and division of cells during the proliferative phase.

1.19Proliferation (growth of new tissue):

In this phase, angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction occur. (Midwood, K.Set al., 2004)In angiogenesis, vascular endothelial cells form new blood vessels.(Chang, HY et al., 2004) In fibroplasia and granulation tissue formation, fibroblast grow and form a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin.(Midwood, K.Set al., 2004) Concurrently, re-epithelialization of the epidermis occurs, in which epithelial cells proliferate and 'crawl' atop the wound bed, providing cover for the new

tissue.(Garg, H.G. et al., 2000) In wound contraction, my fibroblasts decrease the size of the wound by gripping the wound edges and contracting using a mechanism that resembles that in smooth muscle cells. When the cells' roles are close to complete, unneeded cells undergo apoptosis. (Midwood, K.Set al., 2004)

1.20 Maturation (remodelling):

During maturation and remodelling, collagen is realigned along tension lines, and cells that are no longer needed are removed by programmed cell death, or apoptosis. The wound healing process is not only complex but also fragile, and it is susceptible to interruption or failure leading to the formation of non-healing chronic wounds. Factors that contribute to non-healing chronic wounds are diabetes, venous or arterial disease, infection, and metabolic deficiencies of old age. (Enoch, S et al., 2004)Wound care encourages and speeds wound healing via cleaning and protection from reinjury or infection. Depending on each patient's needs, it can range from the simplest first aid to entire nursing specialties such as wound, ostomy, and continence nursing and burn center care.

Phases of Wound Healing

Keywords: Area of interest in present research

1. Optimize production of fungal alkaline protease.
2. To prepare bioactive peptides and study on its potential app
3. Formulation of biocompatible wound healing gel using protease.

2.LITERATURE REVIEW

1.(Kunamneni et al., 2003; Merheb-Dini et al., 2009)Among the bulk of industrial enzymes, proteases from plant, animal, and microbe constitute around 60 % of the total worldwide enzyme sales.

2. (Ashok Pandey et al., 2004) A comparative study was carried out on the production of neutral protease using agro-industrial residues as substrate in solid-state fermentation (SSF) and submerged fermentation (SmF).

3. (M. Chandra et al., 2005) produced, purified and partially characterised protease from marine source, Engyodontium album isolated from marine sediment produced protease, which was active at pH 11. Process parameters influencing the production of alkaline protease by marine E. album was optimized. Particle size of <425 mm, 60% initial moisture content and incubation at 25 8C for 120 h were optimal for protease production. The organism has more than one optimal pH (5 and 10) for maximal enzyme production. Sucrose as carbon source, ammonium hydrogen carbonate as additional inorganic nitrogen source and amino acid leucine enhanced enzyme production during SSF. The protease was purified and partially characterized.

4. (H. Genckal et al., 2005) explained Bacillus strains isolated under extreme alkaline conditions (Izmir, Turkey), were screened and identified for high alkaline protease activity. Strains with high protease yields were optimized with respect to inoculum concentration, temperature, agitation speed, initial medium pH and incubation time.

5. (Mohamed Hajji et al., 2008)Medium composition and culture conditions for the bleaching stable alkaline protease production by Aspergillus clavatus ES1 were optimized. Two statistical methods were used. Plackett–Burman design was applied to find the key ingredients and conditions for the best yield. Response surface methodology (RSM) including full factorial design was used to determine the optimal concentrations and conditions.

6. (Pilanee Vaithanomsat et al., 2008) investigated the method for protein hydrolysate.

7. (Daniela de Araújo Viana et al., 2010) examined cow raw milk from dairy cooperatives for its microbial composition.Although qualitative and quantitative tests for extracellular proteolytic activity were positive for all the species isolated, Candida buinensis showed the highest response (23.5 U/mg); therefore, it was selected for subsequent investigation. The results of fermentations carried out at variable temperature, pH, and soybean flour concentration, according to a 23 full factorial design, demonstrated that this yeast ensured the highest production of extracellular proteases (573 U/mL) when cultivated at 35 °C, pH 6.5, and using soybean flour concentrations in the range 0.1–0.5% (w/v).

8. (Preeti Chanalia et al., 2011) reviewed proteases are potential target for developing therapeutic agents against life-threatening diseases such as bacterial infection, cancer, malaria and AIDS.

9. (RJ. Patel et al., 2011) has collected important information from various research papers that protease has been used for cosmetics, medical, polymer materials and other applications due to its ant oxidative, antibacterial, anti-microbial, anti-wrinkle, wound healing, UV resistant and moisture-absorbing and –desorbing properties.

10. (Jiri MLCEK et al., 2014) review summarizes up-to-date knowledge about using edible insects in human, veterinary medicine and agriculture, especially from the viewpoint of the biological and chemical content of active substances and the possibilities of further use in these areas. Insects in Asian, African, American and South Central American cultures are mainly nutritional components. In Europe and other developed countries, however, insect is used in different ways, and this issue is viewed from a different angle. Insects are mainly used as feed for animals, in the organic waste recycling systems, in human and veterinary medicine, material production (such as silk) etc.

11. (Hamlyn, 1997). During the course of human history fungi have been exploited in many ways. Even before the microorganisms were recognized they were used in making of beer, wine, bread, cheese, milk products and even for simple eating (edible mushrooms). The Babylonians used the yeast S.cerevisiae in the brewing of beer in 6000 BC (Pommerville, 2004). In the eastern world rice was used instead of malt or mashed grapes for ethanol production and Aspergillus oryzae was used for hydrolysis of rice starch (Purohit, 2001)._Mould was later on utilized for enzyme production Penicillium camemberti and P. roqueforti were used for secretion of different enzymes

12. (Satyanarayana, 2006). Enzymes are natures answer to many industrial and environmental challenges. Microbial enzyme plays a key role in microorganism's metabolic activities and ability to survive under various environmental conditions (Germano et.al. (1998). As a biocatalyst enzymes replace harsh chemicals in number of industrial process

13. (Voet, 2006). This capability has launched an initiative in scientific community dedicated to enzyme discovery and development. Because of enzymes very specific catalytic properties only small quantities of them are required to perform the desired conversions and product yields are often higher than those obtained with chemical based routes. Derived from renewable sources, they are fully biodegradable Nester et. a/. (2005).

14. (Prescott, 1996). The study of enzymes is a subject, which has a special interest because enzymes are of supreme importance in biology. In 1833 Payen and Persoz made first clear recognition of an enzyme and found that an alcohol precipitate of malt extract contained a thermolabile substance, which converted starch into sugars. They named them diastase Ineighteenth century many workers used the name 'ferment' for enzymes

15. (Adria and Demain, 2003). During the second half of the nineteenth century Liebig postulated that process of fermentation was due to

the action of chemical substances but according Pasteur the process of fermentation was inseparable from living cells

16. (Stryer, 2008). The name 'unorganized ferment' and 'organized ferment' were also used for extracted enzyme. In order to avoid these unsatisfactory names, Kuhne in 1878 introduced the name 'enzyme'

17. Pelczar et.al. (2002) Towards the end of the nineteenth century increasing knowledge in the structural and organic chemistry of biomolecules made it possible to study the specificity of enzymes. In 1897 Buchner succeeded in obtaining the fermentation system from yeast in a cell free extract. Emil Fisher gave the idea of enzyme specificity).

18. 1922 and 1928 (Laskin and Lechevalier, 1984). The serious purification of enzymes began after 1920. Willstatter and his colleagues carried out purification of enzymes between

19. (Dixon et.al.1979).The next important development was the preparation of enzymes in crystalline form. The first enzyme to be crystallized was urease by Sumner in 1926. This work was soon followed by classical isolation of crystalline proteolytic enzymes by Northrop and his colleagues (

20.(Lowry et.al. 1951) Studies on penicillin from Penicillium were carried out by McKee et.al. discovered a new technique for protein measurement with folin phenol reagent. Glucose was estimated by Miller (1959). Identification of fungal species was reported by Barnett (1969) and Ellis (1949). Methods for estimation of proteolytic activity in an organism is defined by Nakagawa (1970) and also quantitatively measured by Yasunobu and McConn (1970).

21. Margulis (1971). Identification of fungi by using new classification system was suggested by The effect of glucose and manganese on adenosine 3', 5' monophosphate levels during growth and differentiation of Aspergillus nidulans

was studied by Zonneveld (1976). Comparison of a-amylase activity from different assay method was analysed by Yoo et.al. (1987). Fungi have various applications in biotechnology (Wainwright, 1990 and Wainwright, 1995). Process for protein enrichment was analysed by Nigam and singh (1994).

22.Hombergh ven den et.al. (1997). Screening of fungi for enzyme producing ability was performed by Dehran and Davies (1999). Applications of industrial enzyme were reported by Ole Kirk et.a/. (2002). Protein quantitation and its biochemistry were studied by Walsh and Walsh (2002). Effects of process parameters on heterologous protein production in Aspergillus oryzae fermentation was studied by Wang et.al. (2003).

23. Sallam et.al. (2003) measured role of some fermentation parameters on cyclosporine production by Aspergillus terreus. Molecular and cellular biology of filamentous fungi was reported by Talbot (2005). Production of

enzymes from fungi was also reported by Nutan et.al. (2003). Physiology and biochemistry of Aspergillus was studied by Ward et.al. (2006). Shimizu et.a/. (2009) studied the proteomics of Aspergillus niduans.

24. Hashimoto et.al. (1972) In past century number of purified enzymes isolated was very small, whereas now the pure and crystalline enzymes exceeds more than fifteen hundred but still the requirement for purified enzymes are high. Thermostable protease had find major application in detergent industry as they have high temperature and pH tolerance and are able to withstand harsh conditions that occur during washing. found a thermostable acid protease produced by Penicillium duponti K1014, was a true thermophilic fungus isolated from compost.

25. . Gill and Modi (1981) They further characterized the acidic protease as thermo tolerant and acid sensitive suggested extracellular protease activity in Aspergillus nidulans. Many strains of the thermophilic fungus

Thermomyces lanuginosus were screened for their ability to produce proteases by Chuan Li et.a/. (1997). According to their finding selected strain Thermomyces lanuginosus P134, showed high enzymatic activity for proteases in submerged culture. They found two protease isoenzymes by SDS-PAGE which was serine protease.

26. Merheb et.al. (2007) A thermostable alkaline protease was purified and characterized by Kaur et.al. (1998) from a Bacillus polymyxa which was useful in laundry industries. A study on thermostable alkaline protease from alkalophilic Bacillus sp. /S-3 was performed by Purva et.al. (1998). Thermostable alkaline proteases from Bacillus sp. have attracted the attention of many researchers due to their use m detergents (Sumandeep et.al. 1999).partially

27.Sun-Young An et. al (2004).Metarhizium anisopliae cultures. The extracellular serine proteases are imas a silver recovery agent from used X-ray film was evaluated by Warin and

Tanticharoen (1999). Van Kuyk et.a/. (2000) carried out analysis of two Aspergillus nidulans genes encoding extracellular proteases. A gene, isp-b, encoding an intracellular serine protease from Bacillus sp WRD-2 was cloned and characterized by

28. Sandhya et.al. (2005) Proteases are used in laundry detergents for over 50 years to facilitate release of proteinaceous material in stains (blood and milk) and account for approx 25% of total worldwide sales of enzymes. Kumar and Bhalla (2004) had isolated new protease as a laundry additive from Bacillus sp. APR-4. Using a phylogenomic approach with 10 fungi of very different virulence and habitat Hu and Leger (2004) determine proteases in Ascomycetes.) observedneutral protease activity in A. oryzae.

3. NEED OF WORK

1.An enzyme novel fungal alkaline protease was partially purified and characterised.

2.In the present study production of protease has to be increase economically by using various inducers, media constituents, supplements, biological surfactants, etc.study its potential uses.

3.This study being focused on testing biocompatible wound healing gel in-vivo in Wistar rats.

4.To show their efficacy, rate of wound healing, wound size reduction and histopathological evaluation, also in vitro evaluation for physico chemical properties.

4.AIMS AND OBJECTIVES:

Isolation characterization and study the application of protease enzyme from microorganism.

4.1Objective:

1)Screening of fungi species for protease production.

2) Cultural, morphological, biochemical and physiological characterization of bioactive microorganism.

3)Optimization of fermentation parameter for protease production.

4)Isolation and purification of protease enzyme.

5)Characterization of protease enzyme.

6)Development of formulation of protease and characterization of formulation.

7)Evaluation of protease formulation for biological activity.

3) Formulation of wound healing gel and its evaluation.

4) In vivo studies of wound healing gel.

5.PLAN OF WORK

5.1Literature review

5.2. Procurement of material

5.3. Growth and maintenance of organism

5.4. Composition of medium

5.5. Fermentation

5.6. Enzyme assay

5.6.1. Substrate preparation

5.6.2. Buffer preparation

5.6.3. Other stock solutions preparation

5.7. Isolation of protease

5.7.1. Purification of protease by ammonium sulfate precipitation

5.7.2. Dialysis of protease

5.8. Isolation of protease

5.9 SDS-PAGE

5.10 Formulation and evaluation gel

5.10.1. Formula optimization and preparation of gel

5.10.2. Physical examination

5.10.3. Spreadability

5.10.4. pH determination

5.10.5. Rheological studies

5.10.7. Photographic comparison

5.10.8. Histopathological evaluation

6.MATERIALS AND METHOD

Aspergillus oryzae Microrganism purchased from agarkhar research institute,agarkhar road,pune

6.1List of Chemical/solvent:

Sr.no	Chemical/solvent	Name of manufacturer
1	Mannitol	Himedia,india
2	Nacl	Merck,india
3	Peptone	Himedia.india
4	Phosphate buffer	Sigma,india
5	Agar	Himedia,india
6	Ammonium sulphate	Merck,india
7	Beef extract	Himedia,india

8	Bovine serum albumin	Himedia,india
9	Casein	Himedia,india
10	Sodium carbonate	Qualigens india
11	Trichloro acetic acid	Qualigens india
12	Molecular weight marker	Bio-rad laboratories,inc
13	Folin ciocalteu reagent	Sigma-aldrich
14	K2HPO4	S.D Fine-chem ltd Mumbai
15	Nacl	Merck,india
16	Sodium dodecyl sulphate(SDS)	Sigma,usa

17	Sodium alginate	Merck,india
18	Trichloroacetic acid(TCA)	Merck,india

6.2List of equipment:

Sr.no	Name of equipment	Name of manufacturer
1	autoclave	Equitron,india
2	centrifuge	Remi,india
3	Deep freezer	New Brunswick scientific,usa
4	fermentor	New Brunswick scientific,usa
5	Heating mental	Remi.india
6	Hot air oven	Lab hosp,india
7	Incubator	Lab hosp,india
8	Incubator shaker	New Brunswick scientific,usa
9	Laminar air flow	Microfilt,india
10	Ph meter	Systronic,india
11	Uv/visible spectrometer	Model jasco v-530,japan
12	Weighing balance	Shemadzu,india
13	Lyophilizer	Lobconco,Czech republic
14	Inverted microscope	nikon

6.3List of other material:

Sr no	Material	Name of manufacturer
1	Centrifuge tubes(15,50ml)	Tarson
2	Dialysis membrane	Himedia
3	Micropipette variable volume(2-20,20-200,200-1000µl)	Eppendorf
4	Microtitre plates(96 well plates)	Tarson
5	Microtubes(0.5,1.5),2 ml)	Eppedorf

7.0Experimental work:

7.1Maintenance Of Organism

The fungal species was grown at 28˚ C and neutral pH (9.0 – 9.2). It sporulated after 7 days. The fungal species was maintained by periodic subculture on MGYP and PDA slants. The slants were incubated for 7 – 15 days at 28˚C. 7 days old MGYP slants were then used for developing inoculum. allowed grow in peptone water media for 48 hours and added in experimental medium flask for fermentation.

7.2Characterization of fungus:

Fungal Isolates Aspergillus Oryzae as a potent L-methioninase producers (Khalaf and El-Sayed, 2009), were selected for further morphological and molecular characterization. These isolates were identified phenotypically according to the universal key of Kebeish and El-Sayed 15281 Aspergillus identification (Raper and Fennell, 1965; Klich, 2002;Geiser et al., 2007). The fungal isolates were grown on Czapek'sagar for 10 days at 28 ± 1°C, then for 2 days at 37°C to confirm their species purity. The purified fungal isolates were used for further identification.

7.3Morphological identification:

Fungal inoculation were pointedly inoculated to Czapek's and PDA agar as prepared by (Raper and Fennell, 1965) then incubated for 15 days at 28°C. The macroscopic features as colony diameter, conidium color, extracellular exudates, pigmentation and reverse mycelium color were examined daily. Microscopical properties as conidial heads, fruiting bodies, degree of sporulation and identity of conidiogenous cells were examined by light microscope after staining by lactophenol solution according to their instructions of (Raper and Fennell, 1965).

7.4Microscopic observation:

The Peptone water media of the fungal isolate was prepared from their slope cultures after 8 days of incubation at 28°C. sample are taken in slide. then add lactophenol cotton blue indicator and then take in microscopical observation.

7.5Fermentation

All the glassware used for fermentation experiments were washed, dried and plugged with cotton and sterilized at 121° C (15 lbs pressure). Inoculum preparation was done by using 7 days old MGYP slants. A loopful of culture was inoculated in peptone water media and incubated for 48 hours and then this inoculum was further inoculated in experimental flasks. Fermentations were carried out in 500 mL Erlenmeyer flasks containing 100 mL of medium. 10% (v/v) inoculum from inoculum flask was added to the fermentation medium under sterile conditions. Flasks were incubated at 28˚ C on rotary shaker at 120 revolutions per minute (rpm). Fermentations were carried out for 192 hours. Samples were obtained at regular intervals of 72, 96, 120, 144, 168 and 192 hours respectively. The samples were centrifuged at 10,000 rpm for 10 minutes and the supernatant was checked for enzyme activity. The experiments were performed in triplicates and repeated twice. The results stated are mean of the triplicate sets and those in the graphs have been stated along with error bars with percentage.

7.6 Enzyme Assay

Protease activity (caseinolytic) was determined according to Kunitz. The reaction mixture contained an aliquot of suitably diluted enzyme and 10 mg Hammerstein casein in 0.05 M sodium carbonate buffer pH 9.0 in a total volume of 2 mL. After incubation at 50°C for 10 min, the reaction was terminated by the addition of 3 mL of 5% trichloroacetic acid (acidified with concentrated hydrochloric acid). The precipitate formed was filtered through Whatman No.1 filter paper after standing at room temperature for 30 min. The absorbance of trichloro acetic acid soluble fraction was measured at 280 nm. One unit of protease activity was defined as the amount of enzyme required to bring about an increase in absorbance of 1.0 unit per ml of reaction mixture per minute under the assay conditions.

7.7 Substrate Preparation

10% of Hammarsten grade casein added in 100 mL of 0.05 M Sodium carbonate (pH 9.2). .Buffer Preparation Reagents

1. Sodium carbonate, anhydrous

2. Sodium bicarbonate (Step 1) 0.2-M solution of anhydrous sodium carbonate (2.2 g/100 mL) and (Step 2) 0.2-M solution of sodium bicarbonate (1.68 g/100 mL) was prepared. 4 mL of carbonate solution from Step 1 and 46 mL of bicarbonate solution combined from Bring to 200 mL with H2O.

3)Other Stock Solution : 5% Acidified TCA 11.25mL of conc. Sulphuric acid was added in 25mL of 100% TCA and final volume 500mL adjusted by distilled water. .

Isolation of protease Partial Purification protease The first step in the purification of the protease is its partial purification by ammonium sulphate fractionation. To obtain a pure form of protein, a multi-step purification process was followed. Thus; a purer form of protein can be obtained than the preceding step. An attempt to purify the enzyme was made. Steps carried out in the partial purification of the enzyme were centrifugation, ammonium sulphate precipitation, dialysis, SDS-PAGE. At each step of purification, enzyme activity and protein content was estimated.

7.8Ammonium Sulphate Precipitation

The silk pupae protein is extracellular thus ammonium sulphate precipitation was the first step carried out in protein purification. SPP was concentrated using 0–90% precipitation and the additions of ammonium sulphate were done as per the standard table.

The supernatant was taken in an Erlenmeyer flask with a magnetic needle, on a magnetic stirrer. The precipitation was allowed to occur at 4° C overnight. This facilitated in the precipitation of proteins. The precipitated SPP was recovered by centrifugation (10,000rpm, 40C for 30 minutes) and then dissolved in fresh phosphate buffer (10 mM, pH -7) for next stage of purification.

7.9 Dialysis

The above sample obtained was subjected to dialysis. Dialysis tubing of cellulose membrane obtained from Sigma chemicals was cut off molecular weight 12,000 and boiled in a beaker containing distilled water to remove preservatives from the dialysis bags. The sample was dialysed against 10mM phosphate buffer (pH- 7.0) overnight at 4° C (3× buffer changes). Dialysis tubing was sealed with clip at one end and 10 ml of the sample with high salt concentration was loaded. Then it was sealed with clip on the other end. Bags were then placed in container containing 10mM phosphate buffer (pH-7.0) that was 100 times the volume of the sample in the bag. The entire apparatus was incubated overnight in cold. Following this, the molecular weight of protein was estimated.

7.10 SDS-PAGE

Molecular mass of the partially purified SPP was estimated by SDS PAGE, which was performed using 15% polyacrylamide gel by the method described by Laemmli, (1970). Molecular weight was determined using molecular weight markers of range 3 kDa to 96 kDa.

7.11 Lyophilization:

The material is first frozen and transferred to a drying chamber. During the drying stage, the material in the chamber is subjected to a high vacuum. Heat is applied carefully to the material, and a condenser is used in the chamber to collect the water. When water is leaving rapidly, its heat of vaporization is taken from the material and helps to keep it cool and safe. As the material dries, this

cooling diminishes so that it is possible to overheat and damage the material. Heat supplies the energy necessary for sublimation of the water. An ice crystal is composed of pure water that is rather rigidly confined in a crystal lattice. The molecules have natural vibrations, however, so that extra thermal energy increases the probability of breaking free. When the water molecule breaks free, it diffuses through the already dried surface of the solid and sublimes. As the water molecules diffuse and sublime , the thickness of the dry outer surface of the specimen increases, and thus more energy is required to transport the molecules through the dry shell. The actual force driving water vapor from the drying boundary, through the dry shell and to the specimen surface, is a concentration gradient, and not, as some would assume, the vacuum sucking on the sample. One milliliter of ice produces more than 1,000,000 ml. of water vapor at typical lyophilization cycle pressures.

The most important are listed below:

1. Separated drying chamber and ice condenser to reduce cross-contamination

2. Provision of an isolation valve between chamber and ice condenser to allow for end-point determination and simultaneous loading and defrosting

3. Construction of the chamber and ice condenser as pressure valves to allow for steam sterilization at 121 C or higher

4. Cooling and heating of the product -support shelves by a circulating intermediate heat-exchange fluid to give even and accurate temperature

5. Additional instruments to control, monitor, and record process variables

6. Movable product-support shelves to close the slotted bungs used in vials and to facilitate cleaning and loading

7. Automatic control system with safety interlocks and alarms, duplicated vacuum pumps, refrigeration systems, and other moving parts to enable drying to proceed without endangering the product in the event of mechanical breakdown

8.The steps required to lyophilize a product in a batch process can be summarized as follows:

a.Pretreatment / Formulation

b.Loading / Container (Bulk, Flask, Vials)

c.freezing (Thermal Treatment) at atmospheric pressure

d.Primary Drying (Sublimation) under vacuum

e.Secondary Drying (Desorption) under vacuum

f.Backfill & Stoppering (for product in vials) under partial vacuum

g.Removal of Dried Product from Freeze Dryer

7.12 Formulation And Evaluation Of Wound Healing Gel

In order to optimize the concentration of gelling agent to achieve proper consistency of the gel formulations were prepared with different gel gelling or thickening agents, various gums, Carboxy

methylcellulose sodium, sodium alginate, Hydroxypropyl Methylcellulose (HPMC) and different concentration of viscosity enhancer as 1, 2, 3, 4,5,6,7 and 8% were tried and finally gel that showed good spreadability and consistency was selected.

Table 7.13 formula

	F1	F2
Ingredient	Quantity	quantity
Protease	5%	10%
Sodium alginate	2%	2%

7.14Process of preparation

Gelling agent sodium alginate was slowly added to the protease gel with continuous stirring and heating on water bath (Temp. 60-70oC). Stirred continuously till a uniform gel was formed after 15-20 minute. Finally gel was placed in UV light for 20-25 min and stored in plastic container at room temperature.

7.15 Physical examination

The prepared gels were inspected visually for their colour and homogeneity. The spreadability(n=3) of the gel formulations was determined by measuring the spreading diameter of 1 g of gel between two horizontal plates (20 cm × 20 cm) after one min. The standardized weight tied on the upper plate was 125 g.

7.16 PH Determination

The pH was measured, at room temperature, in each gel sample using digital pH meter which was calibrated before each use with standard buffer solutions. The pH of the gel formulations was performed at 1, 10, 45 and 60 days after preparation to detect any pH changes with time.

7.17 In-Vivo Wound Healing Model

Animals

In vivo experiments were performed at Symbiosis school of biomedical sciences, Lavale, Pune. Wister rats (Male) weighing 250 ±20 gm. used and all the study was performed as per CPCSEA guidelines (CPCSEA/ QA/07/2015-16).The animals were housed in a standard individual metal cages and room maintained at temperature 22±1OC and relative humidity 55±5% with an alternating 12 h light–dark cycle. Food and water were provided ad libitum. All the experiments on animals were conducted after obtaining permission from Institutional Animal Ethical Committee of the Institute.

7.18Incision wound model

Animals were divided into four groups (six animals each). Body weights of the animals in grams are shown in following table . All animals of four groups were anesthetized with anesthetics ether, and a

para vertebral long incision of 4.4cm length were made through the skin and cutaneous muscle at a distance about 1.5 cm from the middle on right side of the depilated back. All group of animal were received sufficient amount of preparation applied externally. All the test formulations were applied once a day for 10 days starting from the day of incision. Wound-healing property was evaluated by wound length and wound closure time. The wounded area was measured immediately by placing a transparent paper over the wound and tracing it out on every alternate day.

Table 7.19 Body weight of animal:

		Groups		
Animal	A	B	C	D
1	245	240	243	244
2	269	266	263	268
3	250	254	248	258
4	244	276	248	245
5	270	246	263	256
6	256	254	262	254

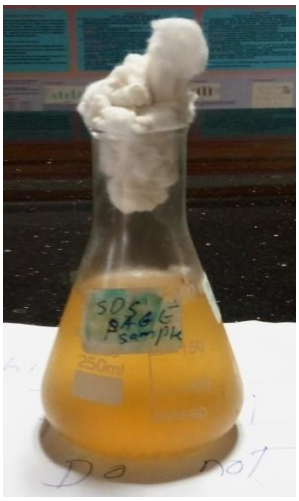
7.20Histopathological Evaluation

The animals were sacrificed on last of experiment day. Skin part of wounds were removed and collected in 10% formalin for histopathological observations. The tissues were processed in a Leica TP 1020 tissue processor, and then embedded in paraffin blocks using Leica EG 1160 paraffin embedder. The paraffin blocks were cut into ribbons of 4 mm using a Microm HM 360 microtome. The slides were stained in hemotoxylin and eosin using a Microm HMS-70 stainer. The permanent slides were made and evaluated for histopathological changes under Olympus BX51 microscope. The slides were coded to avoid possible bias before analysis.

8.RESULTS AND DISCUSSION

8.1Growth and Morphology

The present fungal species is a species. The pH range for growth of isolate was alkaline (9.0-9.8) and temperature 28c maintained.

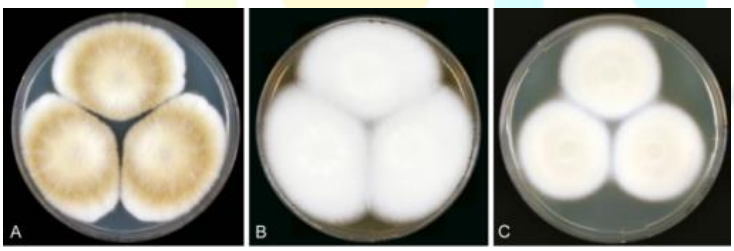


8.2Characterization of fungus:

Fungal Isolates Aspergillus Oryzae as a potent L-methioninase producers (Khalaf and El-Sayed, 2009), were selected for further morphological and molecular characterization. These isolates were identified phenotypically according to the universal key of Kebeish and El-Sayed 15281Aspergillus identification (Raper and Fennell, 1965; Klich, 2002; Geiser et al., 2007). The fungal isolates were grown on Czapek's agar for 10 days at 28 ± 1°C, then for 2 days at 37°C to confirm their species purity. The purified fungal isolates were used for further identification.

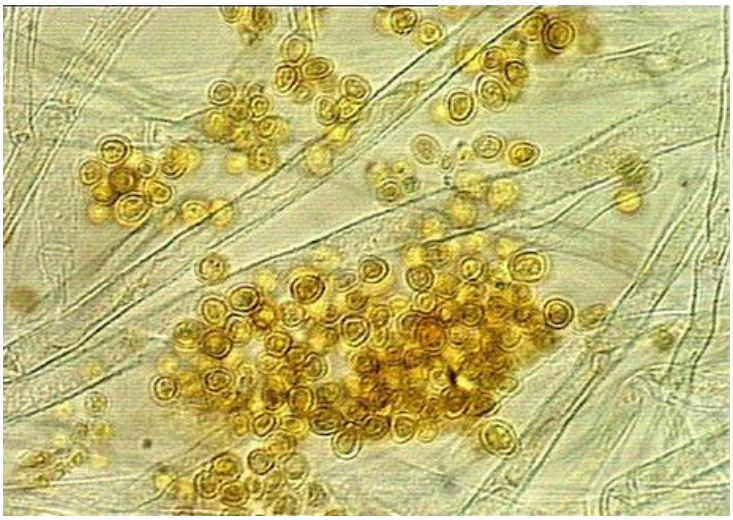
8.3Morphological identification

Fungal inoculation were pointedly inoculated to Czapek's and PDA agar as prepared by (Raper and Fennell, 1965) then incubated for 15 days at 28°C. The macroscopic features as colony diameter, conidium color, extracellular exudates, pigmentation and reverse mycelium color were examined daily. Microscopical properties as conidial heads, fruiting bodies, degree of sporulation and identity of conidiogenous cells were examined by light microscope after staining by lactophenol solution according to the instructions of (Raper and Fennell, 1965).



8.4Inverted microscopic observation

The Peptone water media of the fungal isolate was prepared from their slope cultures after 8 days of incubation at 28°C. sample take in one slide and add lactophenol cotton blue stain one or two drop, then those slide observe under the inverted microscope.



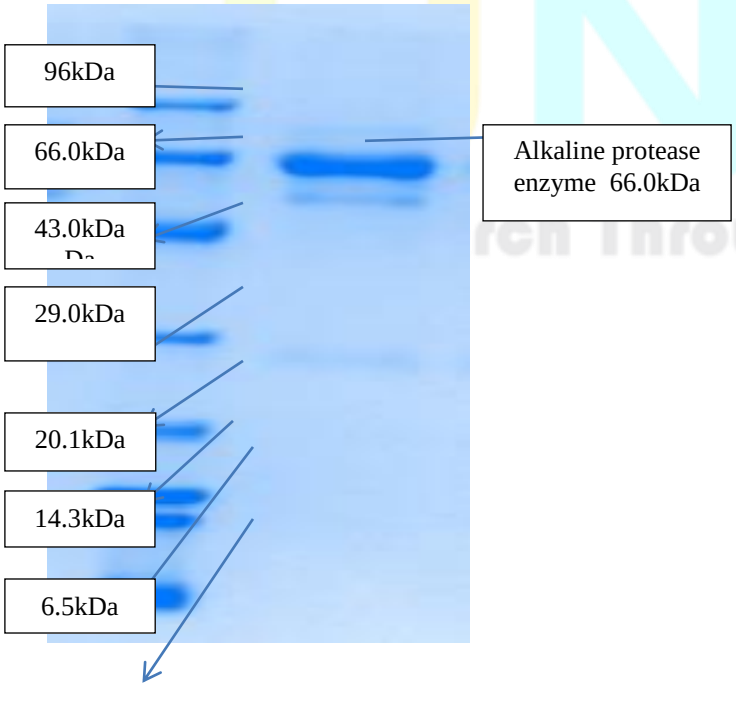
8.5Fermentaion Studies

Fermentations were carried out in 500 mL. Erlenmeyer flasks containing 100mL of medium. 10% (v/v) inoculum from inoculum flask was added to the fermentation medium under sterile conditions. Flasks were incubated at 28˚ C on rotary shaker at 120 revolutions per minute (rpm). Fermentations were carried out for 192 hours. Samples were obtained at regular intervals of 48, 72, 96, 120, 144, 168 and 192 hours respectively. The samples were centrifuged at 10,000 rpm for 10 minutes and the supernatant was checked for enzyme activity. The experiments were performed in triplicates and repeated twice. The results stated are mean of the triplicate sets and those in the graphs have been stated along with error bars with percentage.

8.6 SDS PAGE:

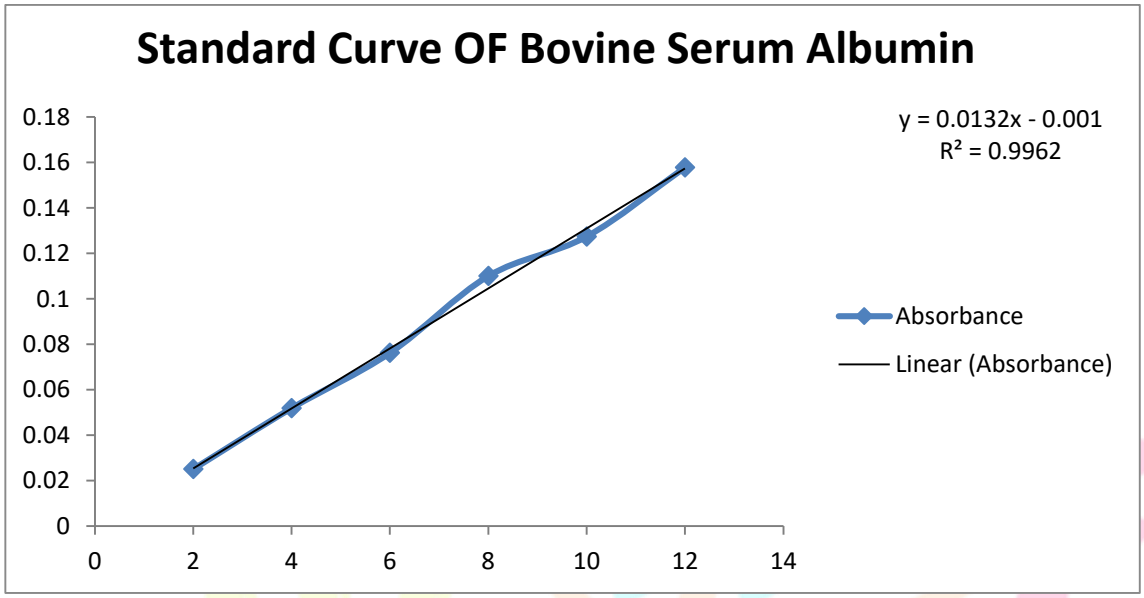
Molecular weight of alkaline protease enzyme was

66ooo Kda



Molecular weight of purified protease was determined by using sodium dodecyl sulphate polyacrylamide gel electrophoresis.

8.7 Total level of Protein concentration:



1oomg protease powder contain 30mg of protein. standard curve bovine serum used those show in above dig.

8.8Assay:

Absorbance 1.110 unit per ml of reaction mixture per minute under the assay condition.

8.9 Lyophilization process:

25 ml liquid sample converted into 1.5 gm powder. those powder sample used in gel formulation. those powder contain protease and mannitol. mannitol was lyoprotecting agent.addition of mannitol as per liquid sample.

8.10Formulation and Evaluation of Wound Healing Gel

In order to optimize the concentration of gelling agent to achieve proper consistency of the gel formulations were prepared with different gel gelling or thickening agents, various gums, Carboxy methylcellulose sodium, sodium alginate, Hydroxypropyl Methylcellulose (HPMC) and different concentration of viscosity enhancer as 1, 2, 3, 4,5,6,7 and 8% were tried. Finally sodium alginate 2% that showed good spreadability and consistency was selected. One of the main ingredients of the formulation is the gelling agent. The concentration of viscosity enhancer or gel former is of immense value as a less concentration will lead to simple solution or lotion with very low consistency, while high concentration may lead to formation of gels with high viscosity leading to non-uniform distribution of drug and problem with handling of gel. Different gel formers were tried in order to select the best gelling agent.

protease with in house produced which has broad antimicrobial properties on wound healing and wound size reduction using rats by generating full- paravertebral long incision on skin. (Aramwit et al., 2007) protease with molecular weight of 1, 00,000 shows an inhibitory action for tyrosinase and lipid per oxidation with rat brain homogenates. The addition of 0.1-2 mg/mL of protease into the aqueous solution shows heat resistant DNA polymerase activity. Protease has been found to possess wound-healing property and can be used as wound healing covering material in the form of film. (Wu C et al., 1999). Preparation of gel (Hidetoshi Teramoto et al., 2008), in the present research work.(Singh K et al., 2014), this helps in killing or inhibiting the growth of bacteria at the site of wound.

8.11Physical examination

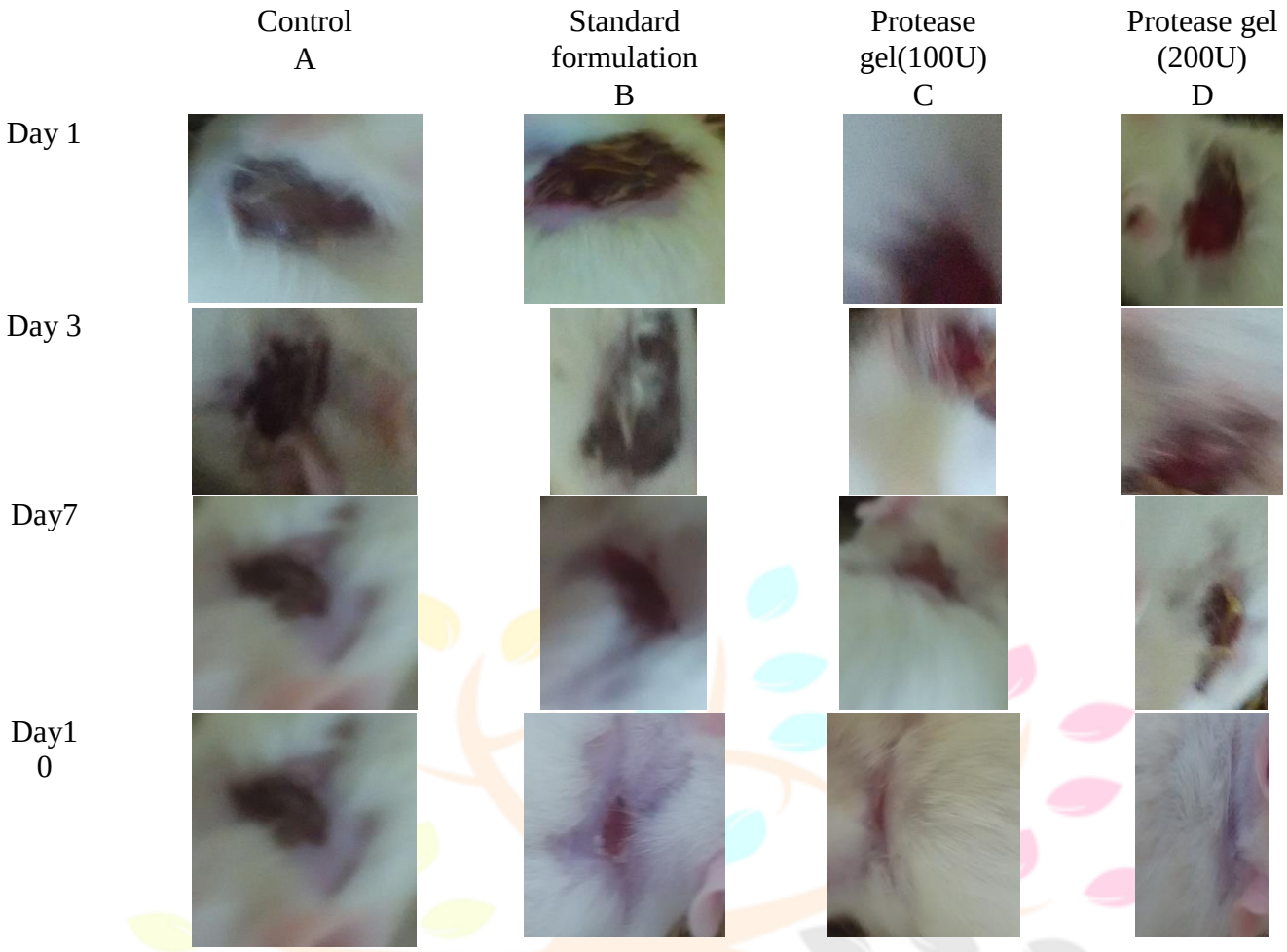
Physical examination of protease (F1), protease (F2) gel formulations are shown in table both the gel formulations showed good homogeneity and spreadability. The physical appearance of formulations was brown. The pH of the gel formulations was in the range of 5.6 ± 0.03 to 6.1 ± 0.12, which lies in the normal pH range of the skin and would not produce any skin irritation. It is important to say that there was no significant change in the pH values as a function of time for both the formulations. The viscosity values of the prepared formulations found to be acceptable.

8.12In Vivo Wound Healing Model

Incision wound healing model followed for study the wound healing potential of the protease.. It took 8 to 10 days for complete healing of wounds of the test group. Photographic comparison and histopathological evaluation of the tissues are shown in following points. Monitoring of inflammatory mediators.(Pornanong Aramwit et al., 2008).

8.13 Photographic Comparison

After creating wound according to the guidelines of the CPCSEA, photographs of the each group of animal‟s wounded part took for the visual comparison and same is shown in figur8.8 A: Control, B: standard gel, C: protease gel. D:protease gel. Standard gel; commercial formulation of povidone iodine ointment was used. Comparing photographs test formulation B group shows rate of wound contraction and healing process is faster than the commercial one as well as other groups also. From this it is clear that the effect of protease intact has wound healing potential but also the protease. After day 7 approximately 90% of the wound was healed in group B and complete wound healed on day 10.



8.14Wound size measurement

Every alternative days wound size contraction was measured. By placing transparent blotting paper carefully on the wounded part of rats marking were done by permanent marker and size of reduction in wound noted.

DAY 2

Sr.no	A	B	C	D
1	31	22	19	39
2	22	28	21	30
3	22	21	30	41
4	31	21	26	38
5	32	20	21	38
6	18	20	21	31
avg	26	22	23	36.16

Size in mm

DAY 4

Sr.no	A	B	C	D
1	30	9	20	30
2	22	20	18	31
3	21	11	24	31
4	20	14	21	32
5	30	11	21	34
6	17	11	20	27
avg	23.33	12.66	20.66	30.83

DAY 6

Sr.no	A	B	C	D
1	28	5	23	30
2	21	9	26	34
3	20	7	21	31
4	19	11	29	30
5	28	13	21	30
6	19	9	20	28
avg	22.5	9	23.33	30.5

DAY8

Sr.no	A	B	C	D
1	25	2	16	29
2	20	3	13	25
3	25	5	15	25
4	23	7	14	20
5	21	3	12	23
6	18	4	12	20

avg	22	4	13.66	23.66

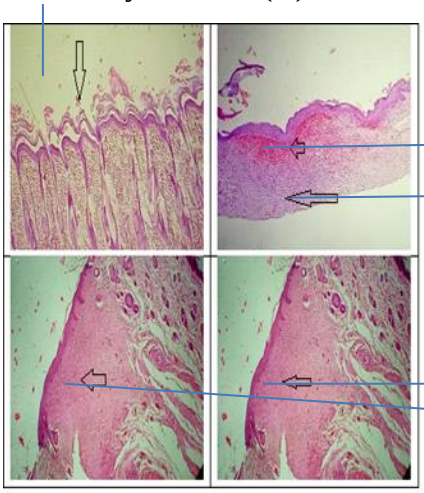
DAY 10

Sr.no	A	B	C	D
1	18	1	8	18
2	13	0	6	20
3	13	0	6	21
4	11	1	5	21
5	14	1	5	18
6	8	2	4	17
avg	12.83	3.33	5.66	19.16

8.15 Histopathological Evaluation

Another widely utilized in-vivo toxicity monitoring test is the examination of histological changes that occurs in cells/tissues/organs after exposure to formulations containing protease with and without nanoparticles. Histological examination was performed on skin art of wound that has been fixed after sacrifice of the exposed animal and changes in the tissue and cell morphology were assessed using a light microscopy. The treatment of rat wounds with protease gel have led to reduce scar formation and enhanced fibroblast proliferation, angiogenesis, keratinization and epithelisation as compared others groups. Any structural changes in the organs are considered to be a good indication of toxicity. The rapid healing and contraction of wound in the treatment group animals may possibly be attributed to the increase in total protein and collagen content in the wound granulation tissue. Fibroblast proliferation, angiogenesis, keratinization is more and faster in protease treated animals as compare to the group treated with standard compound. The healing property appears to be due to increased collagen synthesis via activation of fibroblast (B. M. Min et al., 2004). Wounds dressed with silk film appeared to be kept suitably moist and silk film has the ability to retain water, proteins, and electrolytes (A. Sugihara et al., 200).

normal layer of skin(A)



Re-epithelization ,mild haemmorhages(B)

Infilteration of inflamatory cells and granulation tissue (tissue

Infilteration of collagen fibres and reduced inflammation(C)

Complete re-epithelization (D)

A=control,normal layer of skin

B=standard gel,complete re-epithelialization,mild haemorrhages(arrow head),infiltratioin of inflamatory cell and granulation tisssue(arrow)

C=protease gel(100U) complete re-epitheliazation (arrow)

D=protease gel(200U) infiltration of collagen fibres and reduced inflammation.

The wounds in the group D. 10% formalin fixed Skin tissue of animals microscopically revealed marked exudates with degenerating inflammatory cells, very slight epithelization characterized by few immature epithelial cells along with inflammatory cell infiltration and there was no fibroblasts proliferation, incomplete epithelization, congestion of vessels in the dermis, proliferation of fibroblasts and infiltration of inflammatory cells were noticed but there was no keratinization. The wounds of group B treated with protease were microscopically revealed it showed epithelization with formation of epithelial layer, keratinization was observed, the dermis showed neovascularization, matured fibroblasts, increased collagen synthesis and absence of inflammatory cells In addition group A treated with povidone iodine showed regular arrangement of fibroblasts in the dermis, severe hemorrhages and marked neovascularization.

Excessive transepidermal water loss is one of the causes of dry skin and skin moisturizers have been used to overcome it. Protease gel prepared by using protease solution with pluronic and carbopol as a stabilizer reported to prevent water loss from the upper layer

of the skin (M. N. Padamwar et al., 2005). This retention of fluid may keep the concentrations of important substances such as Transforming Growth Factor (TGF)-α, TGF-β, interleukin (IL)-1, IL-1β, IL-6, keratinocyte growth factor and other cytokines in the granulation tissue at adequate levels (P. W. Finch et al., 1989). The wound healing property of silk protein might be due to stimulation of the expression of Basic Fibroblast Growth Factor (bFGF) which triggers target cells involved in wound

healing like fibroblasts and myofibroblasts (R. Hino et al., 2006). Also 8 % protease(a silk protein) cream promoted wound healing with a significant decrease in wound area as compared to cream base treated wounds (P. Aramvit et al., 2007). Application of silk protein film could protect unhealed skin defect area from external stimuli and help to maintain the proper healing environment and then could promote wound healing process (A.B.Lansdown et al., 2002). In the BF-27 (CA-21) treated animals, the promotion of wound healing could possibly be attributed to promotion of fibroblast proliferation (A.D. Veechai et al., 1984) and stimulation of collagen synthesis (F. X. Maquart et al., 1990). Veechai and colleagues (A.D. Veechai et al., 1984) also reported that the asiaticoside stimulates the collagen synthesis in fibroblasts in vitro.

(Roh et al., 2006) reported that the silk fibroin blended sponge treated animals showed significant increase in collagen deposition in wound granulation tissue. Hydroxyproline and fibroblasts proliferation are direct estimates of collagen synthesis, thus, agents which increases collagen formation throws light on increased hydroxyproline and better fibroblasts proliferation. Also protease and fibroin reported to promote wound healing by facilitating collagenisation at the site of skin injury (J. H. Yeo et al., 2000).

Neo-vascularization plays an important role in wound healing and newly formed blood vessels comprise 60 % of the repair tissue. Neovascularization helps hypoxic wounds to attain the normoxic conditions (H. P. Ehrlich et al., 1972). Silk protein promoted angiogenesis indicated by histological studies. The increased vessel growth can facilitate both the extent and direction of fibroplasias. Improved angiogenesis, therefore, would be contributing significantly to wound healing activity of proteins. The healing property of silk protein film appears to be due to epithelial proliferative action. Reports are available where greater collagen regeneration, less inflammation and less lymphocyte infiltration found in the wound treated with silk film (B. M. Min et al., 2004). Also the silk protein film is safe biomaterial without any skin sensitizing and skin irritating potential and with no effects on serum biochemical profile when applied dermally (A. R. Padol et al., 2011). These findings support increasing evidence of the importance of silk protein in promoting the wound healing. Efforts are being made to analyze the effect of fibroin and on the growth and attachment of fibroblasts in vitro. Results from these studies may provide insight into the clinical applicability of biofilms and dressing for better healing.

9.SUMMARY AND CONCLUSION

In the present study, Protease production from a novel fungal sp. is studied. Since protease is one of the industrially important enzymes, increase in the yield is important aspect to study. Protease production was maximum at 120h and declined after 144h. Protease production was studied.

Hydrolysis was carried out for 120min at 50ºC and with different enzyme concentrations. The hydrolysate showed protein concentration of 3.22 mg/ mL by the Lowry‟s method. Small molecular weight peptides in the hydrolysate

As protease is biocompatible, further study extended towards its wound healing potential. The treatment of rat wounds with protease gel have led to reduce scar formation and enhanced fibroblast proliferation, angiogenesis, keratinization and epithelisation as compared others groups. Also formulation has showed viscosity, pH, spreadability, extrudability in acceptable range. From this it should be concluded that a new biocompatible wound healing gel will have market capture potential in future.

10.FUTURE PROSPECTIVE

a. To study Enzyme kinetics.

b. To check the effect of various on protease activity.

c. Optimization of enzyme production using statistical tools like Plackett – Burman and Response Surface Methodology (RSM).

d. Use of protease for hydrolysis of various crude enzymes to obtain bioactive peptides.

e. Formulation and development of bioactive peptides containing biopharmaceutical product and determine its potential applications.

f. Use of protease for cosmetics preparations and skin care.

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APPENDIX I

I. MEDIUM COMPOSITION

Peptone water media

Ingredient name	Gram/litre
Peptone	10gm
Sodium chloride	5gm

I. REAGENTS :

1. FOLIN LOWRY REAGENTS

Lowry reagent: A - 2% Sodium carbonate (Na_2CO_3) in 0.1N NaOH

Folin-Ciocalteau reagent

II. BUFFERS :

1.K_2H PO4 buffer – Stock Solution

A: 0.2 M solution of monobasic sodium phosphate (NaH_2PO_4)[27.8 g in 1000 mL of distilled water]

B: 0.2 M solution of dibasic sodium phosphate (Na_2HPO_4) [53.65g of $Na_2HPO_4.7H_2O$ or 71.7g of $Na_2HPO_4.12H_2O$ in 1000mL distilled water]

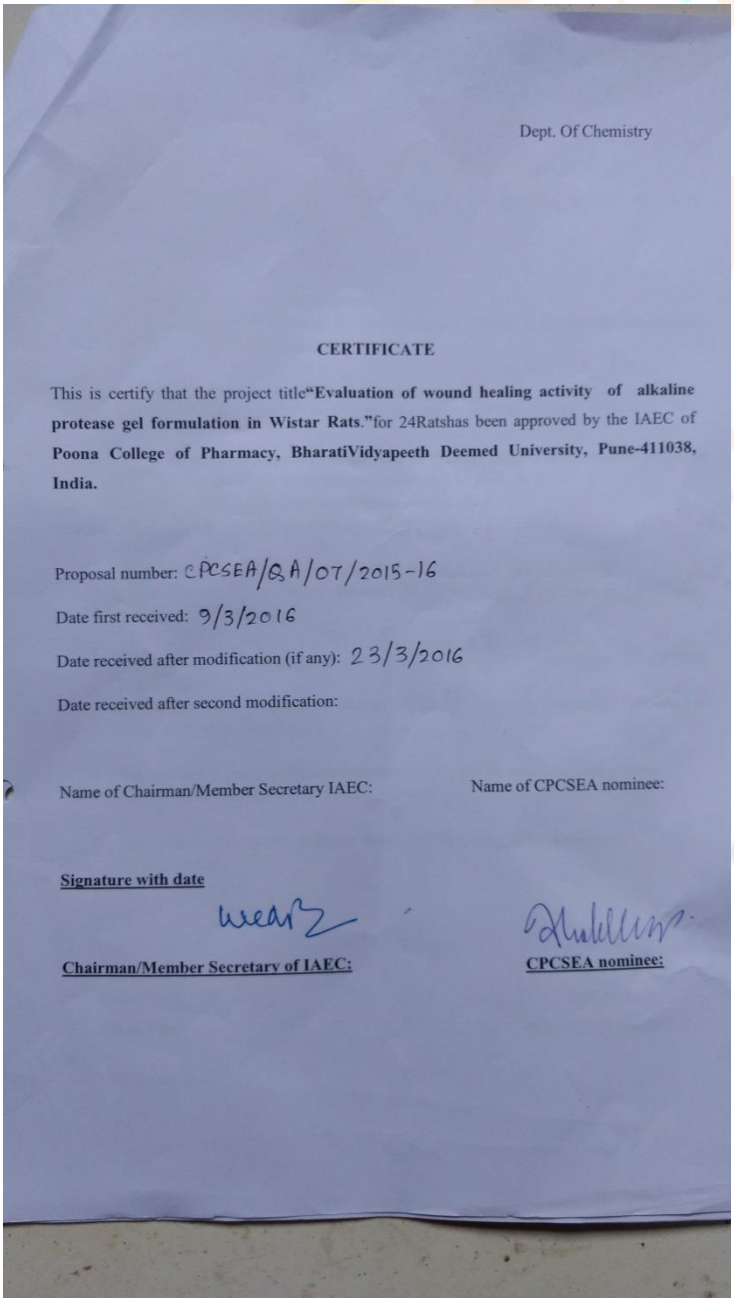
For pH: 7- [39.0ml of A+61.0ml of B+100ml of distilled water] -50mM of buffer.

APPENDIX - II

Table for Ammonium Sulphate Precipitation

Pick a number from the vertical column that corresponds to your current concentration of ammonium sulphate and determine the volume of saturated ammonium sulphate solution that you need to add to achieve the desired increase. Simply add this volume to litre of sample.

%	10	15	20	25	30	33	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	56	84	114	144	176	196	209	243	277	313	351	390	430	472	516	561	610	662	713	767
5		28	57	86	118	137	150	183	216	251	288	326	365	406	449	494	540	592	640	694
10			28	57	88	107	120	153	185	220	256	294	333	373	415	459	506	556	605	657
15				29	59	78	91	123	155	189	225	262	300	340	382	424	471	520	569	619
20					30	49	61	93	125	158	193	230	267	307	348	390	436	485	533	583
25						19	30	62	94	127	162	198	235	273	314	356	401	449	496	546
30							12	43	74	107	142	177	214	252	292	333	378	426	472	522
35								31	63	94	129	164	200	238	278	319	364	411	457	506
40									31	63	97	132	168	205	245	285	328	375	420	469
45										32	65	99	134	171	210	250	293	339	383	431
50											33	66	101	137	176	214	256	302	345	392
55												33	67	103	141	179	220	264	307	353
60													34	69	105	143	183	227	269	314
65														34	70	107	147	190	232	275
70															35	72	110	153	194	237
75																36	74	115	155	198
80																	38	77	117	157
85																		39	77	118
90																			38	77
95																				39



Dept. Of Chemistry

CERTIFICATE

This is certify that the project title**"Evaluation of wound healing activity of alkaline protease gel formulation in Wistar Rats."**for 24Ratshas been approved by the IAEC of **Poona College of Pharmacy, BharatiVidyapeeth Deemed University, Pune-411038, India.**

Proposal number: CPCSEA/IAEC/07/2015-16

Date first received: 9/3/2016

Date received after modification (if any): 23/3/2016

Date received after second modification:

Name of Chairman/Member Secretary IAEC: Name of CPCSEA nominee:

Signature with date

Chairman/Member Secretary of IAEC: **CPCSEA nominee:**

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