BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, September 2018. Vol. **15**(3), p. 509-515

Extraction of Protease Under Solid State Fermentation using Bacterial Isolates from Traditional Leather Processing Waste Water Found Around Wukro Maray

Girma Haile and Birhanu Babiye

Ethiopian Institute of Agricultural Research, National Agricultural Biotechnology Research Center, Microbial Biotechnology Program.

http://dx.doi.org/10.13005/bbra/2656

(Received: 13 August 2018; accepted: 19 September 2018)

Enzymes are important in reducing both energy consumption and combating environmental pollution. Proteases are enzymes which catalyze the hydrolysis of protein molecules.Most of the tannery industries in Ethiopia use chemicals for dehairing that led great environmental and human health problem. The objectives of the present study were,to isolate potential protease producing bacteria from water sample collected from traditional leather processing waste water around Wukro maray;to extract the protease enzyme through SSF using cheap wheat bran, and evaluate the potential activity of the enzyme in leather dehairing. Water samples were serially diluted and 1ml of sample was spread on nutrient agar and kept at 37^{0}C for 24 hrs. Many colonies of bacteria were formed. The colony from C10^{-4} and G10^{-3} were taken by using inoculating loop for sub culturing to get pure colony. Then the pure cultured colony were inoculated into the 250 ml Erlenmeyer flasks containing substrate were fermented after 6 days incubation at 37^{0}C. The results of the unknown concentration of the crude protease enzyme showed successfully used as dehairing agent on hide. The results indicate that these bacteria isolate can be used as biotechnological tool for industrial purpose.

Keywords: Bacteria, Leather dehairing, Protease, Solid state fermentation (SSF), wastes**.**

Enzymes play crucial roles in different applications: in producing the food we eat, the clothes we wear, the drugs we need, the detergents we use, even in producing fuel for our automobiles, etc. Apart from use in various production processes towards greater efficiency, enzymes are also important in reducing both energy consumption and combating environmental pollution. Proteases are enzymes which catalyze the hydrolysis of protein molecules. Microbial proteases are among the most important, better studied enzyme groups since the development of enzymology. They are so far exploited as industrial catalysts in various industrial sectors, leather processing being the most interesting and potential area for large scale application (Abdullah, 2006).

Proteases are hydrolytic enzymes found in every organism to undertake important physiological functions. These include: cell division, regulating protein turnover, activation of zymogenic performance, blood clotting, lysis of blood clot, processing and transport of secretary proteins across membrane, nutrition, regulation of gene expression and virulence

*Corresponding author E-mail: bbirhanu23@gmail.com

factors. Proteases differ in their specific activities, substrate specificities, pH and temperature optima and stability, active site, and catalytic mechanisms. All these features contributed in diversifying their classification and practical applications in industries involving protein hydrolysis.It also possess some characteristics of biotechnological interest due to which these have become the most important industrial enzymes (Barindra*et al.*, 2006). Commercial proteases account for nearly 60% of the total industrial enzyme in the market (Udandi *et al.,* 2009).

Neutralophilic and alkaliphilic microbial alkaline proteases possess a considerable industrial potential due to their biochemical diversity and stability at extreme pH environments, respectively (Moon *et al.,* 1994). However, the demanding industrial conditions for technological applications and cost of protease production required continuous exercise for search of new microbial resources. Enzyme cost is also the most critical factor limiting wide use of protease for different applications. A large part of this cost is accounted for the production cost of the enzyme. Therefore, reduction in the production cost of enzymes could greatly reduce the cost ofthe enzyme. In submerged fermentation up to 40% of the total production cost of enzymes is dueto the cost of the growth substrate (Enshasy *et al.,* 2008). In this regard, SSF which uses cheap agricultural residues have enormous potential in reducing enzyme production cost. However, studies on protease that are produced insolid state fermentation (SSF) by microorganisms are scarce in literature. As a result, it is of great importance to pursue such studies. This type of fermentation process also does not require highly caliber equipment and energy for agitation to provide oxygen.

Ethiopians are believed to have been practicing traditional leather processing since their ancient civilizations. This local knowledge has been transferred through generations and is being widely practiced these days to process leathers of cattle origin for making their shoes, clothes, beds, cushions and many other items primarily among the rural communities. They use small ponds usually on the sides of rivers and embed the leather for a period of time to remove hairs. However, very little has been done to promote this work through the application of biotechnology.

Most of the tannery industries in Ethiopia use chemicals for dehairing that led to great environmental and human health problem. The tannery pollutants are causing heavy damage to water resources, agriculture, fisheries, and so on. Since the shortage of income to import protease enzyme and lack of technology to produce this enzyme are the major problems that lead the industries to use chemicals like sodium sulfide. At present, there are few scientific reports available in Ethiopia on the potential microbial isolates that can be used in the process of dehairing. Therefore, there is a need to investigate the role and contribution of microorganisms during traditional leather processing.

MATERIALSAND METHODS

Time and Place of the Study

The study was conducted at Aksum, University College of Natural and Computational Science Department of biotechnology laboratory from March to June 2017 G.C

Material and Chemical Required

Materials that were used to conduct the research were petri plate, conical flask, micropipette, inoculating loop, electronic balance, autoclave, test tube, hot plate, spatula,filter paper , fridge, measuring cylinder, beaker, laminar air flow, Bunsen burner, Ependroff tube, cotton, bottles, incubator, centrifuge, horizontal shaker, sample tube, ruler, scissor, and funnel.The chemicals used in the laboratory are like ethanol, MgSO4:7H2O, $CaCl_2$, and distilled water.

Media Preparation

Nutrient agar was prepared from commercially available dehydrated base according to manufacture instruction. 22.4 gram of nutrient agar powder was weighted using a clean electronic balance then 800 ml of distilled water was added into bottle containing the measured of nutrient agar and shacked by hand to mix properly and covered with in a cotton, then the bottle sample was boiled on hot plate then placed in to autoclave for better sterilization for 15 min at 121^0C.

Growth and Isolation of Bacteria

Samples were collected from traditional leather processing ponds/wastes (water from stagnate pond used for dehairing, from submerged goat and cow skin in stagnate pond) and was kept

in sterile tubes in refrigerator, at 4°C until used. The sample collected from traditional leather processing pond was serially diluted (10^{-1} to 10^{-5}) andseeded on nutrient agar medium and after 24 hrs the bacteria was cultured and kept in refrigerator, until going to sub cultured. For sub culture goat sample (10^{-3}) and cow sample (10^{-4}) was selected and cultured by spread plate method on nutrient agar.

Preparation of Substrate

Wheat bran was collected from Dejen powder factory and it was sterilized by autoclaving at 121°C for 15 minutes.

Solid State Fermentation

Solid state fermentation medium containing (g/g): wheat bran, 10; MgSO4.7H**2**O, 0.02; CaCl2, 0.01; was prepared in a 250 ml Erlenmeyer flask, moistening agents was added in such a way to given final bran to moisture ratio of 1:1.5, thoroughly mixed, and autoclaved at 121 ºC for 15 minutes. Then, the sub cultured (pure) bacteria was inoculated by using loop into each flask and, was incubated at 37 °C for 5 days. From the fermented substrate, protease was harvested by soaking the fermented solid with ten volumes of distilled water per gram solid substrate (wheat bran), in shaking condition for 30 minutes at room temperature (Ikasari and Mitchel, 1996). At the end of the extraction, the suspension was hand squeezed through a double layered muslin cloth and the particulate materials clarified by centrifugation at 10,000 rpm for 5 minutes.

Analytical Methods

Test for potential use in enzymatic dehairing

The sheep hides was washed and cut into 3x4 cm pieces. Two setswere placed into petri plate containing distilled water and tape water used as a control. The other set was placed into petri plate containing the enzyme solution by keeping liquid (ml) to gram hide proportion one to one (Najafi *et al*., 2005; Macedo *et al.,* 2005; Malathi and Chakraborty, 1991) and it was incubated at 37^0C for 24 hrs. To assess dehairing extent, one petri plate at a time was taken for hair removal trial with fingers. The skin pieces after treatments was examined for dehairing time, dehairing extent.

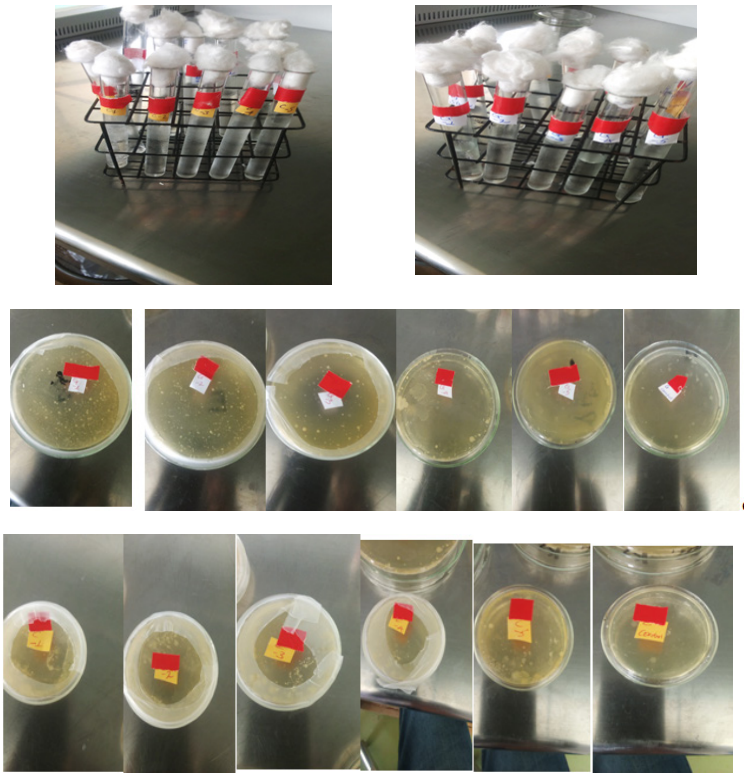


Fig. 1. Serial dilution and bacteria culture

RESULT AND DISCUSSION

The study was conducted to extract industrial protease through solid state fermentation from traditional leather processing wastes, and it has the following results

Isolation of Bacteria

The bacteria were isolated from a sample that taken from traditional leather processing wastes through serial dilution and sub culture.

- Serial dilution of from cow sample (a), serial dilution of from goat sample (b), serial culture of goat sample on nutrient agar (c), and serial dilution of cow sample on nutrient agar (d).

The sub culture were taken from 10^{-3} of Goat sample and 10^{-4} of Cow sample because those levels of serial dilution contains pure colony, and were spread on fresh nutrient agar by using spread plate methods.

Solid state fermentation

Pure cultured colony were inoculated into the 250ml Erlenmeyer flasks containing substrate were fermented after 6 days incubation at 37^0C, and then it was centrifuged.

- Fermentation by inoculating pure colony of goat sample (a), and fermentation by inoculating pure colony of cow sample (b).

Centrifugation

The fermented wheat bran with pure colony were centrifuged at 10000 rpm for 5 min. and it gives supernatant and two layer plate as shown below.

Enzymatic activity on sheep hide dehairing.

The deharing results are shown in the table 1 and figure 5.

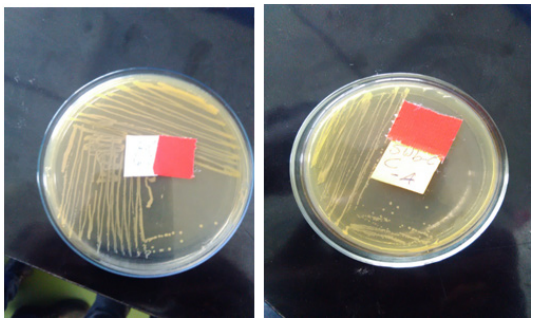
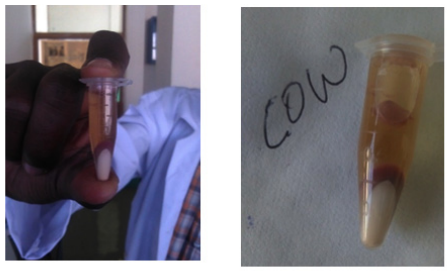


Fig. 2. sub cultures: Subculture from 10^{-3} serially cultured goat sample (a), and sub culture from 10^{-4} serially cultured cow sample (b)

Fig.3. Fermentations

DISCUSSIONS

In this study, microorganisms isolated from traditional leather processing water samples were screened for protease extraction. The microorganisms were protease positive. Enzyme production through SSF has enormous potential in reducing the cost of enzyme production. Since cheap wheat bran as a media substrate can be used, production cost could be minimized that indirectly minimize enzyme cost. Microbial growth medium for enzyme production at industrial scale takes about 30%-40% production cost (Enshay *et al.*,

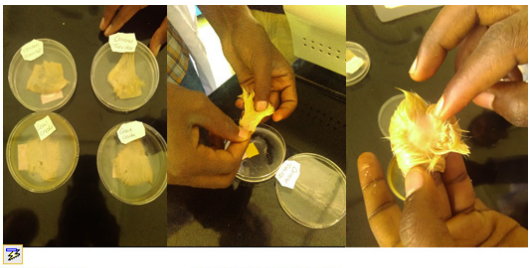


The supernatant were collected into sample tube for dehairing test.

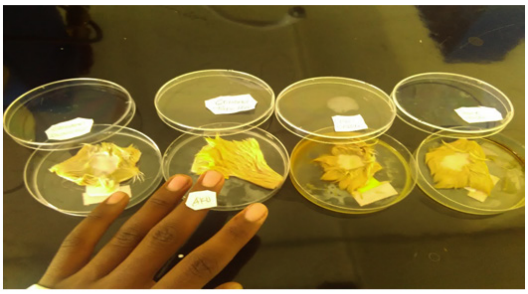
Fig. 4. Collected supernatant

Table 1. Results of enzymatic activity on sheep hide

No	Sample	Temperature in ^{0}C	Time in hrs			
			6	12	20	24
1	Cow crude sample	37	-ve	-ve	+ve	+ve
2	Distilled water	37	-ve	-ve	+ve	+ve
3	Goat crude sample	37	-ve	-ve	+ve	+ve
4	Tape water	37	-ve	-ve	-ve	-ve



a) Before trial b) During trial with finger



c) Collection of results after trial.

Fig. 5. Enzymatic activities on sheep hide

2008), by using wheat bran alone, appreciable amount of protease production can be achieved, implying presences of enough nutrients in wheat bran that supports the growth of the isolate and protease production. The selected isolate from isolate cow sample and goat sample grew well and produced protease under SSF. Thus, growth and extraction of appreciable amount of protease by isolate cow sample and goat sample under SSF offer better option for its large scale production. Many reports showed bacterial and fungus alkaline protease production lower at (d" 25^{0}C) and moderate (30^{0}C-40^{0}C) temperatures, to mention are in *Aspergillus* and *Bacillus* strains preference of such temperature under SSF (Malthi and Chakraborty, 1991; Kumar and Takagi, 1999; Niadu and Davi, 2005; soarese *et al.,* 2005).

Dehairing is also an important operation in tanneries conventionally practiced using lime and sodium sulphide (Thanikaivelan *et al.*, 2004). In this process, the skin/hide is painted with sulphide which helps to reduce the disulphide bond that is responsible for attachment of hair keratin in epidermis. This brings about complete removal of hair, but the hair root remained within skin (Siva Subramanian*et al.,* 2008). However, the use of alkaline protease has proven superior and efficient for selective removal of the non-collagen part of hide/skin (Kamini *et al.,* 1999).

But in the present study was also give the false positive results on distilled water and the tape water was not de haired the hides. Results of enzymatic (crude protease enzyme) on sheep hide dehairing showed successful use of this enzyme as a dehairing agent. Complete dehairing of hide was achieved at 20 hr. Because of specificity to hydrolyse non-collagen protein part at hair roots in hide and the enzyme also was not purified, proteases are very important in shortening hide dehairing time and in production of high quality full gain leather having natural hair pores on the surface (Sivasubramanian et al., 2008). Cow/sheep hide is usually treated with dehairing chemicals in a drum for 24 hr (Thanikaivelan et al., 2004). Shortening of deharing time has been also reported, 20 hr for *Aspergilus flavus* protease by Malathi & Chakraborty (1991), and 9 hr for keratinases of *Bacillus subtilis* S14 by Macedo *et al*., (2005). Thus, the centrifuged cow and goat fermented sample produced crude protease has a potential to substitute environmentally objectionable dehairing chemicals for hide/skin dehairing in leather industries and for production of quality leather products.

CONCLUSIONS

Proteases are one of the most important groups of industrial enzymes with considerable application in the animal feed processing, leather industry, medical activity, detergent additive, protein hydrolysis, silver recovery, management of wastes and other sectors. In this study, protease was successfully produced by bacteria isolated from traditional leather processing ponds for the purpose of dehairing.

Generally the crude protease separated by centrifuge and its enzymatic activity on sheep hide was successfully used as deharing agents.

REFERENCES

1. Abdullah F.M. The production of extracellular protease using *Bacillus subtilis*: effect of temperature and agitation speed. MSc Thesis. University Collegeof Engineering and Technology. Malaysia. 2006; 46p.
2. Agrawal, D., Patidar, P., Banerjee, T. and Patil, S. Alkaline protease production by the soil isolate of *Beauveri felin* under solid state fermentation condition: parameter optimization and application to soya protein hydrolysis. *Proces.Biochem* 2004; **40:**1131-1136.
3. Aguilar, C.N., Gutierrez-Sancez, G., Rado-Barragan, P.A., Rodriguez-Herrera, R., Martinez-Hernandez, J.L. and Contreras-Esquivel, J.C. Perspectives of solid state fermentation for production of food enzymes. *Am.J.Biochem. & Biotech,* 2008; **4**:354-366.
4. Amare Gessesse, Hatti-kaul, R., Berhanu A.Gashe and Mattiasson, B. Novel Alkaline protease from alkaliphilic bacteria grown on chicken feather. E*nz. Microb. Technol***,** 2003; **32:** 519-527.
5. Barindra, S., Debashish, G., Malay, S. and Joydeep, M. Purification and characterization of a salt, solvent, detergent and bleach tolerant protease from a new gamma Proteobacterium isolated from the marine environment of the Sundarbans. *Process Biochem*. 2006; **41(1):** 208-215.
6. Barrios-Gonzalez, J.,Fernandez, F.J., Tomasini, A., and Mejia, A. Secondary metabolites production by solid state fermentation. *Malys.J. microbial.* 2005; **1:**1-6.
7. Chiplonkar, J.M., Gangodkar, S.V., Wagh, U. V., Ghadge, G. D. Rele, M. V. and Srinivasan, M. C. Applications of alkaline protease from *Conidiobolus* in animal cell culture. *Biotechnol. lett.,* 1985; **7:** 665-668.
8. Crout, D.H., Macmanus,D.A., Rica, JM, Singh,S., Critchley, P. and Gibson,W.T. Biotransformation in the peptide and carbohydrate fields. *Pure & Appl.chem,* 1992; **64**: 1079-1084.
9. Da Silva, M. Enzymatic Treatment of Wool with Modified Proteases. *Ph.D Thesis. University of Minho* 2005.
10. Enshasy, E.H., Abuol-Enein, A., Helmy, S. and Azaly, E. Optimization of the industrial 2008.
11. Freddi, G., Mossotti, R. and Innocenti, R. Degumming of silk fabric with several proteases. *J Biotechnol,* 2003; **106:**101-112.
12. Fujiwara, N., Yamamato, K., and Masui,A. Utilization of thermostable alkaline protease from an alkaliphilic thermophile for the recovery of silver from used x-ray film. *J.Fermt.Bioeng* 1991; **72:** 306 -308.
13. Gençkal, H. Studies on Alkaline Protease Production from *Bacillus* sp. MSc Thesis. Ýzmir Institute of Technology, Ýzmir, Turkey**.** 2004; 98p.
14. Gupta, R., Q.K. Beg and P. Lorenz. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl. Microbiol. Biotechnol,* 2002a; **59:**15–32.
15. Gupta, R., Beg, Q.K. and Lorenz, P. Bacterial alkaline proteases: molecular approaches and industrial applications. *Appl.Microbiol. Biotechnol,* 2002b; **59:**15-32.
16. Gupta, R., Q.K. Beg, S. Khan and B. Chauhan. Anoverview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl Microbiol Biotechnol* 2002c; **60(4):** 381-95.
17. Guzman, F., Brberis, S. and Illanes, A. Peptide synthesis: Chemical or enzymatic. *Ejbiotechnol.* 2007; **10:**179-314.
18. HÕlker, U. and Lenz, J. Solid-state fermentation-are there any biotechnological advantages? *Curr. Opin. Microbiol***.** 2005; **8:**301–306.
19. Horikoshi, K. Alkaliphiles: some applications of their products for biotechnology. *Microbiol. Mol. Biol. Rev.* 1999; **63:** 735–750
20. Ikasari, L. and Mitchell, D.A. Leaching and characterization of *Rhizopus oligosporus* acid protease from solid-state fermentation. *Enz. Microb.Technol,* 1996; **19:**171-175.
21. Ikram. N. Enhanced production of thermo stable bacterial proteases and their applications. PhD. Disertation. University of Punjab. Pakistan. 2008; 158p.
22. Kamini, N.R., Hemachander, C., Mala, J.G.S. and Puvanakrishnan, R. Microbial enzyme technology as an alternative to conventional

chemicals in leather industry. *Curr.Sci***.** 1999; **77**: 80-86.

23. Kumar, C.G. and Takagi, H. Microbial alkaline proteases: from bio-industrial viewpoint. *Biotechnol. Adv* 1999; **17**: 561–594.
24. Kumara, M.S. Kashyap,.N. S., Vijay. R., Rahul. T, Anuradha. M. Production and optimization of extra cellular Protease from *bacillus* sp. Isolated from soil. International *Journal of Advanced Biotechnology and Research***.** 2012; **3:**564-569.
25. Kwon, Y.T, Kim, J.O., Moon, S.Y., Lee, H.H. and Rho, H.M. Extracellular alkaline protease from alkaliphilic *Vibrio metschnikovii* strain RH530. *Biotechnol Lett*. 1994; **16:** 413–418.
26. Macedo, A.J., Da Silva, W.O., Gava, R., Driemeier, D., Henriques, J.A.P. and Termignoni, C. Novel keratinase from *Bacillus subtilis* S14 exhibiting remarkable dehairing capabilities. *Appl.Env.Microbiol* 2005; **71:** 594-596.
27. Malathi, S. and Chakraborty, R. Production of alkaline proteases by a new *Aspergillus flavus* isolate under solid state fermentation conditions for use as a depilation agent. *Appl.Environ. Microbiol***.** 1991; **57:** 712-716.
28. Maurer, K. Détergent protéases. *Cur. Opin. Biotechnol*. 2004; **15:** 330–334.
29. Moon, S.Y., Oh, T.K. and Rho, H.M. Purification and characterization of an extra cellular alkaline protease from *Bacillus subtilis* RM **615.***Korean Biochem.J***.** 1994; **27:** 323-329.
30. Mukhtar, M. and Ul-Haq, I. Production of alkaline protease by *Bacillus subtilis* and its application as a depilating agent in leather processing. *Pak.J.Bot***.** 2008; **40:** 1673-1679.
31. Nadeem, M. Biotechnological production of alkaline protease for industrial use. PhD Thesis. University of Punjab, Lahore, Pakistan. 2009; 208p.
32. Najafi, M.F., Deobagkar, D. and Deobagkar, D. Potential applications of protease isolated From*Pseudomonas aeruginosa* PD100. *EJ.Biotechnol*. 2005; **8**: 197-203.
33. Nakiboglu, N., Toscali, D. and Yasa, I. Silver recovery from waste photographic films by an enzymatic method. *TurkJ Chem*. 2001; **25***:* 349-353**.**
34. Niadu, K.S.B. and Devi, K.L. Optimization thermostable alkaline protease production from species *Bacillus* using rice bran. Afr.J.Biotechnol. 2005; **4**:724-726.
35. Nirmal, N.P., Shankar S. and Laxman R.S. Fungal Proteases: An Overview. *J. Biotech and Biosci*, 2011; **1(1):** 1-40.
36. Pandey, A., Selvakumar, P., Soccol, C.R. and Nigam, P. Solid state fermentation for the production of industrial enzymes. *Curr.sci***.** 1999; **77***:*149-162.
37. Pérez-Guerra, N., Torrado-Agrasar, A., López-Macias, C. and Pastrana, I. Main characterstics and applications of solid substrate fermentation. *E.J.Envir. Agric. Food chem* 2003; **2:** 343- 350.
39. Production of alkaline protease by *Bacillus licheniformis* in different production scales. *Aust.J.Bas.Appl.Sci***.2**:583-593
38. Rao, M.B., Tanksale, A.M., Ghatge, M.S. and Deshpande, V.V. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Mol. Biol. Rev.,* 1998; **62**: 597-635.
39. Singhal, P., V. K. Nigam and A.S. Vidyarthi. Studies on production, characterization and applications of microbial alkaline proteases. *International Journal of Advanced Biotechnology and Research* 2012; **3(3):** 653-669.
40. Sivasubramanian, S., Manohar, M. and Puvanakrishnan, R. Mechanism of enzymatic dehairing of skins using a bacterial alkaline protease. *Chemosphere* 2008; **70**:1025-1034.
41. Soarese, V.F., Castilho, L.R., Bon, E.P.S. and Freire D.M.G. High yield Baccilus subtilis for protease production by solid state fermentation: 2005; 311-319. Human press
42. Sumantha, A., C. Larroche and A. Pandey. Microbiology and Industrial Biotechnology of Food-Grade Proteases: A Perspective. *Food Technol. Biotechnol*. 2006; **44 (2)** 211–220.
43. Thanikaivelan, P., Rao, J.R., Nair, B.U. and Ramasami, T. Progress and recent trends in biotechnological methods for leather processing. *Trend. Biotechnol.* 2004; **22:** 181-188.
44. Thanikaivelan, P., Rao, J.R., Nair, B.U. and Ramasami, T. Recent trends in leather making: processes, problems and pathways.*Crit.Rev.Env. Sci.Technol.* 2005; **35:**37-79.
45. Udandi, B., Rajendran, R., Palanivel, K.V. S. and Manoharan, M.J. Optimization of Protease Enzyme Production Using *Bacillus* Sp. Isolated from Different Wastes.*Bot. Res. Intl.,* 2009; **2(2)**:83-87.
46. Underkofler, L.A., Barton, R.R. and Rennert, S.S. Production of microbial enzymes and their applications. *Microbiological Process Report***.** 1958; **6:** 212-221.
47. Vishwanatha, K.S. Acid protease from *Aspergillus oryzae*: Structure stability and enhancement of the activity by physical, chemical and molecular biological approaches. PhD. Thesis. Central Food Technological Research Institute. Karnataka, India. 2009; 266p.
48. Yust, M., Pedroche, M., Megías, J.C., Girón-Calle, J., Alaiz, M., Millán, F. and Vioque, J. Rapeseed protein hydrolysates: a source of HIV protease peptide inhibitors. *Food chem*. 2004; **87**:387-392.