International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 10 (2014) pp. 1063-1080**
http://www.ijcmas.com

Original Research Article

Production of alkaline thermostable protease by immobilized cells of alkalophilic *Bacillus* sp. NB 34

Deepak kumar[1], Prakram Singh Chauhan[1], Neena Puri[2] and Naveen Gupta[1*]

[1]Department of Microbiology, South Campus Panjab University, Sec-25, Chandigarh, India
[2]Deapertment of Industrial Microbiology, Guru Nanak Khalsa College, Yamunanagar, Haryana, India
**Corresponding author*

Keywords

Alkaline protease, Immobilization, Adsorption, Entrapment, immobilized cells

A B S T R A C T

In recent years there has been potential increase in the use of alkaline protease as industrial catalysts. Many major industrial and commercial applications, such as food, textile industries, and medical diagnoses, are highly dependent on the protease enzyme. In the cell immobilization technique, the free movement of microorganisms is restricted in the process, and a continuous system of fermentation can be used. In the present work, this technique has been used for alkaline protease production using different carriers, such as alginate, keiselguhr and agar agar. The immobilization of alkalophilic *Bacillus* sp NB 34 in agar agar was seen to be best at 2% agar concentration, a bead size of 62.5 mm^3, inoculum size of 10%, increased upto 36h, a column length of 10 cm and flow rate of 2.5 ml/h. The best adsorption of alkalophilic *Bacillus* sp NB 34 cells at activated charcoal and keiselguhr was seen at a concentration of 75mg/ml for both the matrices inoculum size of size of 20%was seen to be the best for the production of protease by the activated charcoal and keiselguhr adsorbed cells

Introduction

Proteases are probably the most important class of industrial enzymes worldwide, accounting for nearly 60% of total enzymes sales. The two third of proteases produced commercially are by microorganisms (Joshi et al., 2013). Protease finds their application in a wide range of industrial process viz. detergent, brewing, baking, and pharmaceuticals. Leather tanning, meat tenderization, peptide synthesis and medical diagnosis (Saeki et al., 2007; Rai et al., 2010; George et al., 2014a, Chauhan et al., Though plants and animals also produce extracellular protease, microorganisms are the preferred source of protease because of their rapid growth, limited space required for their cultivation, longer shelf life, the ease with which they can be genetically manipulated to generate improved enzymes (Chauhan et al., 2012; Kalisz, 1998; Zhou et al., 2007). Microorganisms elaborate a large array of proteases that are intracellular and /or extracellular. Intracellular proteases are important for different metabolic functions

like sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of cellular protein pool whereas extracellular proteases help in hydrolysis of protein in the cell free environment and their and their cellular uptake. The hydrolytic property of extracellular proteases has been commercially has been exploited in various industrial processes (Sondhi et al., 2014; George et al., 2014b; George et al., 2014c). Although alkaline proteases are produced by bacteria, fungi, actinomycetes and yeast yet bacteria are the most dominant group of alkaline protease producers with the genus Bacillus being the most predominant source followed by *Psuedomonas* (Dutta and Banerjee, 2006). Beside these *Flavobacterium* and *Arthrobacter* are also known to produce alkaline serine protease . In fungi *Aspergillus* is the most exploited group. Whereas stains of *Streptomyces* are the preferred source among actinomycetes (George et al., 2014b).

Immobilization of whole cells for the production of extracellular enzymes offers many advantages such as ability to separate cell mass from bulk liquid for possible reuse, facilitating continuous operation over a prolonged period and enhanced reactor productivity. However, proper selection of immobilization technology for alkaline protease production too is on the rise and the initial reports are encouraging (Mishra, 2007; Nadeem et al., 2008; Joshi et al., 2013).

Alkalophilic *Bacillus* sp. NB 34 previously isolates in our lab is known to give alkaline thermo stable protease (Cheema, 2008). The purpose of present investigation is standardization of cell immobilization techniques for alkalophilic *Bacillus* sp. NB 34 for the production of alkaline protease.

Materials and Methods

Bacterial strain

The bacterial strain in this work, alkalophillic *Bacillus* sp. NB34 strain was already isolated in our lab. The organism was grown and maintained at 37^0C and sub cultured on Horikoshi medium

Methods

Inoculum preparation

A loopful of cells from the culture of alkalophilic *Bacillus* sp. NB34 was inoculated into 250 Erlenmeyer flasks containing 50 ml of sterile production medium.

The flasks were kept on shaker incubator at 150 rpm, 37°C for 24 hours. This cell suspension was used as inoculum for immobilization as well for free cell enzyme estimation

Whole cell entrapment

Entrapment of cells in calcium alginate

Sodium alginate solution was prepared in sterilized distilled water. To the alginate slurry, inoculum was mixed and stirred for 10 minutes to get a uniform mixture taking care that no bubbles are entrapped inside. The slurry was filled in sterile hypodermic syringe and an injection needle was fixed over its end .the alginate solution was dropped into a 0.2 M ice cold solution of calcium chloride from 5cm height. Beads were formed in CaCl2 solution that was incubated overnight for curing. The cured beads were washed with sterile distilled water 3 to 4 times and used to inoculate enzyme production media. When the beads were not being used, they were preserved in

0.9% sodium chloride in the refrigerator. All the operation were preserved in 0.9% sodium chloride in the refrigerator. All the operation were carried out aseptically on a laminar flow bench

Entrapment of cells in Agar-agar

A 2% solution of agar-agar was prepared in 45ml of 0.9% NaCl solution and was sterilized by autoclaving. The 24 hr old inoculum (5ml) was added to the molten agar, shaken well for few seconds (without forming form or entrapped bubbles), poured into 2 sterile flat bottomed (100mm) diameter petriplates and allowed to solidity. The solidified agar blocks were cut into equal size cuboids (62.5mm^3) and were added to sterile 0.1M carbonate – bicarbonate buffer (pH 9.5). The buffer was decanted and cuboids were washed with sterile distilled water 3 to 4 times

Effect of concentration of immobilized material on the entrapment

Agar –agar and sodium alginate were used as entrapment material and their concentration was varied between 2-4%. The immobilized beads prepared with different concentration of agar /alginate was used for inoculation of enzyme production medium. The inoculated flasks were incubated under shake flask condition at 37°C and samples were drawn periodically.

Effect of bead size of entrapped cells for the production of protease

The calcium alginate bead size was varied by changing the gauge (pore size) of the injection needles to drop the alginate cell slurry into 0.2 M calcium chloride solution. Injection needles of 18-22 gauge were used that produced alginate beads of different sizes (4.18, 1.76, 0.52 mm3).similarly, agar blocks having different sizes (62.5, 250, 562. 5 mm^3) were used for inoculation of enzyme production media. The inoculated flasks were incubated under shake flask condition at150 rpm, 37°C and samples were drawn periodically for the protease assay.

Effect of inoculum size on entrapment

The inoculum size was varied between 2 to 10% (v/v).For 2% inoculum size 1 ml of overnight grown culture of alkalophilic *Bacillus sp*. NB34 was added to 49ml of agar and sodium alginate solution. For 4% inoculum size 2ml of overnight grown culture was added to 48 ml of agar and sodium alginate solution. Similarity, different inoculum sizes were prepared and the entrapment was carried out. The entrapped cells were then used to inoculate enzyme production media. The flasks were incubated under shake flask conditions at 150 rpm, 37°C and samples were drawn periodically .The latter was used crude alkaline protease to analyze the enzyme activity.

Effect of bacterial cells in activated charcoal and keiselghur

The use of charcoal and keiselghur for the adsorption alkialophilic *Bacillus sp*. NB34 cells was stuied by incubating the cell and adsorption material mixture in water bath (37°C) for 2h under shaking conditions. The mixture was centrifuged at 600 rpm and the supernatant was plated in different dilutions on Horikoshi medium to determine free cells.

Effect of concentration of immobilization material of adsorbed for the production of protease

Different amount of charcoal and keiselguhr, 50-100mg/ml were added to innoculum and adsorption capacity was studied as explained above.

Effect of inoculum size adsorbed cells for the production of protease

The activated charcoal and Keiselguhr optimally adsorbed with alkalophilic *Bacillus sp*. NB 34 was added in different concentration (5-20%) in the production media. The samples were incubated and assayed as above.

Production of alkaline protease with immobilized cells

Batch production of alkaline protease

The calcium alginate beads, agar-agar blocks and adsorbed cells were used as inoculum for alkaline protease production (50ml in 250 ml Erlenmeyer flasks) the composition of production medium was already optimized in the lab. The flasks were used as incubated at 37°C for 72h. Samples were withdrawn intervals of assayed for alkaline protease activity.

Production of alkaline protease by repeated batch process

The immobilized biocatalysts can be used repeatly and continuously. Therefore the reusability of alkalophilic *Bacillus sp*. NB 34 entrapped cells was examined. After attaining the maximum production of alkaline protease in the batch fermentation, the spent medium was replaced with fresh production medium and the process was repeated for several batches until the enzyme activity was optimal.

Semi continuous production of alkaline protease

Alkalophilic *Bacillus* sp. NB 34 entrapped in agar agar cuboids was filled in column upto different lengths (5-20 cm) to optimize the column length. The packed columns were filled with enzyme production medium and the flow rate was regulated and spent medium was collected for every 4 h and used crude enzyme extract. The spent medium was replaced with fresh medium continuously. The samples collected every 4 h were assayed for alkaline protease activity

Results and Discussion

In the present study, alkalophilic *Bacillus* sp. NB34 giving alkaline thermo stable protease already isolated in our lab was used. The production of alkaline protease by free cells of alkalophilic *Bacillus* sp. NB34 was carried out in Horikoshi broth using already optimized conditions of pH 10, temp 37°C, agitation 150 rpm, gelatin as nitrogen source, 2% sodium carbonate and glucose as carbon source.

The same conditions were used with immobilized cells, however with calcium alginate, K_2HPO_4, and Na_2CO_3 were omitted from the production media because these tend to dissolve the alginate beads.

Alkaline protease production by Alginate entrapped cells in batch process

The 24 h old cells of alkalophilic *Bacillus* sp. NB 34 were used for entrapment in calcium alginate as explained in material and methods section.

The cells of alkalophilic *Bacillus* sp. NB 34 were suspended in sodium alginate and dropped from a height of 5cm in ice cold solution of 0.2M of $CaCl_2$ using a hypodermic syringe needle to form calcium alginate beads. The effect of alginate concentration, bead size, and inoculum size was determined to optimize the protease production process.

Effect of alginate concentration

In order to find out the optimum alginate concentration for akalophilic *Bacilius* sp. NB 34 whole cell immobilization, sodium alginate of different concentration 2, 3 and 4% (w/v) were used. At low alginate concentration of 2% however, the beads were relatively soft and showed rapid disintegration. On the other hand, beads of 3 5 and 4% concentration of alginate remained stable.

The production of alkaline protease reached a maximum yield of 6.0U/ml in 48 h at 3% alginate concentration (Fig.1). Protease production by entrapment of cells of alkalophilic *Bacillus* sp. NB 34 was 2 fold higher than that of free cells but free cells could give maximum protease production at 24 h

In similar results, reported that 3% sodium concentration gave the maximum yield of alkaline protease production by *Bacillus subtilis* PE-11, they reported that the enzyme production was gradually decreased, where as maximum enzyme titer was observed at 48 h in case of free cells, it was also reported that the alkaline protease production by immobilized cells was 1.2 folds higher than that of free cells. Potumarthi et al., (2007) also reported 2.78% of sodium alginate concentration as optimum for the production of alkaline protease by *Bacillus licheniformis* NCIM-2042.

In contrast, Mishra, (2007) reported 4% of alginate concentration as optimum for alkaline protease production by *Bacillus circulans* . Maximum protease production was observed at 96 h and the protease production by immobilized cells was 1.3 folds higher than that of free cells.

Nougeria, (2004) reported that 2% of alginate concentration gave maximum protease activity from *T .turnirae* cells and its was observed that the free cells gave 2 folds higher activity than immobilized cells. So, for further experiment, alginate concentration of 3%was used.

Effect of bead size

The alginate entrapped cells tends to grow near the bead surface which makes them influenced by surface available for growth of cells. Therefore, the beads size was varied by using hypodermic syringe needles of different gauge sizes i.e. 18, 20 and 22 and the protease production were studied. The beads produced by 18, 20 and 22 gauge syringe needles had bead volumes of 4, 18, 1.76 and 0.52mmm^3 respectively. The results of protease production by different sized beads revealed a maximum protease activity of 5.6 U/ml in 48 h with 4.18 mm^3 formed by 18 gauge needle, however, protease production with other bead sizes was not much different (Fig.2). There are reports of higher enzyme activities with small sized

Mishra, (2007) reported that with *Bacillus circulans* maximum protease production was obtained from beads formed by 20 gauge needle which was significantly higher than other bead sizes as well as that of free cells. In contrast, Elibol and Moreira, (2003) reported that there was no significant difference in the protease production by *T. urnirae* cells with different beads sizes. For further experiments, beads of 4.18 mm^3 were used

Effect of inoculum size on entrapment

The effect of inoculum size on entrapment was studied using 2- 10% of inoculum as explained in materials and methods by

preparing alginate (3%) beads of 4.18 mm3 size. The results revealed that increase in inoculum size increases the protease production with a maximum yield of 5.6 U/ml at 10% inoculum size in 48 h of incubation (Fig.3) which is significantly higher than other inoculum sizes.

In similar results, Potmarthi et al. (2007) reported maximum protease activity of *Bacillus licheniformis* NCIM -2042 with 8.10% inoculum size. Mishra, (2007) reported that increase in inoculum size for entrapment of *Bacillus circulans* increase the protease production with a maximum yield at 10% inoculum size in 96 h of incubation

Enzyme production by repeated Batch cultivation with alginate entrapped cells

The reusability of alkalophilic *Bacillus* sp. NB4 cells immobilized in calcium alginate was examined for several batches (as explained in materials and methods section). The results revealed that the protease production capacity of the cells could be retained even after 3 cycles (Fig. 4)

These findings were in accordance with those obtained previously for the protease production by immobilized *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate beads (Vuillemard et al., 1988). It was found that protease production by immobilized *S. marcesens* increased with repeated growth cycles, and reached a maximum after S cycles.

Earlier, breshay, (2003) and Elibol and Moreira (2003) Also reported the protease production by repeated batch cultivation and the 3rd and 4th batches were observed to be the high protease yielding batches with T. turniare respectively. Adinarayana et al (2005) studied alkaline protease production by calcium alginate entrapped cells of *Bacillus subtilis* for 10 cycles and reported that after 9th cycle beads started disintegrating leading to decrease in activity.

Agar Entrapment

The alkalophilic *Bacillus* sp. NB 34 was entrapped in agar –agar beads (as explained in material method section)

Effect of agar concentration

The optimum concentration of agar agar for alkalophlic *Bacillus* sp. NB 34 immobilization was determined by immobilizing bacterial cells in different concentration (2, 3 &4% w/v) of agar agar . Results presented in Fig.5 reveal the maximum enzyme production was with 2% agar concentration after 24g and in contrast to calcium alginate entrapment; there was n increase in protease production with respect to free cells. The less protease production at higher agar concentrations may due to diffusion limitation arising out of reduced pore size of beads at increased concentration of entrapment material (Elibol and Moreria, 2003)

Adinarayna et al. (2005) also reported 2%concentration of agar agar to be optimum for the entrapment of *Bacillus subtilis* for protease production.They reported that alkaline protease production was started from 6 hours onward and reached a maximum level by 24 hours . It was also observed that the alkaline protease production with immobilized cells in agar-agar was less than the immobilized cells in agar-agar were less than the immobilized cells with other matrices

Mishra, (2007) reported no significant difference in the protease production from *Bacillus circulans* between 2 and 3% agar

concentrations, however at 4% concentration of agar-agar the enzyme production was significantly less than 2 and 3% concentration and the maximum production by agar immobilized cells was observed to be at 96h.

Effect of Bead size

Alkaline protease production was carried out in different volumes of agar beads (62.5,250,562.5 mm^3) and the results obtained revealed maximum protease production with 62.5mm3 size at 24 h and it started decreasing sequentially at large bead sizes.(Fig. 6)

Mishra, (2007) also reported that maximum protease production by *Bacillus circulans* was obtained with a bead volume of 62.5 mm^3 which was significantly higher than those obtained with the other two large bead sizes. Adinarayana et al. (2005) reported 4 mm^3 agar beads to be optimum for alkaline protease production by *Bacillus subtilis* cells.

Effect of inoculum size on entrapment

The effect of inoculum size on entrapment was studied using 2-10 inoculum for preparing agar- agar beads as explained in materials and methods section. The results revealed that increase in inoculum size increases the protease production with a maximum enzyme yield of 2.6U/ml at 10% inoculum size in 24h of incubation (Fig.7)
Mishra, (2007) reported that increase in inoculum size for entrapment of *Bacillus circulans* increases the protease production with a maximum enzyme yield at 5% inoculum size in 96h of incubation.

Effect production by repeated batch cultivation with agar entrapped cells

The reusability of alkalophilic *Bacillus sp*. NB34 cells immobilized in agar-agar matrix for repeated batch cultivation was evaluated in a similar manner as with alginate entrapped cells .The results revealed that the maximum yield of protease was obtained in 1^{st} cycle at 24h, in the subsequent cycles, the protease production could be achieved however it started decreasing sequentially (Fig. 8).

In similar results, Adhinarayan et al. (2005) carried out the repeated batch fermentation for the production of alkaline protease by agar entrapped cells of *Bacillus subtilis* and the results revealed that there was a gradual decrease in the alkaline protease titer from the first batch onwards.

In contrast, Mishra , (2007) reported that there was no significant difference between different cycles with respect to protease production by agar entrapped cells of *Bacillus circulans* and the activity was maintained throughout the 5 cycles tested.

Alkaline protease production in semi – continuous process

The alkaline protease production by semi continuous process was studied by agar agar entrapped cells of alkalophilic *Bacillus* sp. NB34. The protease production was optimized with respect to column length and flow rate

Effect of column length

The columns were packed with agar bead size (62.5 mm^3) for 5-20cm column length and enzyme production media was poured over the beads. The columns were incubated at room temperature for overnight (16h), followed by flushing the spent production media and fresh medium was then poured over the beads, samples were collected every 4 h at a flow rate of 2 ml/h

Results presented in Fig.9 reveal that enzyme production increases up to 36h and started decreasing thereafter. The enzyme production was comparable with all the column lengths however, at 10 cm length it was little better. The enzyme production was also significantly better the batch production. The enzyme product was significantly better than batch production the production of 5.0 U/ml in 36h could be achieved with semi-continuous process vis–a-vis 2.6u/ml under batch production in 48h Similar results have been reported by Mishra, (2007) where a column length of 10cm has been reported to be the optimal length for the production of protease in semi-continuous system by agar agar entrapped cells of *Bacillus circulans.*

Effect of flow rate

The flow rate in semi-continuous process was varied between 1.5-5ml/h. Enzyme production was in the same range upto a flow rate of 3.75 ml/h. However at a higher flow rate of 5 ml/h a decrease in the enzyme production was seen. (Fig.10)

In similar results, Mishra, (2007) has also reported a flow rate of 2.5 ml/h to be ideal for the production of protease by agar immobilized cells of *Bacillus circulans* in semi –continuous system. With increase in flow rate, the reduction levels of protease production have been reported.

Alkaline protease production by adsorbed cells

The adsorption of alkalophilic *Bacillus* sp. NB34 was standardized using different concentrations of activated charcoal and keiselguhr. Then the production of alkaline protease was optimized using different amounts of adsorbed cells.

Effect of concentration of activated charcoal and Keiseluhr

The use of charcoal and keiselguhr for the adsorption of alkalophilic *Bacillus* sp. NB34 cells was studied by incubating the cell and adsorption material mixture in a water bath 37° C for 2h under shaking conditions. The mixture was centrifuged at 600rpm and the supernatant was plated in different dilution on Horikosh medium to determine free cells. Different amounts of charcoal and keiselguhr, 50-100mg/ml were added to the inoculum and adsorption capacity was studied

Results presented in Fig.11 and 12 revealed that 75mg/ml concentration of activated charcoal as well as keiselguhr production minimum number of free cells hence the maximum adsorption. Therefore this concentration was chosen for the adsorption of the cells of alkalophilic *Bacillus* sp. NB34.

Mishra, (2007) also reported that 75mg /ml of concentration of activated charcoal and keiselguhr gave maximum adsorption of *Bacillus circulans* cells.

Effect of inoculum size of adsorbed cells for the production of protease

The activated charcoal and keiselguhr optimally adsorbed with alkalophilic *Bacillus* sp. NB34 was added. In different concentrations (5-20%) in HK medium. The results revealed that the maximum activity (2.6 U/ml) with activated charcoal could be achieved when it was added at a concentration of 205 9 Fig.13 in the medium. Similarly, with keiselguhr also, maximum activity (3.0U/ml) was obtained when it was added at a concentration of 20% Fig. 14.

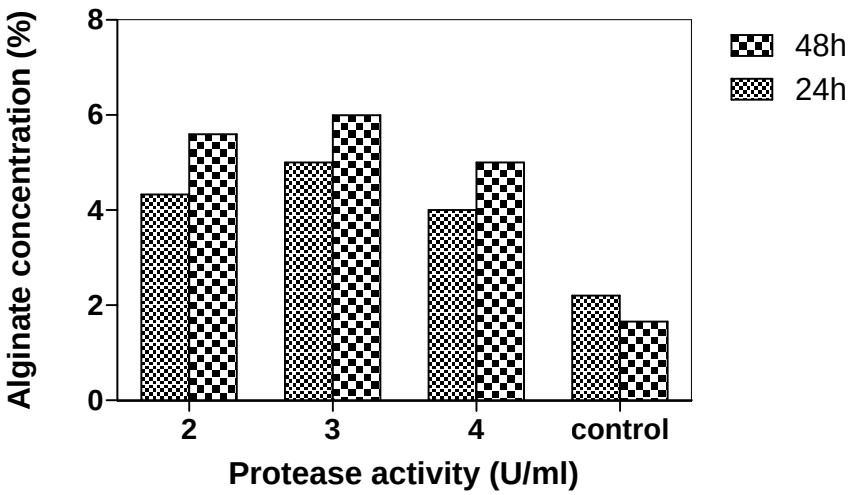


Fig.1 Effect of alginate concentration on alkaline protease production by calcium alginate entrapped cells of alkalophilic *Bacillus* sp. NB34

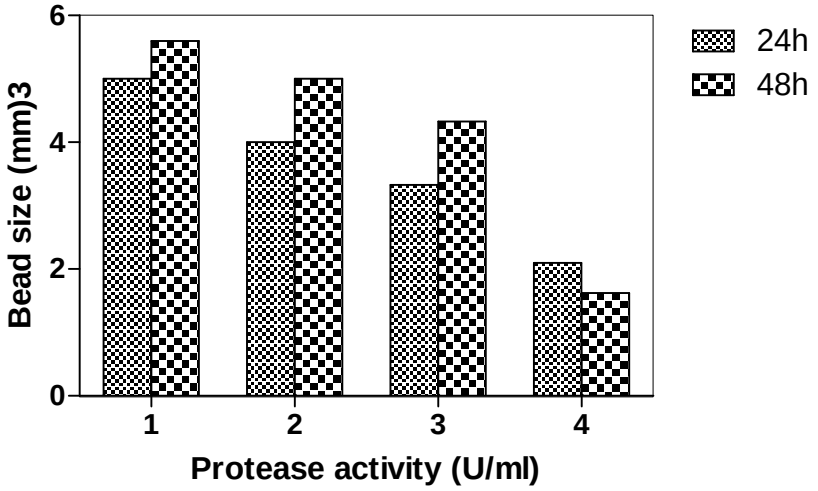


Fig.2 Effect of alginate bead size on alkaline protease production by calcium alginate entrapped cells of alkalophilic *Bacillus* sp. NB34

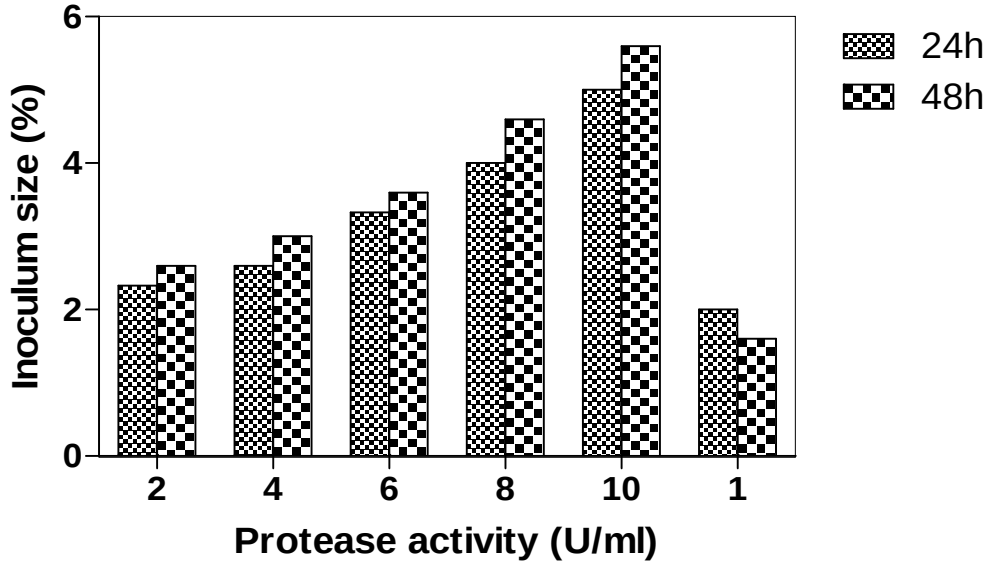


Fig.3 Effect of inoculums size on alkaline protease production by calcium alginate entrapped cells of alkalophilic *Bacillus* sp. NB34

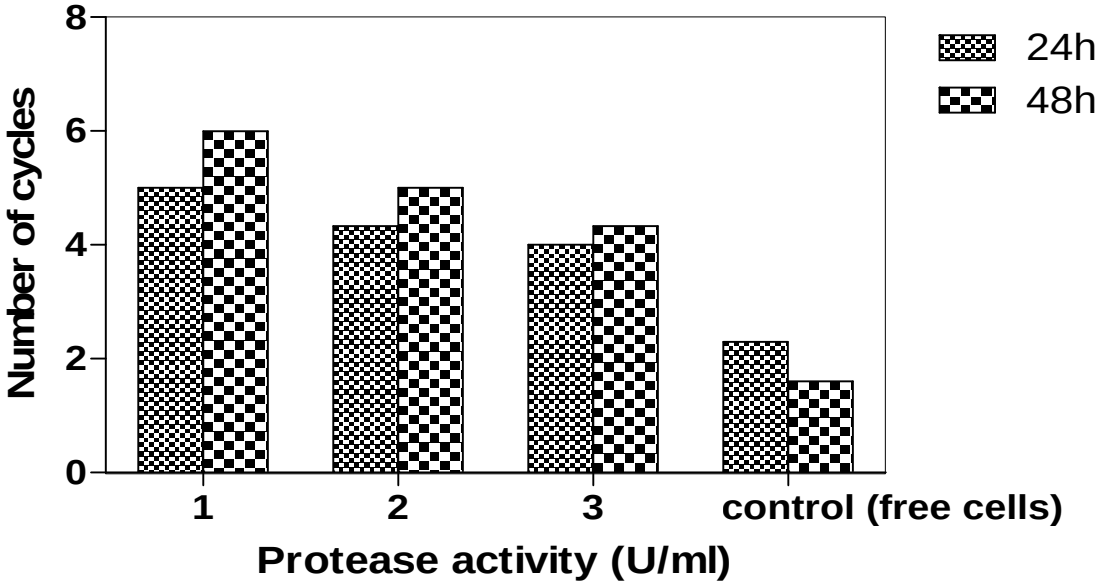


Fig.4 Alkaline protease production in repeated batch cultivation by calcium alginate entrapped cells of alkalophilic *Bacillus* sp. NB 34

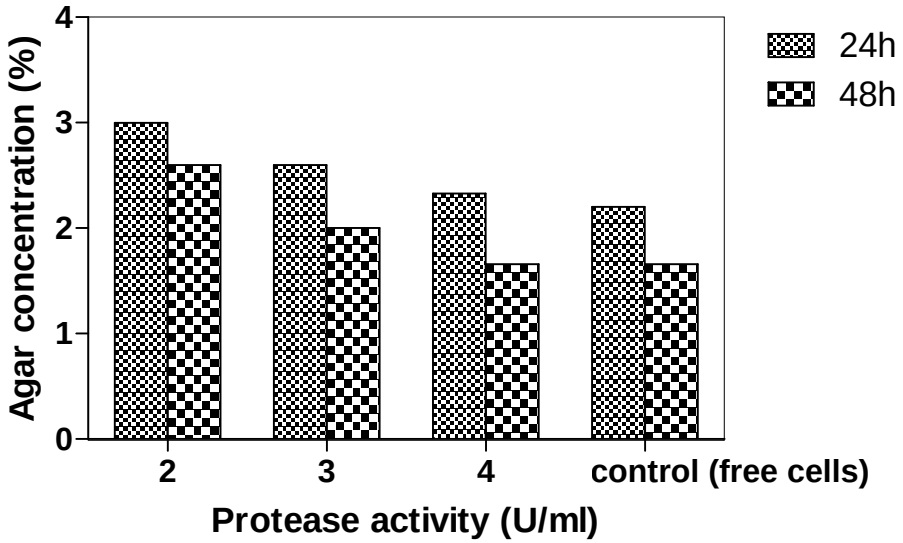


Fig.5 Effect of agar concentration on alkaline protease production by agar-agar entrapped cells of alkalophilic *Bacillus* sp. NB 34

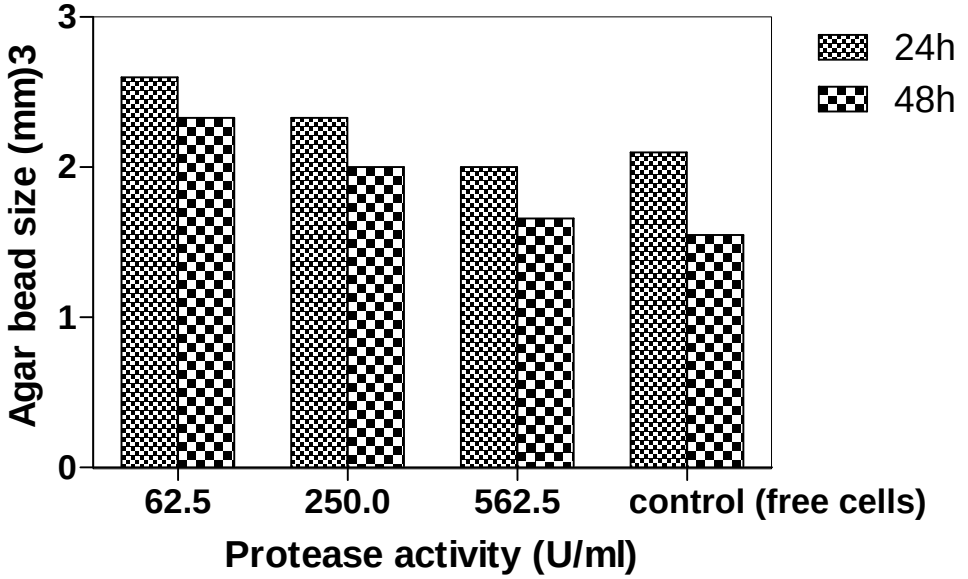


Fig.6 Effect of agar bead size on alkaline protease production by agar- agar entrapped cells of alkalophilic *Bacillus* sp. NB 34

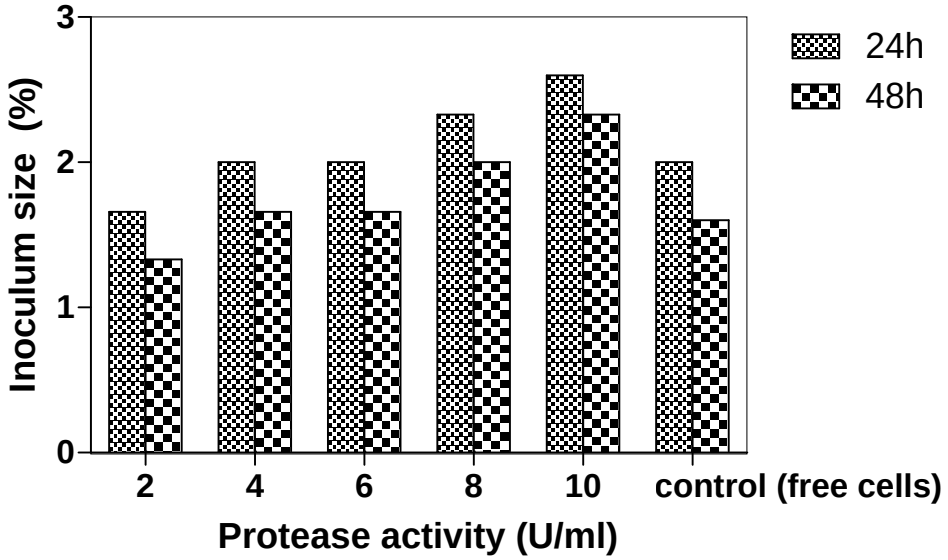


Fig.7 Effect of inoculum size on alkaline protease production by agar-agar entrapped cells of alkalophilic *Bacillus sp.* NB 34

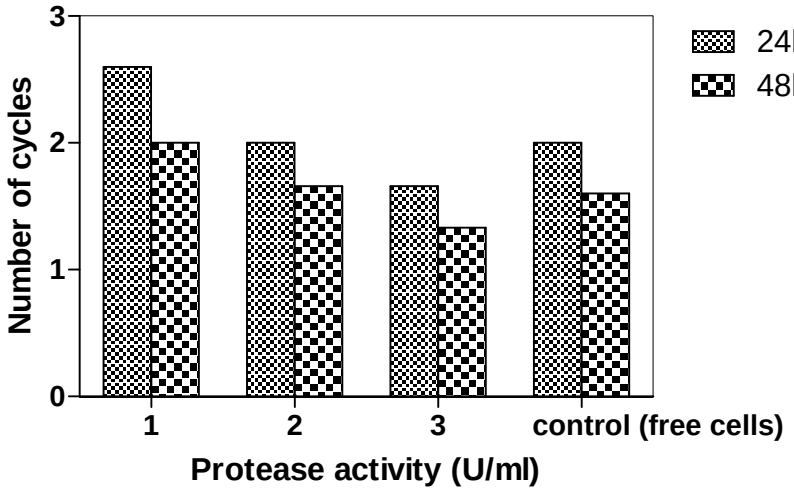


Fig.8 Alkaline protease production in repeated batch cultivation by agar agar entrapped cells of alkalophilic *Bacillus* sp. NB 34

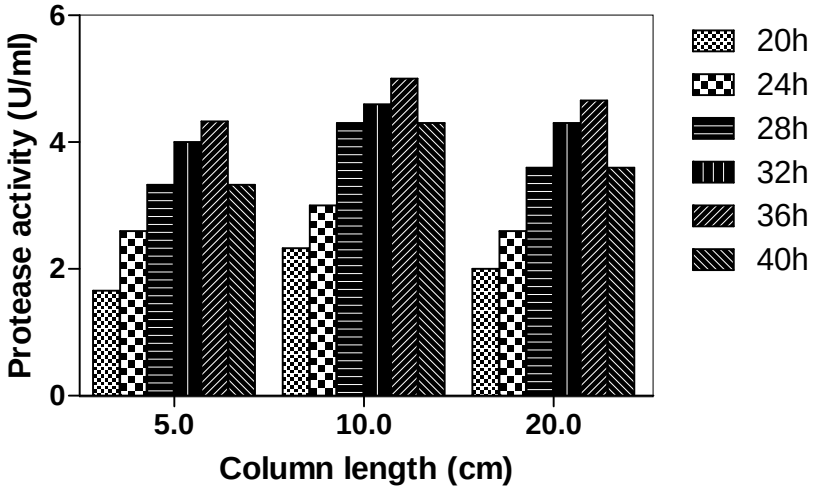


Fig.9 Effect of column length on alkaline protease production by agar agar entrapped cells of alkalophilic *Bacaillus* sp. NB34 in sem continuous process

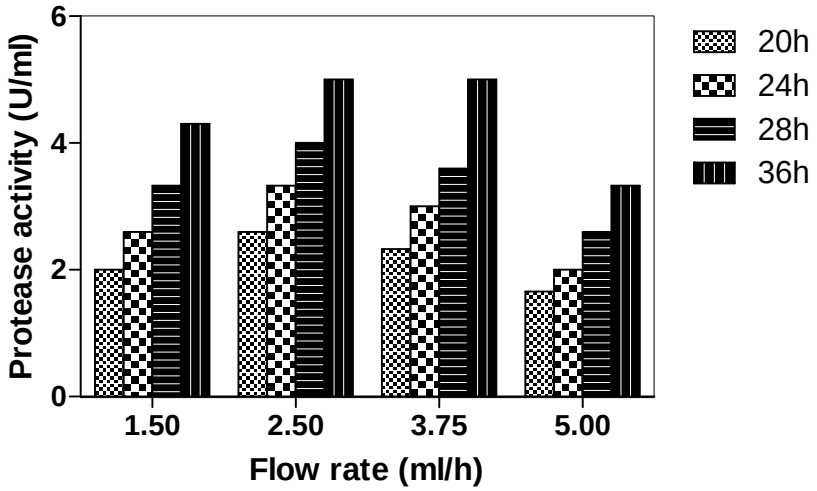
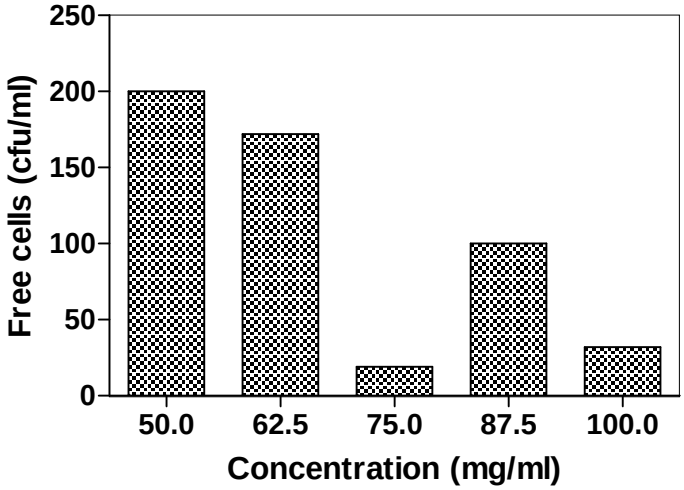
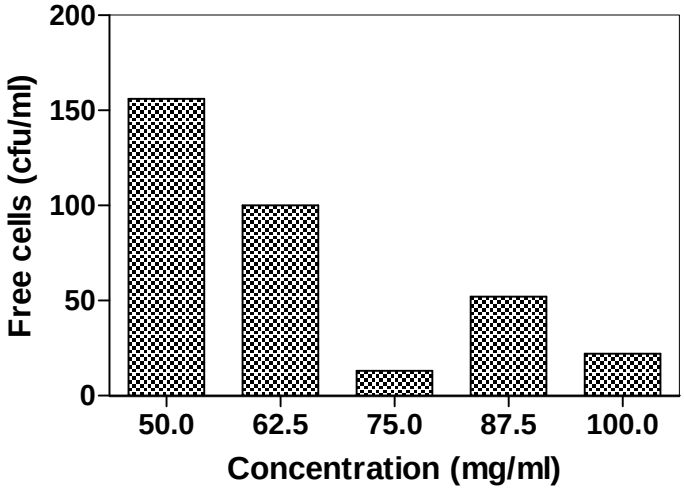


Fig.10 Effect of flow rate on alkaline protease production by agar agar entrapped cells of alkalophilic *Bacillus* sp. NB34 in semi continuous process



Initial cell count =320*10^6 cfu/ml

Fig.11 Effect of different concentration of activated charcoal on adsorption of alkalophilic *Bacillus* sp. NB 34 cells



Initial cell count =320*10^6 cfu/ml

Fig. 11 Effect of different concentration of keiselguhr on adsorption of alkalophilic *Bacillus* sp. NB 34 cells.

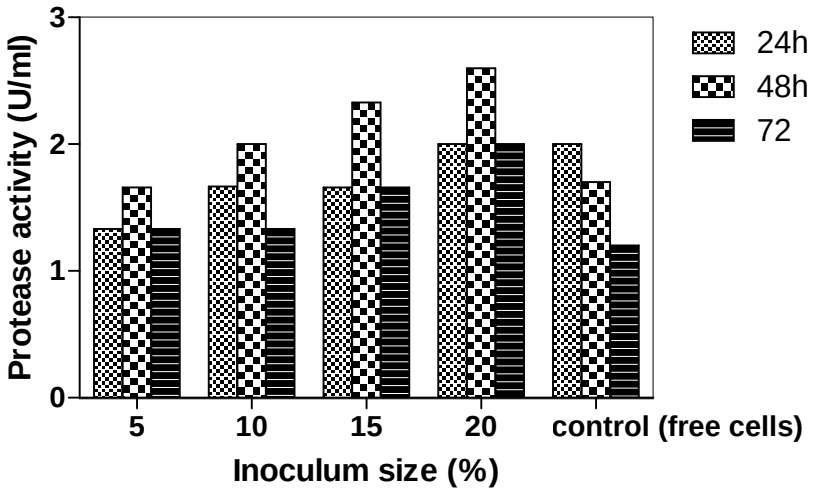


Fig.13 Effect of inoculums size on alkaline protease production by alkalophilic *Bacillus* sp. NB34 strain adsorbed on activated charcoal

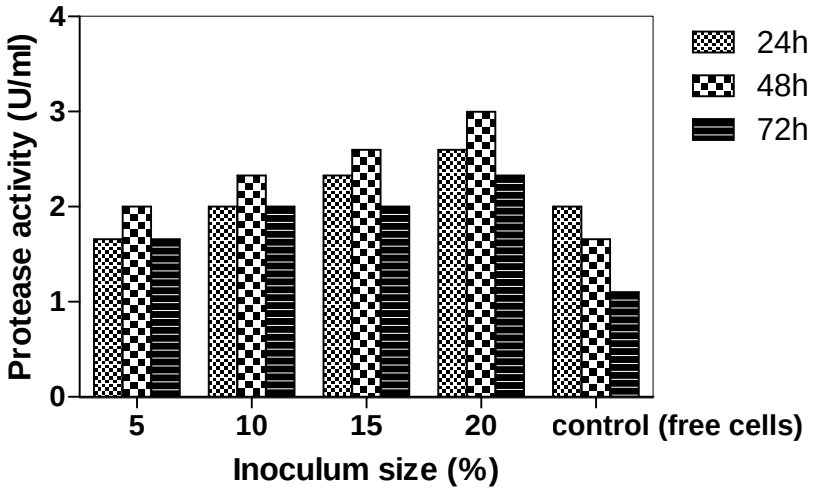


Fig.14 Effect of inoculums size on alkaline protease production by alkalophilic *Bacillus* sp. NB34 strain adsorbed on keiselguhr

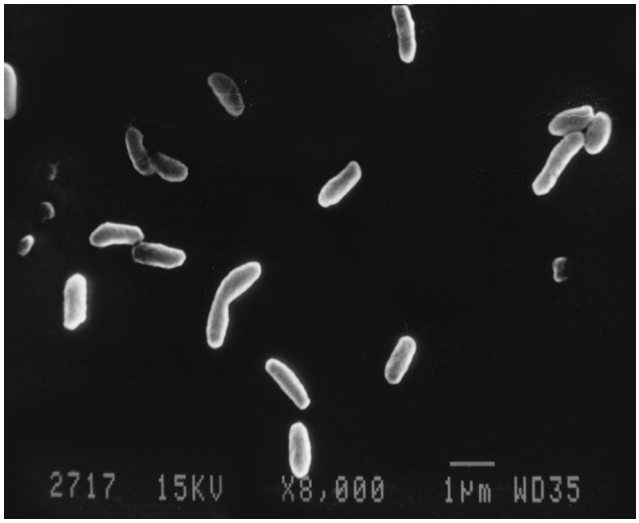


Fig.A SEM observation of free cells of Alkalophilic *Bacillus* sp. NB34 (X 8000) 1µm

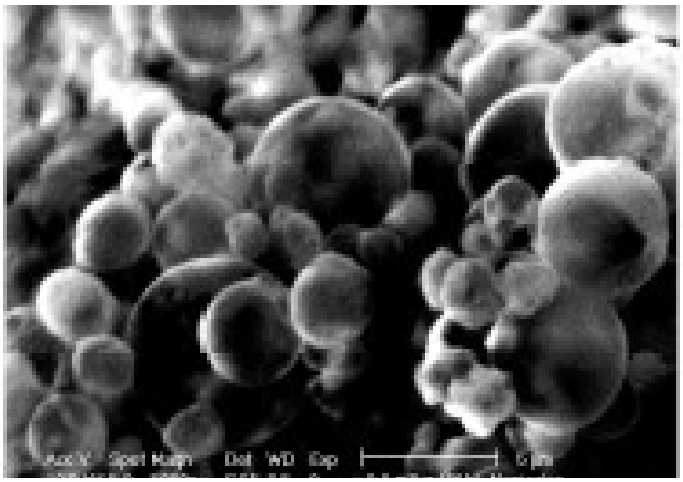


Fig.B SEM observation of alginate entrapped cells of Alkalophilic *Bacillus* sp. NB34 (X 7500) 1µm

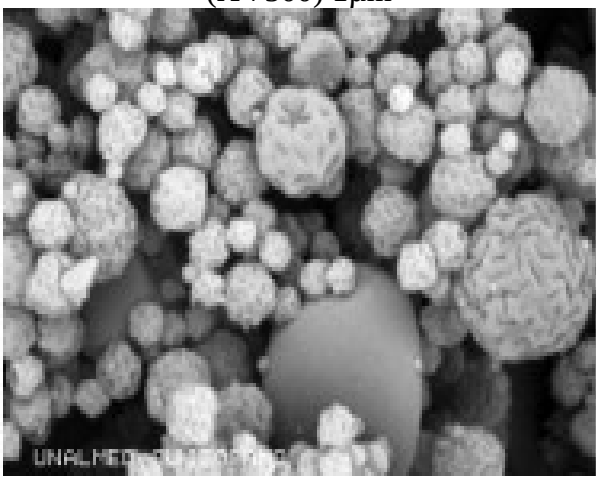


Fig.C SEM observation of agar entrapped cells of Alkalophilic *Bacillus* sp. NB34 (X 8000) 1µm

In both cases the maximum production was given at 48 h of incubation and the enzyme production was in same range at that of free cells. Similarly, Mishra (2007) reported 20% inoculum size as optimum for protease production by *Bacillus circulans* adsorbed on activated charcoal and keiselguhr. However, in this case, the adsorbed cells could give better production than free cells

Scanning electron microscopy

In scanning electron microscopy, free cells were seen to be typical cells of Bacilli. (Fig. A). The internal structure of calcium alginate and agar –agar beads in the presence of immobilized alkalophilic *Bacillus* sp NB34 was studied under a scanning electron microscope. It was observed from the SEM photographic plates that the cells were randomly distributed in alginate and agar beads (Fig. B and C).The region in the periphery of the beads appeared to be more densely packed as compared to the central region due to higher growth rate aiming to better the supply of nutrients. In literature, SEM of alginate entrapped cells is available showing similar observation of random cell distribution (Beshay, 2003 and Beshay and Moreira, 2003).

References

Adinarayana, K., Jyothi, B., and Ellaiah. P. 2005. Production of alkaline protease with immobilized cells of *Bacillus subtilus* PE-11 in various matrices by entrapment technique. AAPS Pharm Sci Tech 6, (3): 91-97.

Ahmed, S.A.,Saleh, S.A. and Abdel-Fattah, A.F. 2007. Stabillization of Bacillus licheniformis ATCC 21415 Alkaline Protease by immobilization and Modification; J. Basic and Appl. Sci. 1, (3): 313-322.

Beshay, U., friehs, K., Azzam, AE., Flaschel, E.2003 Analysis of thee behavior of Dicytostellum disciedium in immobilized state by means of continous cultivation. Bioprocess Biosyst. Eng.26:117-112.

Beshay, U.and Moreira. A.2003 Repeated batch production of alkaline protease by using porous sintered glass as carriers. Process Biochem.38(10):1463-1469.

Chauhan, PS., George, N., Sondhi, S., Puri, N., Gupta. N.2014a. An overview of purification strategies for microbial mannanases.Int J Pharma Bio Sci 5 (1): 176-192.

Chauhan, PS., Soni, SK., Sharma, P., Saini, A., Gupta. N.2014b. A mannanase from Bacillus nealsonii PN-11: Statistical optimization of production and application in biobleaching of pulp in combination with xylanase. Int J Pharma Bio Sci 5 (1): 237-251.

Chauhan, PS., Sharma, P., Puri, N., Gupta, N.2014c Purification and Characterization of an Alkali-Thermostable β-Mannanase fromBacillus nealsonii PN-11 and Its Application In Manno-oligosaccharides Preparation Having Prebiotic Potential Eur Food Res Technol 238 (6) 927-936.

Chauhan, PS., Sharma, P., N, Gupta., N, Puri.2014d A Process for Reduction in Viscosity of Coffee Extract by Enzymatic Hydrolysis of Mannan. Bioproc and Biosyst Eng, 37 (7): 1459-67.

Chauhan, PS.,Puri, N., Sharma, P., Gupta. N.2012 Mannanases: microbial sources, production, properties and potential biotechnological applications. Appl Microbiol Biotechnol 93 (5): 1817-1830.

Cheema, B.S. 2008 Studies on alkaline thermostable protease from an alkalophilic bacterium : Production, Characterization and application. M.Sc Thesis, Panjab University, Chandigarh.

Djamel, C., Ali, T., and Nelly, C. 2009 Acid Protease Production by Isolated Species of *penicillium.* European J. Sci Res. 25 (3): 469-477.

Dutta, J.R. and Banerjee R.2006 Isolation and characterization of a newly isolated *Pseudomonas Mutant* for protease

production. Brazillian J. Biol. Technol. 49 (1): 37-47.

Elibol, M. and Moreira A.R.2003.Production of extracellular alkaline protease by immobilization of marine bacterium Teridinobacter turnnirae. J Process biochem.38:1445-1450.

Genckal, H., Tari, C. 2006 Alkaline protease production from alkalophilic Bacillus sp. isolated from natural habitats'', Enzyme and Microbial Technology.39: 703–710.

George, N., Chauhan, PS., Kumar, V., Puri, N., Gupta. N.2014a. An approach to ecofriendly leather: lime and sulphide free dehairing of skin and hide using bacterial alkaline protease at industrial scale. J Clean Prod.

George., N. Chauhan, PS., Puri, N., Gupta, N.2014b Statistical optimization of process parameters for production of alkaline protease from Vibrio metschnikovii NG155 having application in leather industry. Int J Pharma Bio Sci. 5 (1): 509-517

George, N., Chauhan, PS., Sondhi, S., Saini, S., Puri, N., Gupta, N.2014c Biodegradation and Analytical Methods for Detection of Organophosphorous Pesticide: Chlorpyrifos. Int J Pure Appl Sci Technol 20 (2) 79-94.

Gupta, A., Joseph, B., Mani, A., and Thomas, G.2007 Biosynthesis and properties of an extracellular thermostable serine alkaline protease form *Virgibacillus pantothenticus*. World J. Microbiol. Biotechnol. 104: 946-957.

Joshi, S. and Satyanarayana. T.2013 Characteristics and applications of a recombinant alkaline serine protease from a novel bacterium Bacillus lehensis. Bioresource Technology.131: 76-85.

Kalisz. H.M.1988. ''Microbial proteinases'', in A Fietcher, Ed., In Advances in Biochemical Engineering/ Biotechnology. 36: 3–61.

Khosravi- Darani, K., Falahatpishe, H.R. and Jalali, M.2008. Alkaline protease production on date waste by an alkalophilic Bacillus sp. 2-5 isolated from soil. African J. Biotechnol. 7(10): 1536-42.

Mishra, S. 2007 Production of alkaline protease by immobilized cells of Bacillus sp M.Sc. Thesis., Panjab Agricultural University, Ludhiana.

Nadeem, M., Qazi, J.I., Baig, S., Syed. Q. 2008 Effect of medium composition on commercially important alkaline protease production by Bacillus licheniformis N-2. J. Food technol. Biotechnol. 46 (4): 388-394.

Portmarthi, R., Subhakar, C,H., Pavani, A.,.Jetty, A.2007Evaluation of various parameters of calcium alginate immobilized method for enhanced alkaline protease production by Bacillus licheniformis NCIM-2042 using statistical methods J.Bioresource Technol.99(6) 1776-1786.

Rai, S.K., Roy, J.K. and Mukherjee, A.K. 2010 Charaterization of a detergent-stable alkaline protease from a novel thermophilic strain Paenibacillus tezpurensis sp nov. AS-S24-II. Applied Microbiology and Biotechnology. 85:1437-1450.

Saeki, K., Ozaki, K., Kobayashi, K.2007 Detergent alkaline proteases: Enzymatic properties, genes and crystal structures.J.Biosci. Bioeng.103(6) 501

Sondhi, S., Sharma, P., George, N., Chauhan, P.S., Puri, N., Gupta, N. 2014 A Novel Extracellular Thermo-Alkali-Stable Laccase from *Bacillus tequilensis* SN4 having potential to biobleach softwood pulp. 3Biotech, DOI.10.1007/s13205-014-0207-z.

Zhu, W. , Cha, D., Cheng, G. , Peng, Q. , Shen, P. 2007 Purification and characterization of a thermostable prote-ase from a newly isolated Geobacillus sp YMTC 1049. Enzyme and Microbial Technology. 40: 1592-1597.