Eco. Env. & Cons. 26 (1) : 2020; pp. (193-198)
Copyright@ EM International
ISSN 0971–765X

An efficient de-hairing by Keratinase from *Streptomyces badies var. Shashi*

Shashikant Kamble*[1], Anandrao Jadhav[2] and Kailas Sonawane[3]

[1] Derartment of Microbiolgy Miraj Mahavidyalaya, Miraj (Maharashtra)
[2] Department of Microbiology K.R.P. Kanya Mahavidyalaya, Islampur (Maharashtra)
[3]Department of Microbiology Shivaji University Kolhapur, (Maharashtra)

(Received 30 August, 2019; accepted 6 October, 2019)

ABSTRACT

Currently the research is focused on finding new compounds, bioactive molecules and enzymes from the bacterial sources. Kertinse is a very useful enzyme targeted by many industries like poultry, textile and leather etc. Hence isolation and further application of keratinase is a very demanding task. In the present work we isolated 22 keratinolytic actinomyctes from soil. The potent keratinse producers like *Streptomyces badies, Srteptomyces parvus* and *Streptomyces graminerus* were further characterized. The most prominent keratinase producer species *Streptomyces badies* var. *Shashi* was used further for knowing its de-hairing capacity. Firstly the isolated species was grown on minimal medium and then extraction of keratinase was carried out. These results showed that *Streptomyces badies* var. *Shashi* showed good ability to produce keratinase and this further showed good ability to degrade keratin and remove hairs. Hence this isolated species *Streptomyces badies* var. *Shashi* might be useful for industrial production of keratinase and its further application.

Key words: *Streptomces, Streptomyces badies var. Shashi, Keratinase, De-hairing*

Introduction

In a world daily large quantity of keratin is being wasted in the form of hairs, feathers. The feathers are protenitious waste product of poultry produced in poultry in large quantity (Brandelli *et al.,* 2008; Gupta *et al.,* 2006; Vasileva-Tonkova *et al.,* 2009; Onifade *et al.,* 1998). The feather are disposed routinely by land filling or burning but by this way it may create tremendous environmental problems like increasing BOD, COD and TDS to the effluent (Vasileva-Tonkova *et al.,* 2009). Feather after degradation form feather meal and it may be used as animal feed and organic fertilizers (Brandelli *et al.,* 2010; Coward-Kelly *et al.,* 2006). Meal are produced at high temperature and pressure resulted into destruction of essential amino acids such lysine, tyrosine, methionine, tryptophan which reduces nutritional value and has poor digestibility (Wang *et al.,* 1997; Papadopolous *et al.,* 1986). Hence considering all these facts microbial degradation of feather has gained prime importance. Currently new microbes are screened having high feather degradation capacity. Several bacteria's like Vibrio, Bacillus, Cheyseobacterium and Streptomyces in consortium carry out degradation of feathers (Wang *et al.,* 1997; Brandelli *et al.,* 2010; Zaghloul *et al.,* 2011; Papadopolous *et al.,* 1986). Proteolytic enzymes are found to be 40% worldwide and mainly used in industries (Sanghvia *et al.,* 2016). The keratinase (EC 3.4.99.11) are from protease family having great importance because it cleans the environment (Agrahari *et al.,* 2010). The keratinase plays a useful role in different fields based on their capability to

***Corresponding author's email:** *kamblesha@gmail.com; anandrao_jdhv@yahoo.com ; kd_biochem@unishivaji.ac.in

act on rigid and cross-linked polypeptides in insoluble forms also. The keratinase can be used in production of biodegradable lms, coatings, glues and detergents.The main application of keratinase in leather industry is in pre-tanning and dehairing which is an ecofriendly technology. The decomposition of gelatin, casein, collagen, silk and wool can be accomplished by using keratinase (Gupta and Rammani, 2006).

Keratinases are the enzymes capable of degrading insoluble keratins more efficiently than other proteases. Keratinase included in hydrolyses group which are capable of degrading keratin more efficiently than other protease (Kanmani *et al.,* 2011; Vigneshwaran *et al.,* 2010). The keratinase hydrolyses keratin and produce rare amino acids such as serine, cysteine and proline and essential amino acids like lysine, histidine, tyrosine, valine, methionine, threonine, isoleucine and leucine (Ali *et al.,* 2011; Mousavi *et al.,* 2013). Overall these enzymes are having great importance in differents fields like detergent formulation, textile processing, animal feed production, medicine and leather manufacture. Hence certain keratinase producing actinomycetes, fungi and bacteria's are screened.

The present work is focused on the isolation of keratinase from different bacterial species and its further application. Actinomycetes are able to produce many bioactive molecules like antibiotics, acids and various enzymes. In this study we screened *Streptomyces graminearus* (Acc. No. MF278648), *Srteptomyces parvus* (Acc. No.MF281087) and *Streptomyces badies* Var. *Shashi* (Acc. No. KU763187). The isolated species showed ability to produce keratinse which further showed good dehairing capacity. Hence these isolated species may be very useful in production of keratinase having various industrial applications.

Materials and Methods

Screening of keratinase producing species

The soil sample form Miraj, India region was used to isolate keratinase producers. The isolated species were further grown on minimal medium containing animal hairs (1%). The colonies on minimal medium were further streaked on glycerol asparagine agar slants to get pure culture. Finally 22 different isolates were purified. The keratinolytic ability of isolated species was accessed on basal mineral medium with animal hairs (1%). The potential keratinolytic organism was used for further studies. The isolated species *Streptomyces badies* var. *Shashi* was identified and used for further studies.

Keratinase Production from isolated species

The production medium (pH-7.2) containing KH_2PO_4-0.7 gm, K_2HPO_4-1.4 gm, $MgSO_4$-0.1 gm, NaCl- 0.5 gm and animal hairs- 10 gm was used to inoculate isolated species. The enzyme production was carried out in Erlenmeyer flasks for 24 hrs at RT. The enzyme separation was done by filtering the crude medium through Whatman No.1 paper. The filtered crude enzyme was used for further studies.

Enzyme assay of keratinase

The methodology described by Rayudu *et al.* was referred to do modified keratinase assay (Rayudu *et al.* 2014). In an assay 2 mL reaction mixture with 1 ml keratin and 1 mL crude enzyme was incubated for 10 min at 40 °C. 2 mL chilled TCA (10%) was added and finally the mixture was kept for 20 min n shaking water bath with 100 rpm agitation. Further centrifugation at 5000 rpm was done for 10 min. Then 0.1 mL of supernatant was add with 0.9 ml D/W and 0.5 mL of 500 mM sodium carbonate and incubated 350 C for 10 min. Further 0.5 mL FCR reagent was added and incubated for 20 min. Finally absorbance for developed blue color was taken at 660 nm. The control was processed by adding enzyme after incubation and TCA added immediately. The graph was generated using tyrosine standard (10-100 µg/µL). The one unit of keratinolytic activity is defined as the amount of enzyme that librates 1 µg of tyrosine equivalent per min. Under specified assay condition. The tyrosine standard was added in different concentration in different test tubes diluted to 1 mL with D/W. Then 5 mL alkaline copper reagent was added with 0.5 mL FCR reagent. Finally the reaction mixture was allowed to incubate for 20 min and absorbance was recorded at 660 nm. The graph was plotted and result was interpreted.

Partial purification of keratinase from *Streptomyces badies Var. Shashi*

The crude keratinase enzyme from *Streptomyces badies* Var. *Shashi* was partial purified further.

Ammonium sulphate precipitation

The crude keratinase enzyme was precipitated with ammonium sulphate at different concentration (30%, 40%, 50%, 60%, 70%, 80% and 90%) with constant stirring under cold condition. Then the beaker was incubated at 4 ^{0}C for 24 hrs. After incubation the enzyme was retrieved by centrifuging at 10,000 rpm for 10 min at 4 ^{o}C. Further the pellets of enzyme were dissolved in Phosphate buffer (50 mM, pH 7.2). Used further for dialysis.

Dialysis

The precipitated crude keratinase enzyme was loaded into pre-activated dialysis membrane (cut-off-11kD). The dialysis membrane bag was hanged further in 500 mL Phosphate buffer (pH-7.2). The beaker was incubated at 4 ^{0}C for 12 hrs by changing buffer two or more times.

Gel filtration chromatography

The gel Sephadex G 75 in Phosphate buffer (50 mM, pH 7.2) was activated by heating at 50 ^{0}C for 30 min with constant shaking. The activated gel was packed in column and then washing was carried out with buffer for equilibration. After proper packing and equilibration of column the dialyzed keratinase enzyme was loaded on column and 25 fractions of 1.5 mL were collected. Then all the fractions were analyzed for keratinase activity.

Ion-exchange chromatography

The DEAE-Cellulose matrix was activated by mixing thoroughly with Phosphate buffer (50 mM, pH 7.2) and loaded onto glass column fitted with glasswool. The pH of matrix within column was adjusted to 8.0 usingappropriate buffer component. The column equilibration was carried out by running 500 mL of Phosphate buffer (50 mM, pH 7.2). Finally the active fraction of keratinase enzyme from gel-filtration column was loaded on to the activated ion-exchange column. The sample was eluted and 30 fractions were collected. Each fraction was accessed for keratinase activity.

Protein Estimation

The protein content of all the fractions was determined by the Bradford (1976) method with standard Bovine serum albumin (BSA). The standard BSA solution was taken in different concentrations in different test tubes and diluted to 5 mL with Bradford's reagent. The optical density was read at 595 nm. The standard BSA curve was plotted on OD against concentration of BSA.

Keratinase separation using Polyacrylamide Gel Electrophoresis (PAGE)

PAGE plates were washed to make grease free, clean and dry and then assembled using spacers and clips. The bottom of plates was sealed with 5-10 % agar. Resolving and stalking gel was casted and then immediately comb was inserted. The comb was removed carefully from polymerized gel. The gel was assembled into PAGE unit and then running buffer (Tris- 3 gm, Glysine- 14.3 gm, SDS- 2.0 gm D/W- 1000 mL) was added into upper and lower tank. Partially purified keratinase mixed with sample buffer [1.5 M Tris-HCL-0.625 ml (pH 8.0), 20% SDS-1.0 mL, Glycerol-1.0 mL, 2-mercaptoethanol-0.5 ml, 0.2% Bromophenol blue) in 1:1 ratio and boiled for 2-10 min. The purified keratinase was loaded into gel and then constant current of 50-150 Volts was applied and allowed to run for 3-4 hrs (Laemmli, 1970).

Silver Staining of Purified keratinase

The purified keratinaseprotein was visualized by silver staining (Blum *et al.*, 1987). The gel was soaked in destaining solution for 10 min and then D/Wwas used constantly to wash the gel for 5 min. The washing was repeated five times until removal of stain. The gel was kept in pretreatment solution (Sodium thiosulphate- 0.08 gm in 100 mL) for 5 min and washed for 5 times in D/W with 5 min interval. Then gel was incubated in silver nitrate solution (AgNO3- 0.2 gm, Formaldehyde- 75 µL in 100 mL D/W) for 20 min in dark followed by D/W wash for 2 min. Finally the gel was immersed in developer (Na_2CO_3- 2 gm and formaldehyde- 75 µL in 100 mL D/W) until the brown bands are developed. The reaction was stopped before gel gets brown color.

Biochemical characterization of purified keratinase

Determination of optimum pH for keratinase activity

The optimum pH of purified Keratinase was determined with 1% keratin (w/v) as substrate dissolved in different buffers (acetate buffer pH 4.0, Citrate buffer pH 5.0 and 6.0, phosphate buffer pH 7.0, Tris-HCl buffer pH 8.0, glycine-NaOH buffer pH 9.0, 10.0 and 11.0). The keratinase enzyme activity at

different pH range was calculated by using method described earlier.

Determination of optimum temperature for keratinase activity

The optimum temperature of purified keratinase was determined by incubating the reaction mixture at different temperature range from 20-60 ^{0}C in 50 mM Tris-HCL buffer (pH 8.0) for 10 min by the method mentioned earlier.

Metal ion effect on keratinase activity

The EDTA, PMSF, Phenanthroline, SDS (1%), H_2O_2 (1%) and salt solution NaCl (1M) were tested for their effects on purified keratinase activity. The various metal ions like $HgCl_2$, $FeCl_3$, $AgNO_3$, $ZnSO_4$, $MgSO_4$, $MnSO_4$, and $CuSO_4$ were tested for their effects on purified keratinase activity.

Testing purified keratinase *Streptomyces badies Var. Shashi*for its hair degradation capacity.

The isolated species was further used to access its hair degradation ability. The hair degradation ability of isolated species was accessed on minimal medium having only hairs as a carbon source. The isolated species was further tested for its ability to produce keratinase. The enzyme assay was done using keratin azure as a substrate (Tapia *et al.,* 2007).

Results and Discussion

Screening of keratinase producers from soil

The microbial culture techniques were used to isolate keratinase producers from soil. As per the method discussed in earlier section total 12 isolates were purified. After getting pure cultures all the cultures were tested for their keratinase producing ability. In all these 22 isolates *Streptomyces badies* var. *Shashi* showed highest keratinase activity. Hence this *Streptomyces badies* var. *Shashi* was used further for keratinase production. The purification of keratinase from *Streptomyces badies* var. *Shashi* was done using different purification steps.

Extraction and purification of keratinase from *Streptomyces badies Var. Shashi*

The ammonium sulphate precipitation was used to partialy purify keratinase*Streptomyces badies Var. Shashi*. Highest activity of keratinase was observed at 70 % saturation. Hence the partialy purified keratinase at 70 % saturation was further dialyzed in Phosphate buffer. The dialyzed keratinase was further loaded on to the Sephadex G 75 column. The 25 different fractions were collected and checked for keratinase activity. Fraction no 12^t and 13 showed highest keratinase activity (Fig. 2). Hence these two fractions were mixed and again loaded on the ion-exchange column. 30 different fractions were collected and then each fraction was used to access the presence of pure keratinase (Fig. 3). The fraction number 18 showed keratinase activity. Hence it may be observed that in 18th number fraction there is presence of keratinase in pure form.Finally the purified keratinase from *Streptomyces badies* Var. *Shashi* was further loaded on PAGE to determine its molecular weight. Finally all these purification steps yield 30 fold purified keratinase from *Streptomyces badies* Var. *Shashi* (Table 1).

Fig. 1. Plate showing growth of isolated species.

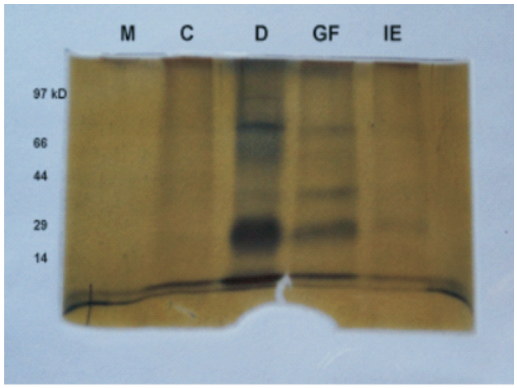

Fig. 4. PAGE analysis of keratinase enzyme M-Molecular weight marker, medium range; IE-Ion exchange; GF-Gel filtration.

Molecular weight determination of purified keratinase from *Streptomyces badies* Var. *Shashi*

The purified keratinase from *Streptomyces badies* Var. *Shashi* was loaded on PAGE to determine its molecular weight. This experiment revealed that purified keratinase from *Streptomyces badies* Var. *Shashi* has molecular weight of 29 kda (Fig. 4). Similarly the silver staining technique was also done to detect the exact molecular weight of purified keratinase from *Streptomyces badies* Var. *Shashi.* This study confirmed that exact molecular weight of keratinase from *Streptomyces badies* Var. *Shashi* was 29 kda (Fig. 5).

Parameter optimization for keratinase activity

The purified keratinase from *Streptomyces badies* Var. *Shashi* was further used and its activity was accessed in different pH ranges. These studies revealed that keratinase from *Streptomyces badies* Var. *Shashi* have highest activity at pH 8.0 (Fig. 6). Similarly the purified keratinase from *Streptomyces badies* Var. *Shashi* was kept in different temperature ranges and its activity was checked. It showed that purified keratinase from *Streptomyces badies* Var. *Shashi* has highest activity at temperature 40 °C (Fig. 7). After knowing the optimum conditions of purified keratinase activity it was further used to know different metal ion effects on it.

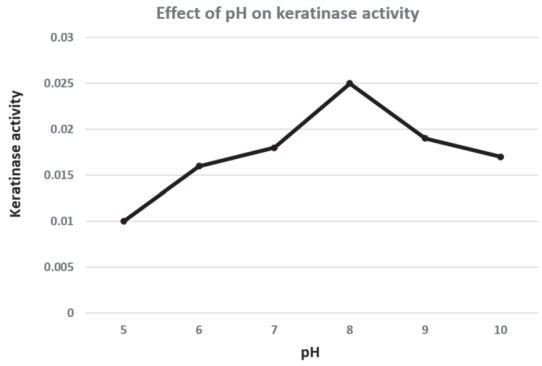

Fig. 6. Effect of pH on purified keratinase activity.

Effect of different metal ions on purified keratinase from *Streptomyces badies* Var. *Shashi*

The purified keratinase from *Streptomyces badies* Var. *Shashi* was used to check the effect of different metal ions on it. This study showed that metal ions such as Hg, Ag, have inhibitory effect while Fe, Zn, Mg, Mn and Cu has enhanced the activity of keratinase (Figure 8).

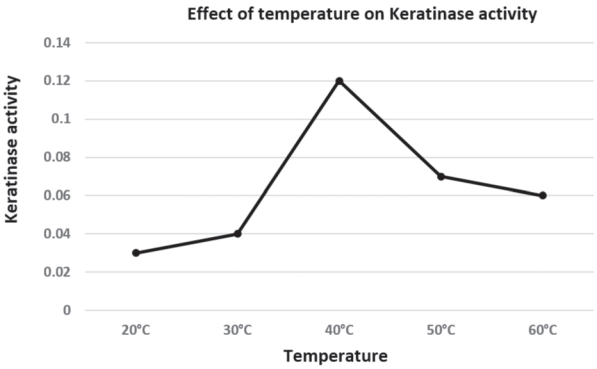

Fig. 7. Effect of temperature on purified keratinase activity.

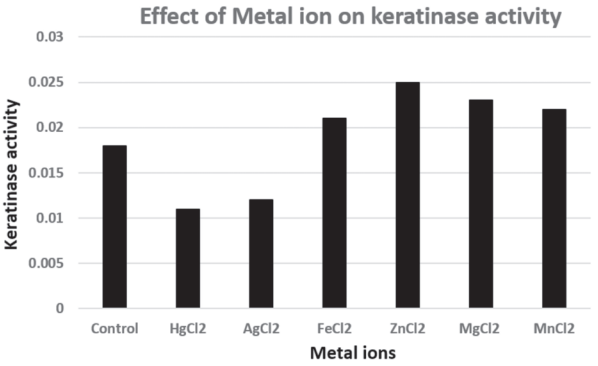

Fig. 8. Effects of metal ion on purified keratinase activity.

Hair degradation by purified keratinase from purified keratinase from *Streptomyces badies* Var. *Shashi*

The purified keratinase from *Streptomyces badies* Var. *Shashi* was used to know its hair removal capacity. This assay revealed that purified keratinase from *Streptomyces badies* Var. *Shashi*able to remove the hair with high efficiency (Figure 9).

Conclusion

The keratinase producing *Streptomyces badies* Var. *Shashi* was successfully isolated and purified from soil. The keratinase from *Streptomyces badies* Var. *Shashi* was successfully purified by differetent purification steps such as Ammonium Sulphate, Size Exclusion and Ion-Exchange chromatography. The purification steps yields 30 fold purified keratinase from *Streptomyces badies* Var. *Shashi.* PAGE analysis revealed that keratinase from *Streptomyces badies* Var. *Shashi* has molecular weight 29 kda. Then purified kerainase from *Streptomyces badies* Var. *Shashi* showed highest activity at pH 8.0 and temperature

40 °C. Similarly metal ions like Hg, Ag, have inhibitory and Fe, Zn, Mg, Mn and Cu has enhancing effect on keratinase activity. Finally the purified keratinase from *Streptomyces badies* Var. *Shashi* showed good efficiency of hair removal. Overall the isolated *Streptomyces badies* Var. *Shashi*showed ability to produce keratinase which has been successfully purified and showed good capacity to remove hairs. Hence this isolated keratinase *Streptomyces badies* Var. *Shashi* might be very useful in further studied to develop effective strategy in clinical and industrial fields.

Authors' contributions

SK and AJ designed the experiments; SK performed the research experiments; MD, and KD helped in the experiments. MD helped in manuscript preparation; SK and MD analysis the data; SK and MD wrote the manuscript. All authors read and approved the final manuscript**.**

Acknowledgements

We thank toDepartment of Microbiology, Miraj Mahavidyalaya, Miraj for kindly providing us the infrastructure facility to carry-out this research work. Also we are thankful to Department of Microbiology Shivaji University Kolhapur for providing the facility to conduct the experiments.

References

Agrahari, S. and Wadhwa, N. 2010. Degradation of chicken feather a poultry waste product by keratiniolytic bacteria isolated from dumping site at Ghazipur poultry processing plant. *Int. J. Poul. Sci.* 9 : 482-489.

Ali, T., Nadia, H. and Latifa, A. 2011. Production, Purification and Some Properties of Extracellular Keratinase from Feathers-Degradation by Aspergillus oryzae NRRL-447. *Journal of Applied Sciences in Environmental Sanitation.* 6 : 123-136.

Blum, H., Hildburg, B. and Gross, H. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis.* 8 : 93-99.

Brandelli, A. 2008. Bacterial keratinases: Useful enzymes for bioprocessing agroindustrial wastes and beyond. *Food Bioprocess. Technol.* 1 : 105-116.

Brandelli, A., Daroit, D. and Riffel A. 2010. Biochemical features of microbial keratinases and their production and applications. *Appl. Microbiol. Biotechnol.* 85 : 1735-1750.

Coward-Kelly, G., Agbogbo, F. and Holtzapple, M. 2006. Lime treatment of keratinous materials for the generation of highly digestible animal feed: 2. animal hair. *Bioresour. Technol.* 97 : 1344–1352.

Gupta, R. and Ramnani, P. 2006. Microbial keratinases and their prospective applications: an overview. *Appl. Microbiol. Biotechnol.* 70 : 21–33.

Kanmani, P., Karuppasamy, P., Pothiraj, C., Venkatesan, A. 2011. Studies on Lignocellulose Biodegradation of Coir Waste in Solid State Fermentation Using Phanerocheate chrysosporium and Rhizopus stolonifer. *African Journal of Biotechnology*. 8 : 6880-6887.

Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227 : 680-685.

Mousavi, S., Salouti, M., Shapoury, R. and Heidari, Z. 2013. Research Article: Optimization of Keratinase Production for Feather Degradation by *Bacillus subtilis*. *Jundishapur J Microbiol.* 6 : 7160.

Onifade, A., Al-Sane, N., Al-Musallam, A., Al-Zarban, S. 1998. A review: Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. *Bioresour. Technol.* 66 : 1–11.

Papadopolous, M., El-Boushy, A., Roodbeen, A. and Ketelaars, E. 1986. Effects of processing time and moisture content on amino acids composition and nitrogen characteristics of feather meal. *Animal Feed Sci. Technol.* 14 : 279-290.

Rayudu, K. and Jayaraj, Y. 2014. Biodegradation of poultry feather by novel Keratinolytic bacteria isolates of North-East Karnataka Ph.D. Thesis.

Sanghvia, G., Patela, H., Vaishnava, D., Ozaa, T. and Daveb, G. 2016. A novel alkaline keratinase from Bacillus subtilis DP1 with potentialutility in cosmetic formulation. *Int. J. Biologic. Macromol.* 87 : 256-262.

Tapia, D. and Simões, M. 2008. Production and partial characterization of keratinase produced by a microorganism isolated from poultryprocessing plant wastewater. *African Journal of Biotechnology.* 7: 296-300.

Vasileva-Tonkova, E., Gousterova, A. and Neshev, G. 2009. Ecologically safe method for improved feather wastes biodegradation. *Int. Biodeterior. Biodegrad.* 63: 1008–1012.

Vigneshwaran, C., Shanmugam, S. and Sathish, K.T. 2010. Screening and Characterization of Keratinase from *Bacillus licheniformis* Isolated from Namakkal Poultry Farm.

Wang, X. and Parson, C. 1997. Effect of processing systems on protein quality of feather meal and hog hair meals. *Poultry Sci.* 76 : 491-496.

Zaghloul, T., Embaby, A. and Elmahdy, A. 2011. Biodegradation of chicken feathers waste directed by Bacillus subtilis recombinant cells: Scaling up in a laboratory scale fermentor. *Bioresour. Technol.* 102: 2387-2393.