

# Investigation of keratinase activity and feather degradation ability of immobilised *Bacillus* sp. Khayat in the presence of heavy metals in a semi continuous fermentation

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## ABSTRACT

Tonnes of feathers generated from slaughterhouses and those that were being used for the removal of heavy metals in wastewaters are often contaminated with high heavy metals and other chemical agents. The toxic nature of these chemicals makes it difficult for feather degrading bacteria to degrade the feathers. An investigation was made on the effect of different concentration of heavy metals on the keratinase activity and feather degrading ability of immobilised cells of *Bacillus* sp. khayat. PH 8, temperature 27°C, gellan gum concentration of 0.8% and 250 beads per 100 ml of heavy metals free media were the optimum conditions to achieve highest keratinase yield and complete feather degradation within 18 h. Immobilised cells were able to secrete higher keratinase enzyme in the presence of 30 ppm Ag, 20 ppm Co and 15 ppm Cu. While feather degradation and keratinase yield were inhibited by above 5 ppm of Hg, Pb and Zn, up to 10 ppm of As and Cd have no effect on the duo. Immobilised cells were successfully used to degrade 5 g/L of feathers in the presence of individual 30 ppm Ag, 20 ppm Co, 15 ppm Cu, and combination of 5 ppm each of Ag, As, Co, Cu and Ni for 10 and 6 consecutive cycles respectively in a semi-continuous mode of cultivation without desorption. The beads used to degrade feather in the presence of a particular heavy metals were successfully used to degrade other feather laden with other metals. Enhanced keratinase activities and 90-100% feather degradations by gellan gum entrapped *Bacillus* sp. khayat in the presence of various concentrations of chemically known toxic heavy metals showed that the *Bacillus* has a relatively high tolerance to heavy metals while still maintaining its feather degrading ability. This property makes the *Bacillus* a potential tool not only in the bioremediation and waste management of heavy metal laden feathers, but also in the production of keratinase protease for industrial usage.

**Keywords:** Keratinase, Feather degradation, Heavy metals, Immobilisation, Fermentation, *Bacillus* sp. khayat

## INTRODUCTION

The use of biotechnological approaches to manage millions of tonnes of chicken feather wastes generated annually has been receiving more attentions because feather has constituted nuisance to the environment. Microorganisms with enhanced capacity to produce keratinase enzyme in the presence of tough keratin containing feathers are being utilised in an environmentally friendly way to replace the conventional environmentally disastrous methods. Different microbes belonging to group of bacteria, fungi and *Streptomyces* have been isolated and now being used industrially to produce keratinase protease, protein and other valuable essential amino acids (Kumar, 2011; Sahoo, 2012; Muhsin and Hadi, 2002; Tatineni, 2008). The use of detergents and surfactants in many industrial processes has led to the contamination of wastes generated from such industries. The toxic nature of the chemicals makes it more difficult for both transient and resident microorganisms to degrade them effectively. Similarly, continual use of feather as economical and easy biosorption material to remove heavy metals from surface water has led to the accumulation of feather-laden heavy metals. These feather-laden feathers were usually disposed by incineration technique (Das, 2008), a technique that also pollutes the environment with toxic fumes (Sharma and Sharma, 2013). These feathers are more recalcitrant to biological action due to the toxic nature of heavy metals to microorganisms and also as the inhibitors of enzymatic activities (Cabreraet, 2006). Microorganism that is capable of degrading these feathers in the presence toxic chemicals will be very useful in the management of such hazardous wastes. Even though some heavy metals like copper, zinc, nickel and iron at trace level are essential for metabolic processes of some organism, but at elevated concentration, they are also toxic (Seifert, 2005). On the other hand, metals like mercury, silver and cadmium have no biological role and are harmful to organisms, even at very low concentrations (Lima de Silvaet al., 2012). However, many organisms either in free

or immobilised states have been reported to also bio-absorb heavy metals in aquatic and terrestrial environments (Piccirillo and Pereira, 2013; Pires, 2011).

Immobilisation of cells in the field of biotechnology for continual production of microbial end product has been used successfully by different researchers. The advantages of immobilised cells include high catalysis efficiency, high cell density retention, high stability, ability to re-use for different cycles unlike the free cells (Prakash, 2010), less tedious, minimal chance of contamination, and high ease of product separation (Suvase et al., 2010). From literature, bacteria, fungi and *Streptomyces* immobilised in the entrapment matrix such as alginate, chitosan, agar, K-carrageenan have been used to produce keratinase and degrade feathers (Cheethamet, 1979; Vuilleumardet, 1988; Adinarayanaet, 2005; Tosa, 1979; Jouenneet, 1933; Ravi Kumar, 2000). However, there is hardly any report regarding the usage of gellan gum as a carrier for the immobilisation of feather degrading bacteria for the production of keratinase. Gellan gum has several advantages over other matrices as it is non-toxic, has a wide pH stability range (2-10) and has high mechanical and thermal stability (Moslemy, 2003).

Our laboratory has isolated a novel bacterium, capable of efficiently degrading different feather types with high keratinase production at pH 8, temperature 27°C within a short period of time. Both free and gellan gum entrapped cells of the *Bacillus* have degraded feather efficiently. Free cells were able to tolerate up to 20 ppm of some toxic heavy metals in batch fermentation. However, the ability of the immobilised cells of the *Bacillus* to degrade feather and produce keratinase enzyme in the presence of heavy metals in semi continuous bioprocess is still unknown. To the best of our knowledge, this is the first report of immobilised cells of bacteria that are capable of degrading feathers and produce high keratinase activity at elevated concentration of toxic heavy metals. The aim of this study was to investigate the ability of immobilised cells of *Bacillus sp. khayat* to degrade feathers contaminated with heavy metals in a semi continuous fermentation.

## MATERIALS AND METHODS

**Medium and cultivation conditions:** The bacterial strain used in this study was *Bacillus sp. khayat*. It was isolated from feather dumpsite in Johor Bahru, Malaysia. The bacterium was grown and maintained in an optimised feather meal basal medium. The medium composed of 0.5% (w/v) NaCl, 0.7% (w/v) KH<sub>2</sub>PO<sub>4</sub>, 1.4% (w/v) K<sub>2</sub>HPO<sub>4</sub>, 0.001% MgSO<sub>4</sub> and 0.5% (w/v) chicken feathers in 100 ml of distilled water. The initial pH of the medium was adjusted to 7.5 using 0.1 M NaOH. Media were then autoclaved for 15 min at 121°C. To obtain the bacterial mass for the immobilisation studies, the whole bacterium was grown in large volumes of nutrient broth (2 L) and centrifuged in a refrigerated high speed centrifuge at 4°C, 10,000 xg for 15 minutes to obtain the pellets.

**Immobilisation of *Bacillus sp. khayat*:** Cells of *Bacillus sp. khayat* were immobilised in gellan gum (Gelzan gum, Sigma) by emulsification–internal gelation technique described by Moslemy et al., (2003). In brief, 0.75 g of gellan gum powder was suspended in 100 mL of deionised water. The insoluble suspension of gellan gum was heated to 75°C on a hot plate stirrer to completely dissolve them. A 0.06 g of calcium chloride (Fisher Scientific) was added to the suspension and was allowed to cool down to 45°C. The pH of the solution was carefully adjusted to 7.0 using 0.1 N NaOH. Pellets of *Bacillus sp. khayat* (3.5 g) was added and stirred continuously until it was mixed thoroughly. Beads with the same sizes were produced by means of peristaltic pump in which the suction tube was inserted into the solution and the other tube releases the beads into canola oil containing 0.1% Span 80 (Sigma). The beads were carefully collected, added to 500 ml of 0.1% (w/v) CaCl<sub>2</sub> and allowed to stand for 2 h. The beads were then rinsed several times with 0.1% (v/v) Tween 80 solution.

**Keratinase activity and feather degradation:** Keratinase production and feather degradation using the gellan gum entrapped cells was carried out in 250 mL capacity Erlenmeyer flask containing 100 mL of media that contains 0.5% feather concentrations. In order to maximise keratinase yield, pH of the medium, temperature of incubation, size of the beads and gellan gum concentration were optimised. The flasks were incubated on a rotary shaker set at the speed of 150 rpm. The number of cycles that the beads can be used to repeatedly produce optimal keratinase and feather degradation was also investigated. After every 18 h, beads were collected and thoroughly rinsed with distilled water and transferred into a new medium containing 0.5% feathers. Loss of feather mass (feather degradation) and keratinase activities were then measured. This is repeated until feather degrading ability of the immobilised cells showed a significant decrease in activity. Keratinase activity was assayed using azokeratin as substrate following the method demonstrated by Joshi et al. (2007). Feather degradation was estimated from the washed residual feather that remained after incubation when compared with control. Cell leakages were estimated by plate counting method and reported as colony forming units (CFU/ml).

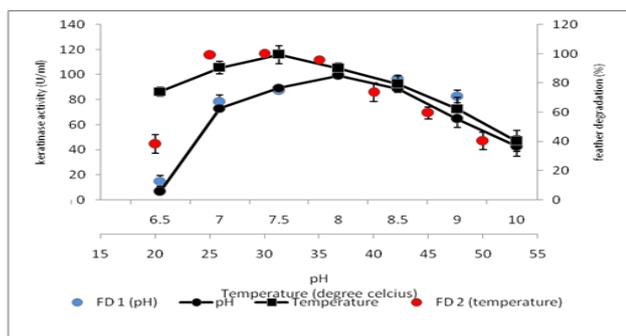
**Experiments with heavy metals:** Nine different stocks of heavy metals (lead, nickel, silver, mercury, tin, cadmium, zinc, cadmium and copper) were used for the study. All metal stocks are of analytical grade. Different concentrations (1-30) in part per million (ppm) of different heavy metals were prepared from the stock and added into the media containing immobilised cells and feathers. The metal concentrations were chosen based on previous study on the effect of metals on free cells (data not shown), from where we established the lower concentration of each metal that feather degradation occurred. The medium was adjusted to pH 8. The metal solutions were sterilised by membrane filtration (pore size 0.22  $\mu$ m).

For analytical purpose, if the immobilised bacterium produces keratinase enzyme and degrades feather within 18 h in the presence of a particular metal concentrations compared to control, we defined the bacillus to be tolerant to the particular heavy metal. Heavy metal that prevents the secretion of keratinase and feather degradation at lowest concentration (1 ppm) was classified as highly toxic to the bacteria. Two types of controls were setup, which are (1) medium containing heavy metals and blank beads (without bacteria) (2) medium without heavy metals and beads of immobilised *Bacillus sp. khayat*. All the experiments were conducted in triplicate and data presented are means of the triplicates.

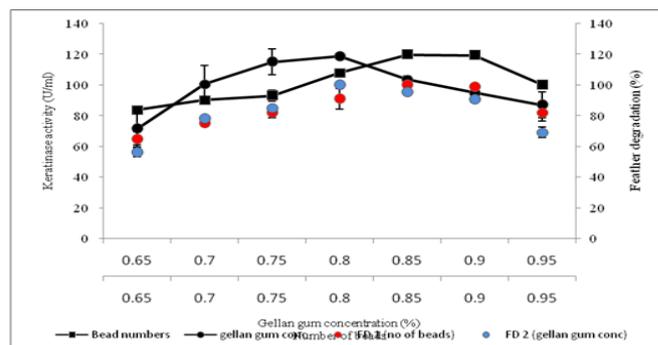
## RESULTS AND DISCUSSION

The success of biological degradation of organic compounds such as feather depends on the interaction of different factors within the environment such as physical, chemical and biological factors. Optimal keratinase protease yield and feather degradation by immobilised cells of *Bacillus sp. khayat* in heavy metals free media occurred at pH 8, temp 27°C, gellan gum concentration of 0.8 g and initial cell load of 250 beads/100ml of media (Figure 1). A higher keratinase of  $119.75 \pm 2.3$  U/ml was observed following the optimisation when compared with initial  $89.3 \pm 1.9$  U/ml.

Following optimisation, the entrapped cells of *Bacillus sp. khayat* completely degraded feathers in the medium to fine fibres within 18 h of incubation at 150 rpm. This 18 h period also corresponded relatively with the time that maximum keratinase activity was recorded.



**Figure.1. Optimisation of pH and temperature in maximizing keratinase activity and feather degradation by immobilised cells of *Bacillus sp. khayat*. Data represents mean of replicates.**



**Figure.2. Optimisation of gellan gum concentration and number of beads in maximizing keratinase activity and feather degradation by immobilised cells of *Bacillus sp. khayat*. Data represents mean of replicates.**

The use of gellan gum as a matrix for entrapment was considered because it is more stable and robust than others like calcium alginate (Moslemy, 2002). However, since the mechanical strength and pore size of the beads were all affected by the concentration of gellan gum, which in turn affects the diffusion of substrates and leakage of cells, its concentration, was optimised. Maximum feather degradation and keratinase enzyme yield was observed at 0.8% (w/v) concentration (Figure 2). The duo reduced significantly below 0.7% (w/v) and above 0.8% (w/v). Bead size of 3 mm was the optimum for the *Bacillus* to degrade the feathers (result not shown) as it was used throughout the studies. The effect of numbers of 3 mm sized beads on feather degradation and keratinase yield by the immobilised bacteria showed that 250-300 beads resulted in faster degradation and higher keratinase activities. However, at lower and higher bead numbers, the degradation was slow and keratinase activity was reduced. This may be as a result of the role cell density played in feather degradation. Low concentrations have lesser bacterial mass to do the job and higher cell density leads to a greater demand for oxygen and nutrient (Ahmad, 2012).

At the optimum conditions, 100% feather degradation and keratinase activity of 119.75 U/ml at 18 h was set as control to measure the effect of heavy metals on the process of feather degradation and keratinase activities by the immobilised cells of *Bacillus sp. khayat*. Different concentrations (1-30 ppm) of heavy metals were incorporated into the optimised feather media and incubated with 250 beads of immobilised bacteria. Several

aspects of the result showed that the actions of immobilised cells on feather degradation and keratinase depended on the metal's type and its concentration in the medium. Studies using live or dead immobilised cells of bacteria and fungi in different matrixes have shown greater ability to either tolerate or remove higher concentration of heavy metals in aqueous environment when compared to free cells (Piccirillo and Pereira, 2013; Pires, 2011; Rani and Hemambika, 2010). Tolerance level ranges from few to high ppm for selected heavy metal types by different organism. Selectivity for absorption or tolerance depends on several factors, which are type of the isolate, site of organism isolation and the nature of other pollutants present in the isolation area (Hassenet al., 1988). The result in this study showed that heavy metals at different concentration have different effects on both keratinase activities and feather degradations (Table 1).

Some metals such as Ag, Co, Cu increases keratinase activities while others like Hg, Ni, Pb, Zn decreases or completely stop keratinase activities and feather degradation at more than 5 ppm concentration, and others like Cd neither increase nor decrease the duo at 10 ppm when compared with the control. Mechanisms like structural and physical/ physiological means have been reported to play significant role in bacterial metal absorption (Lima de Silva et, 2012) and the possession of metal sequestration properties (Volesky, 1990). While higher concentration of Ag, Cu and Co promotes keratinase activity and feather degradations, metals like Pb, Ni and Zn better supported keratinase yield and feather degradation at lower concentrations. The mechanism of heavy metal tolerance or removal in the whole experimental setup was not well understood yet, since both chicken feathers and immobilised cells of bacteria especially bacillus have been reported to remove heavy metals from aqueous solution. However, the increase in feather weight from the initial weight of feather introduced into the media by some undigested feathers and un-inoculated media after 18 h of incubation showed that the feather adsorbed the heavy metals in the medium. The inability of the entrapped cells to degrade feathers or produce enzyme for example in 10 ppm and above of Hg, Pb but completely degraded at 5 ppm and below, indicated that the concentration was beyond what it can tolerate. However, it is important to note in the case of metals that affect keratinase yield and feather hydrolysis negatively that the inhibition may be related to the deleterious effect of metals on the bacterial cell or the enzyme produced (Lima de Silva et, 2012). Nevertheless, where the production of keratinase was not affected by the presence of heavy metals in the solution, it has been suggested that enzyme that is protected does not loss its functional activity in the presence of metals. Further studies involving molecular studies are needed to explore the mechanism.

**Table.1. Effect of different concentration of heavy metals on keratinase activity (KA) and feather degradation (FD) by immobilised cells of *Bacillus sp. khayat***

Metals	Concentrations of heavy metals												30 ppm	
	1 ppm		5 ppm		10 ppm		15 ppm		20 ppm		25 ppm			
	KA (U/ml)	FD (%)	KA (U/ml)	FD (%)	KA (U/ml)	FD (%)	KA (U/ml)	FD (%)	KA (U/ml)	FD (%)	KA (U/ml)	FD (%)	KA (U/ml)	FD (%)
<b>Pb</b>	78.9	92	87.5	93	29.4	21	7.6	15	12.4	10	2.4	0	0	0
<b>Ni</b>	116.1	99	86.3	78	50.6	56	12.1	32	0.6	10	0	0	0	0
<b>Ag</b>	112.7	100	108.9	100	135.3	100	132.9	100	115.3	100	111.3	100	108.8	97
<b>Hg</b>	108.6	100	106.8	98	9.4	14.7	1.9	0	0.4	0	0	0	0	0
<b>As</b>	102.4	99	100.4	97	83.2	91	70.4	66	29.1	17	0.7	0	0	0
<b>Cd</b>	117.2	100	104.0	100	113.2	100	66.4	85	43.2	76	3.2	6.0	0	0
<b>Zn</b>	111.3	100	103.8	98	24.1	43.2	21.4	10	0	0	0	0	0	0
<b>Co</b>	132.4	100	122.8	100	124.3	100	116.2	100	114.3	100	93.6	94	0	0
<b>Cu</b>	123.5	100	135.4	100	127.1	100	107.5	100	83.1	96	50.9	71.9	0	0
<b>Control</b>	Keratinase activity 109±2.4, feather degradation 98 ± 1.3 after 18 h of incubation													

**KA represents keratinase activity, FD represent feather degradation**

Result have also showed that the immobilised cells did not take part in the bio absorption of heavy metals in the solution since the same beads were used for several cycles to degrade fresh feathers laden with heavy metals without desorption (Table 2). The recycling of immobilised cells for continual removal of heavy metals in aqueous solution requires desorption with mineral acids to avoid reaching the saturation point (Pandey et al., 2007). In the presence of Cd, Zn and Pb, after 4<sup>th</sup> cycle, the beads size significantly reduced so also keratinase activities and feather degradation. However, in Co, Cu and Ag the beads remained stable with insignificant size reduction up to 10 cycles. In the case of Ni and Hg at 5 ppm the beads were re used for 6 and 7 cycles respectively. Interestingly, beads used to degrade feathers with particular heavy metals were reused and were able to completely degrade feather laden with other heavy metals for at least 5 cycles. This observation can further explain the tolerability of the organism to multiple of heavy metals. In addition, beads that were unable to degrade higher concentration of Hg and Pb were able to degrade feathers laden with four other heavy metals completely. This shows that the toxicity of Hg and Pb at more than 5 ppm only affects either the keratinase secretion by the organism or inactivates the already produced keratinase but not the immobilised cells. Furthermore, about 86% of feather was degraded when 250 beads were place in feather meal medium containing 5 ppm each of 5 different heavy metals (Cd, Co, Cu, As and

Ag) and the same beads were used for at least 4 cycles. It can be assumed that the bacteria possess specific genetic mechanisms of resistance to many heavy metals. The presence of some metals e.g. Ag, Hg and Co resulted in the changes in colour of beads during the fermentation.

For Hg, the change in colouration was observed at 10 ppm and above. The changes in colour of beads observed were probably because of chemical modification of metals when interacting with the bacteria. For instance, since the medium contains NaCl, the formation of silver chloride that may later photo-convert to metallic silver is possible or reduction of  $\text{Ag}^+$  to  $\text{Ag}^0$ , or to its sulphide form is possible. In the case of Hg, since it occurred only at higher concentration where degradation occurred, it could be as a result of reaction of Hg with some substance released by the bacteria in the environment (Lima de Silva et al., 2012).

**Table.2. Concentrations of heavy metals tolerated and number of times the beads were re used**

Heavy metals	Maximum concentration tolerated (ppm)	Number of cycles
Lead (Pb)	5	4
Mercury (Hg)	5	7
Zinc (Zn)	5	4
Arsenic (As)	10	9
Cadmium (Cd)	10	4
Nickel (Ni)	10	6
Cobalt (Co)	20	10
Copper (Cu)	15	10
Silver (Ag)	30	10
Co, Cu, Cd, As, Ag	5 each	4

## CONCLUSION

In conclusion, immobilised cells of *Bacillus* sp. khayat were able to produce keratinase protease and efficiently degrade chicken feather in the presence of elevated concentration of different heavy metals ions in a submerged fermentation. Beads of the immobilised cells can be used for many cycles in the process of continual degradation of heavy metal laden feather and keratinase enzyme production. The stability of the cells in gellan gum and the ability of the beads to be used for degrading different types of heavy metal laden feather make it a potential to be utilized in the bioremediation of feather wastes and industrial production of keratinase protease.

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## REFERENCES

- Adinarayana K., B. Jyothi and P. Ellaiah, Production of alkaline protease with immobilized cells of *Bacillus subtilis* PE-11 in various matrices by entrapment technique, *AAPS PharmSciTech*, 6, 2005, 391–397.
- Ahmad S., N. Shamaan and N. Arif, Enhanced phenol degradation by immobilized *Acinetobacter* sp. strain AQ5NOL 1, *World Journal of Microbiology and Biotechnology*, 28, 2012, 347–352.
- Cabrera G., R. Pérez, J. M. Gómez, A. Abalos and D. Cantero, Toxic effects of dissolved heavy metals on *Desulfovibrio vulgaris* and *Desulfovibrio* sp. Strains, *Journal of Hazardous Material*, 135, 2006, 40–6.
- Cheetham P. S. J., K. W. Blunt and C. Bocke, Physical studies on cell immobilization using calcium alginate gels, *Biotechnology and Bioengineering*, 21, 1979, 2155–2168.
- Das N., R. Vimala and P. Karthika, Biosorption of heavy metals—an overview, *Indian Journal of Biotechnology*, 7, 2008, 159–169.
- Hassen A., N. Saidi, M. Cherif and A. Boudabous, Resistance of environmental bacteria to heavy metals, *Bioresource and Technology*, 64, 1988, 7–15.
- Joshi S., M. Tejashwini and N. Revati, Isolation, identification and characterization of a feather degrading bacterium, *International Journal of Poultry Sciences*, 6, 2007, 689–693.
- Jouenne T., H. Bonato, L. Mignot, and G. A. Junter, Cell immobilization in composite agar layer microporous membrane structures: growth kinetics of gel-entrapped cultures and cell leakage limitation by a microporous membrane, *Applied Microbiology and Biotechnology*, 38, 1933, 478–481.

Kumar, E. Biodegradation of poultry feathers by a novel bacterial isolate *Bacillus altitudinis* GVC 11, Indian Journal Biotechnology, 10, 2011, 502–507.

Lima de Silva A.A., M.A.R. de Carvalho, S.A.L. de Souza, P.M.T. Dias, R.G. da Silva Filho, C.S. de Meirelles Saramago, C.A. de Melo Bento and E. Hofer, Heavy metal tolerance (Cr, Ag and Hg) in bacteria isolated from sewage, Brazilian Journal of Microbiology, 43, 2012, 1620–1631.

Moslemy P., R.J. Neufeld and S.R. Guiot, Biodegradation of gasoline by gellan gum-encapsulated bacterial cells, Biotechnology and Bioengineering, 80, 2002, 175–184.

Moslemy P., R.J. Neufeld, D. Millette and S.R. Guiot, Transport of gellan gum microbeads through sand: an experimental evaluation for encapsulated cell bioaugmentation, Journal of Environmental Management, 69, 2003, 249–259.

Muhsin T.M., and R.B. Hadi, Degradation of keratin substrates by fungi isolated from sewage sludge, Mycopathologia, 154, 2002, 185–189.

Pandey A., D. Bera, A. Shukla and L. Ray, Studies on Cr (VI), Pb (II) and Cu (II) adsorption–desorption using calcium alginate as biopolymer, Chemical Speciation and Bioavailability, 19, 2007, 17–24.

Piccirillo C., and S. Pereira, Bacteria immobilisation on hydroxyapatite surface for heavy metals removal, Journal of Environmental Management, 121, 2013, 87–95.

Pires C., A. Marques, and A. Guerreiro, Removal of heavy metals using different polymer matrixes as support for bacterial immobilisation, Journal of Hazardous Materials, 191, 2011, 277–286.

Prakash P., S.K. Jayalakshmi and K. Sreeramulu, Production of keratinase by free and immobilized cells of *Bacillus halodurans* strain PPKS-2: partial characterization and its application in feather degradation and dehairing of the goat skin, Applied Biochemistry and Biotechnology, 160, 2010, 1909–1920.

Rani M., B. Hemambika, Hemapriya, J. and Kannan, V.R. Comparative assessment of heavy metal removal by immobilized and dead bacterial cells: A biosorption approach, African Journal of Environmental Science and Technology, 4, 2010, 77–83.

Ravi Kumar M.N. A review of chitin and chitosan applications, Reaction and Functional Polymer, 46, 2000, 1–27.

Sahoo D.K., A. Das, H. Thatoi, K.C. Mondal and P.K. Das Mohapatra, Keratinase production and biodegradation of whole chicken feather keratin by a newly isolated bacterium under submerged fermentation, Applied Biochemistry and Biotechnology, 167, 2012, 1040–1051.

Seifert F.D.K. Inhibiting effect of surfactants and heavy metal ions on the denitrification process, Polish Journal of Environmental Studies, 14, 2005, 87–93.

Sharma R. and M. Sharma, The impact of incinerators on human health and environment, Reviews on Environmental Health, 28, 2013, 67–72.

Suvase S.A., U.S. Annapure and R.S. Singhal, Gellan gum as an immobilization matrix for the production of cyclosporin A, Journal of Microbiology and Biotechnology, 20, 2010, 1086–1091.

Tatineni R., K.K. Doddapaneni, R.C. Potumarthi, R.N. Vellanki, M.T. Kandathil, N. Kolli and L.N. Mangamoori, Purification and characterization of an alkaline keratinase from *Streptomyces* sp., Bioresource Technology, 99, 2008, 1596–1602.

Tosa T., T. Sato, T. Mori, K. Yamamoto, I. Takata, Y. Nishida and I. Chibata, Immobilization of enzymes and microbial cells using carrageenan as matrix, Biotechnology and Bioengineering, 21, 1979, 1697–709.

Volesky B., Biosorption of heavy metals, CRC Press, 1990, 408.

Vuilleumard J.C., S. Terré, S. Benoit and J. Amiot, Protease production by immobilized growing cells of *Serratia marcescens* and *Myxococcus xanthus* in calcium alginate gel beads, Applied Microbiology and Biotechnology, 27, 1988, 423–431.