Enzymes have unlimited applications in various industrial sectors viz. food and feed, detergent, pharmaceutical, organic synthesis, paper & pulp, leather, waste treatment, textile, etc. (Fogarty et al., 1974 a,b; Zaks and Klibanov, 1987; Takami et al., 1989; Outtrup et al., 1998; Priest, 1992). The enzyme-based processes are advantageous as they can be carried out at moderate reaction conditions with greater specificity in contrast to chemical-based processes. In this scenario application of various enzymes viz. protease, amylase, glucoamylase, pectinase, lipases etc. have been realized. Market for enzymes in industrial processes is fast growing. The total enzyme sales in 1995 was US $ 1 billion and is expected to go up to US $ 1.7 - 2 billion in 2005.(Ellaiah et al., 2002).
Recently proteolytic enzymes have also gained considerable attention in the industrial community. Estimated world sales of enzymes indicate that protease represents the largest market and account for nearly 60% of the total industrial enzyme market. Among the various proteases available, alkaline proteases are the most significant representing 31% of the total enzyme sales, offer several advantages over alternative physical and chemical manipulations in industrial processing. Applications of alkaline proteases are most widely used as additives to synthetic detergents (Eriksen, 1996). Alkaline proteases also find use in food industry (Nakadai et al., 1977), in production of soya products (Lahl and Braun, 1994) and solubilization of fish protein (Mackie, 1992). Also alkaline proteases are used in leather tanning during the soaking and bating stages (Godfrey, 1996a). In textile industry, as a tool for Backstaining removal in case of denim washing (Yoon, 2000), antifelting finishing on wool (Heine and Hocker, 1995), in natural raw silk for removing the adhesive protein sericin (Shukla et al., 1982) etc. In photographic film for recovering silver (Fujiwara et al., 1989) as well as in industrial production of glue & cosmetics (Vanishing creams, lotions, tooth pastes) etc.

From an analysis of their in-vitro properties, proteases have been classified in a number of ways, e.g., on the basis of pH optima (acid, neutral or alkaline), their ability to hydrolyze specific proteins (Keratinase, elastase, collagenase, etc.), and their similarities to well-characterized enzymes (trypsin, chymotrypsin, cathepsin, chymosin etc.). The most satisfactory classification is based on their mechanism of action. This classification, which is used by the Enzyme Commission, consists of four groups-serine, thiol, metal (or metal chelator sensitive), and acid proteases which are distinguished from each other by their sensitivity to various inhibitors, nature of the active site, catalytic mechanisms, pH and temperature optima (Hartley, 1960; Morihara, 1974; Kelly and Fogarty, 1976; Frost and Moss, 1987; Kalisz, 1988).

The current commercial sources of protease include animals (stomach), plants (saps, juices) and microorganisms. Many fungi, bacteria and actinomycetes produce extracellular alkaline protease. The most important producers belong to *Bacillus spp* (*B. subtilis, B. firmus, B. megaterium, B. pumilis*), Streptomyces strains (*S. fradiae, S. griseus* and *S. rectus*) and *Aspergillus spp* (*A. niger, A. oryzae, A. flavus* and *A. sydowi*).
Bacillus spp. are prolific producers of extracellular hydrolytic enzymes (Fogarty et al., 1974a,b; Boer et al., 1994). There are several reasons for the predominance of the genus Bacillus in the area of enzyme production. First, they are a group of chemoorganotrophs that can be easily maintained and cultivated and yet are markedly heterogeneous in character (Prest, 1977). Psychrophiles, mesophiles and thermophiles, in addition to alkaliphilic, neutrophilic and acidophilic species are well represented. Also, nearly all species of Bacillus secrete extracellular enzymes. Enzymes of bacteria from such diverse habitats exhibit unique properties. Proteases from Bacillus species may be thermostable (Takii et al., 1987; Kubo et al., 1988; Takami et al., 1989) and stable in alkaline conditions (Horikoshi, 1971; Fujiwara & Yamamoto, 1987; Takami et al., 1990; Giesecke et al., 1991).

This investigation entitled Studies on alkaline protease from Bacillus Spp. was undertaken realizing the importance of alkaline protease for the commercial market.

Results described during the course of investigation are summarized and concluded below:

I Isolation & Identification of Alkaline protease producing bacteria.

In this investigation seventy five alkaline protease producing bacterial strains were isolated from Cambay soil samples and selection was done based on zone of proteolysis on milk agar plate. Total of sixteen isolates were selected for assessing alkaline protease production ability. Final selection of strain was done by step wise screening procedure, and alkaline protease producing organism referred to as NSM-3 (Isolate number NSM-3 showing 350 IU/L protease activity) strain that active at pH 9.5 & 55°C temperature was selected. The organism was identified as Bacillus subtilis on basis of criteria laid down in Bergeys Manual of Systematic Bacteriology and the identification was confirmed at Institute of Microbial Technology, Chandigarh.

II Parametric optimization for alkaline protease production by submerged fermentation

Optimization of parameters for maximum alkaline protease production by submerged fermentation was carried out in shake flask. Alkaline protease was maximally produced at 40°C, pH 7.0 with 4% level of 15 hrs old inoculum using Tapioca starch-soyabean meal-calcium phytate medium after 60 hours under shaking condition at 225rpm.
Tapioca starch was found as the best carbon source for alkaline protease production at concentration 4%. Among various nitrogen sources examined, defatted soyabean meal (SBM) at 1% level serve as best nitrogen source for alkaline protease production followed by Corn-steep liquor. In presence of inorganic nitrogen sources, (NH₄)₂HPO₄ with 1% level supported better protease production than organic nitrogen sources but due to higher specific activity of alkaline protease obtained in presence of SBM favors organic nitrogen source, SBM as part of medium component. Secretion of alkaline protease was enhanced in presence of Calcium phytate (1%), phosphate and sulfate ions while oil cake supplement did not enhance alkaline protease production. Alkaline protease produced under optimized conditions was 1605 IU/L, indicating about 4.6 fold increase in protease production after optimization.

II Parametric optimization for alkaline protease production by solid state fermentation (SSF)

Alkaline protease production was also carried out by solid state fermentation where substrates with 8:2 ratio of wheat bran:groundnut cake supported maximum growth & production of enzyme. Tape water was used as moistening agent. The optimum conditions for maximum alkaline protease production by SSF were growth temperature 40°C, 10% inoculum, 60% moisture content in substrate and process period 72h, with maximum activity 145 IU/gram.

IV Partial purification and Characterization of Alkaline protease

The protein fractions collected at 30 - 60% ammonium sulfate saturation level were assayed for proteolytic activity at pH 9.5. The protein fraction precipitated at 40% saturation level gives good proteolytic activity and 10.55 fold more activity was found as compared to crude enzyme.

Alkaline protease concentrated by Iso-propyl alcohol precipitation (ratio 1:3.5 v/v) is possessing better proteolytic activity than that obtained by ammonium sulfate precipitation. Proteolytic activity is about 23.07 fold more than crude alkaline protease sample.

Partially purified enzyme was further studied for the characterization of the alkaline protease enzyme derived from *B. subtilis* NSM-3

A single protein band is visible in a 10% SDS-PAGE and exhibited a molecular weight of 30 kDa of electroeluted alkaline protease.
The km value of alkaline protease is 1.45mg/ml for casein with Vmax 123.50 micromole tyrosine/ml/min.

Alkaline protease enzyme was found to be active over wide temperature range 35°C to 65°C, with optimally active at 55°C temperature, further increasing the temperature to 70°C, alkaline protease activity decline. The enzyme retain 100% of its activity at 55°C after 1 h. Alkaline protease was found to be active over wide pH range from pH 6.5 to pH 11 with maximum proteolytic activity towards casein was observed at pH 9.5 and retain 100% activity at pH 9.5 even after 1 h incubation.

The *B. subtilis* NSM-3 protease was not inhibited by EDTA, indicating that it is not a metalloprotease while inhibited by PMSF indicating that it is serine protease. The metal ions did not greatly affect the enzyme activity.

The alkaline protease has half life of more than 90 min. at 55°C and shelf life of at least one month at 5°C (Storage stability).

In presence of glycerol and polyethylene glycol, alkaline protease retains thermostability more than 80% up to temperature 90°C. Also, the effect of various polyethylene glycols on thermostability was investigated at 80°C temperature with three polyethylene glycol having molecular weight ranging from 400 to 8000 and results indicate that there exists positive correlation between thermostabilizing efficiency & molecular weight of polyethylene glycol.

VI Applications of alkaline protease derived from *B. subtilis* NSM-3.

Different applications of alkaline protease derived from *B. subtilis* NSM-3 were undertaken, realizing the importance of alkaline protease in the commercial market.

1. As Biodetergent

Pollution in any manner has become a critical problem of the day. This is true in case of conventional chemical-based detergents, which causes several pollution problems. Microbial enzymes-based detergents today occupy a place of prominence among different categories of detergents. Therefore, presently detergents-compatible enzymes have the largest share of the World market. In order to assess the suitability alkaline protease enzyme derived from *B. subtilis* NSM-3 for use in laundry detergents, its suitability over a range of alkaline pH and temperature and its compatibility with commercial detergents were determined. It was not inactivated in the presence of most of the detergents tested and appear to be potentially useful for inclusion in washing powder as biodetergent.
2. **Soaking & Bating process in leather industry.**

The soaking and bating processes create effluent disposal problem which can be partially solved by rationalizing the beam house processes, using enzyme and enzymatic formulations for leather processing. Enzymatic soaking process can ensure softer leather with cleaner gain, reduce duration of soaking time and need for limming chemicals, as compared to conventional soaking process. While enzymatic bating process is done where conventional enzyme (bate) used is extracted from pancreas. Due to less availability, the leather industry cannot afford to depend on pancreas, a byproduct of meat industry.

The condition of goat skin leather after using alkaline protease for soaking was found to be soft, smooth with some gain in total area. Alkaline protease derived from *B. subtilis* NSM-3 was found to be good as compared to conventional soaking. The alkaline protease enzyme was also found very good (Grade No.7) for bating of goat-skin leather.

3. **Degumming of raw silk.**

The silk gum sericin is a protein compound, yellowish in color and serve as cementing agent to the silk filament fibroin. Its harsh feel and dull appearance are undesirable in silk materials. Also the presence of sericin interferes with efficient bleaching and also lead to uneven dyeing. Therefore, removal of silk gum (i.e. sericin) from the raw silk materials is one of the most important step in processing of silk in textile industry and this process is known as degumming of silk. Conventional degumming of raw silk with chemical treatment at high temperature for prolonged time period produce adverse effect on silk materials due to improper controls. With alkaline protease (1000 IU/L) degummed silk sample shows better luster, whiteness index and better feel as compared to conventional process.

4. **As a Tool for Denim Washing**

Cellulase enzymes were introduced in 1980s as a denim washing aid to achieve fashionably aged looks. Although whole acid-stable cellulases have been widely used for efficient and economical denim finishing, the final look provided by whole acid-stable cellulase was characterized by a flat and low contrast abrasion due to high backstaining. To avoid backstaining problem, denim processors use conventional method carried out at high pH/ high temperature or with bleaching agent that damage garments and alter shade of the denim. Literature reports suggested that backstaining is caused...
by many proteins present in the conventional whole acid-stable cellulase. So, the use of alkaline protease offers flexible alternative process for backstaining clean up, improved contrast of denim finishes and reduce residual cellulase on fabric. For the backstaining clean up with alkaline protease derived from *B. subtilis* NSM-3, laboratory scale wash performance tests were conducted under different conditions and positive results were observed that indicate 9.5 pH, 50°C temperature, alkaline protease (0.1 %owg) dosage with 1% detergent for 20 minutes rinse time exhibited best performance for backstaining clean up processes for denim washing.

5. **Extraction of genomic DNA from bacterial cells**

Alkaline protease produced from *B. subtilis* NSM-3 has broad protein cleavage specificity and stable even in presence of 0.5% SDS & EDTA. So, alkaline protease can be applied for genomic DNA extraction from various Gram positive and Gram negative bacteria, thereby establishing its candidature as a substitute to the proteinase K, purified alkaline serine protease commercially available with high costs.

6. **Recovery of silver from used X-Ray films**

X-Ray films contain a good amount of silver as compared with other types of films and even used X-Ray films still contain 1.5% to 2% silver (by weight) in its emulsion layers. Since the emulsion layer possessing silver also contain gelatine, it is possible to break it down using proteolytic enzymes. We have successfully recovered 60-70% silver by breaking down gelatine layer within 10 minutes by alkaline protease produced by *B. subtilis* NSM-3 and qualitative as well as quantitative presence of recovered silver was detected by scanning electron microscopy with EDAX method.

7. **Induction of Banding Patterns in Cattle & Buffalo Chromosomes**

G-bands are defined as a system of alternating dark & light bands throughout length of the euchromatic part of chromosomes. These bands are useful to pair the homologous chromosomes and to identify the structural chromosomal aberrations, which are usually associated with reproductive failure in animals as well as in human beings. Alkaline protease derived from *B. subtilis* NSM-3 is acting as tool for generating G-banding pattern on chromosomes of cattle and buffalo, thereby establishing its candidature as a substitute to the traditional use of Trypsin, a protease of animal origin.
8. Gelatine extraction

Gelatine is finding wide applications in food, cosmetics, pharmaceutical as well as gelatine-based photographic film preparation industries. Gelatine is produced from collagen from animal bones (Ossein) and it is produced by alkaline treatment. In conventional chemical method for gelatine extraction, crushed animal bones (Ossein) as collagen sources are soaked for two months in lime solution. The treatment and proper disposal of lime solutions are serious, economical and social problems. This step can be replaced by ecofriendly enzymatic process using alkaline protease to replace lime, reduce duration of processing time and save the production costs. Ecofriendly application of alkaline protease from *B. subtilis* NSM-3 for gelatine extraction was found to be better as compared to conventional chemical method.

Alkaline proteases are important in view of their activity and stability at alkaline pH. Since our alkaline protease is produced extracellularly from the selected Gram positive **bacilli** and producing alkaline protease in inexpensive medium and hence its production is economical. Our alkaline protease enzyme is suitable to be used for different industrial applications because of its unique characteristics, which are the hallmarks of an ideal industrial enzyme. So, further efforts should be made to improve the protease production by implementing strain improvement programmes. Industrial up-scaling of submerged and solid state fermentations is required to produce large volumes of alkaline protease.