Biodegradation (2011) 22:111–128
DOI 10.1007/s10532-010-9381-9

ORIGINAL PAPER

Key determinants affecting sheep wool biodegradation directed by a keratinase-producing *Bacillus subtilis* recombinant strain

Taha I. Zaghloul · Amira M. Embaby · Ahmed R. Elmahdy

Received: 23 December 2009 / Accepted: 21 June 2010 / Published online: 21 July 2010

Abstract OVAT (one variable at a time) approach was applied in this study to screen the most important physicochemical key determinants involved in the process of sheep wool biodegradation. The process was directed by a keratinase-producing *Bacillus subtilis* DB 100 (p5.2) recombinant strain. Data indicate that, sheep wool could be degraded efficiently in cultures incubated at 30°C, with initial pH of 7 with agitation at 150 rpm. Two times autoclaved alkali treated and undefatted chopped sheep wool is more accessible to biodegradation. *B. subtilis* recombinant cells could utilize sheep wool as a sole source of carbon and nitrogen. Sheep wool-based modified basal medium II, lacking NH_4Cl and yeast extract, could greatly support the growth of these bacterial cells. Sheep wool biodegradation was conducted efficiently in the absence of kanamycin consequently; high stability of the recombinant plasmid (p5.2) represents a great challenge upon scaling up this process. Three key determinants (sheep wool concentration, incubation time and inoculum size) imposing considerable constraints on the process are highlighted. Sheep wool-based tap water medium and sheep wool-based distilled water medium were formulated in this study. High levels of released end products, produced from sheep wool biodegradation are achieved upon using these two sheep wool-based water media. Data indicate that, sheep wool hydrolysate is rich in some amino acids, such as tyrosine, phenylalanine, lysine, proline, isoleucine, leucine, valine, aspartic acid and glutamic acid. Moreover, the resulting sheep wool hydrolysate contains soluble proteins of high and intermediate molecular weights. The present study demonstrates a feasible, cheap, reproducible, efficient and rapid biotechnological approach towards utilization of raw sheep wool waste through a recombinant bacterium.

Keywords Sheep wool waste · A keratinase-producing *Bacillus subtilis* recombinant strain · OVAT · Physicochemical key determinants · Sheep wool-based tap/distilled water medium · Sheep wool hydrolysis end products

T. I. Zaghloul (✉) · A. M. Embaby
Department of Biotechnology, Institute of Graduate Studies and Research, Alexandria University, 163 El Horreya Aven., EL Chatby, P.O. Box 830, 21526 Alexandria, Egypt
e-mail: tzaghloul@hotmail.com

A. M. Embaby
e-mail: amira_mohamed_2000@yahoo.com

A. R. Elmahdy
Department of Food Science and Technology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt
e-mail: elmahdy200966@yahoo.com

Introduction

Keratinous wastes (feathers, wool, hair, nails, hooves, horns, claws and beaks) are considerable proportion

of the globally produced environmental solid wastes. Incremental increases in the poultry, tanneries and other meat processing industries all over the world have resulted in the generation of millions of tons of these wastes annually (Williams et al. 1991; Zaghloul et al. 1998; Sangali and Brandelli 2000a; El-Refai et al. 2005; Vasileva-Tonkova et al. 2007; Khardenavis et al. 2009; Zheljazkov et al. 2009). The inefficient utilization of such wastes contributes greatly to their accumulation. The world market for wool has dropped dramatically consequently; an excess quantity of wool could not be processed (Zheljazkov 2005). Post consumer carpets also represent a large amount of sheep wool waste (McNeil et al. 2007). Consequences resulted from the above aspects and others would increase the quantity of sheep wool waste. Some environmental pollution problems will raise as a consequence of accumulation of these wastes (Onifade et al. 1998; Zaghloul et al. 1998; Balint et al. 2005; Gousterova et al. 2005; CAI et al. 2008). Keratin, the main component of these above wastes, represents about 90% of these wastes on solid weight bases (Gessesse et al. 2003; Ramnani et al. 2005; CAI et al. 2008; Oulad Haddar et al. 2009). Moreover, keratin is an insoluble hard to degrade animal protein. It is characterized by high resistance to hydrolysis by common proteolytic enzymes (e.g., papain, pepsin and trypsin). Resistance to enzymatic hydrolysis is mainly attributed to its unique complex structure; since, keratin polypeptide chains are tightly packed in two forms α-helix and ß-sheets. These polypeptides chains are further supercoiled due to their high degree of cross-linking via disulfide bonds, hydrogen bonds and hydrophobic interactions (Böckle et al. 1995; Bressollier et al. 1999; Ignatova et al. 1999; Mitsuiki et al. 2004; Gradisar et al. 2005; Gupta and Rammani 2006; Radha and Gunasekaran 2008; Khardenavis et al. 2009). Despite the high protein content of these wastes, they cannot be utilized efficiently, as fodder additives for animals in their native state due to their poor digestibility (Moran et al. 1996; Evans et al. 2000).

Traditional methods, currently used to retrieve benefits from these wastes in the animal feed industry, apply mainly physical and chemical treatments. However, these methods suffer from some drawbacks (e.g., energy loss, destruction of some amino acids resulting in products with low nutritional values (Papadopoulos et al. 1986; Wang and Parson 1997; Sangali and Brandelli 2000a; Mortiz and Latshaw 2001; Anbu et al. 2005). Due to an increase in environmental awareness, a push to reduce the cost of animal feed production, and the drawbacks of the physicochemical methods, much attention has been turned toward the valorization of these wastes through an alternative method (Shih 1993; Friedrich and Antranikian 1996; Sangali and Brandelli 2000b; Suzuki et al. 2006). Despite the complex nature of keratinous wastes, they could be degraded by the action of keratinolytic microorganisms. These microorganisms possess specific unique hydrolytic enzymes called keratinolytic serine protease (in majority) and keratinolytic metalloproteases (in minority). Several reports discussed the isolation, purification and characterization of keratinolytic enzymes from bacteria, actimoycetes and to lesser extent from fungi (Williams et al. 1990; Gradisar and Friedrich 2000; Riessen and Antranikian 2001; Mitsuiki et al. 2002; Brandelli and Riffel 2005; Suntornsuk et al. 2005; Hossain et al. 2007; Ionata et al. 2008; Cao et al. 2009; Fakhfakh et al. 2009; Syed et al. 2009; Brandelli et al. 2010; Mazotto et al. 2010; Xie et al. 2010). The role of such enzymes in keratin hydrolysis greatly facilitates the efficient utilization of these wastes on biotechnological bases (Lahl and Braun 1994; Onifade et al. 1998; Riffel and Brandelli 2002; Odetallah et al. 2003; Ramnani and Gupta 2004; De Azeredo et al. 2006; Deivasigamani and Alagappan 2008). Few attempts have been made to clone keratinase genes from keratinolytic microorganisms (Lin et al. 1995; Kluskens et al. 2002; Mitsuiki et al. 2004; Carolin et al. 2005; Radha and Gunasekaran 2008; Lin et al. 2009a; Lin et al. 2009b). Low expression as well as low stability of the recombinant plasmids carrying keratinase genes are both considered obstacles to maximizing the amount of keratinase produced by genetically modified cells (Ramnani et al. 2005). High expression level of a keratinolytic serine alkaline protease (*apr E* gene) from the *B. subtilis* DB 100 (pS1) and *B. subtilis* DB 100 (p5.2) recombinant strains (Zaghloul et al. 1994; Zaghloul 1998; Zaghloul et al. 2004; Oulad Haddar et al. 2009) was reported. Biodegradation of keratin in the form of chicken feathers by the *B. subtilis* DB 100 (p5.2) recombinant cells was accompanied with the production of considerable levels of keratinolytic alkaline protease, soluble proteins and NH_2-free amino groups (Oulad Haddar et al. 2009). This alkaline protease enzyme (*aprE*), a key enzyme in

the keratin degradation process, is a dual enzyme. It is working as a protease and as a keratinase at the same time as reported earlier by Zaghloul 1998.

Since 1990, researchers have given a great attention to the valorization of chicken feather waste through biotechnological methods. On the other hand, a very little attention has been paid to another protein rich unutilized keratinous waste; sheep wool waste. High expression of the cloned *apr E* gene in *B. subtilis* cells, high keratin capability of these cells and the unutilized abundant amounts of sheep wool waste all greatly address the indispensable need for a step wise optimization of sheep wool biodegradation. Moreover, each microbial strain has its own peculiar physicochemical and nutritional requirements (Puri et al. 2002; Bernal et al. 2006) and no uniform defined medium for keratin biodegradation is reported till now. Improving keratin biodegradation is a prerequisite for designing biotechnological processes through developing efficient genetically modified bacterial strains and optimizing the media composition of the producers (Greasham 1983). Actually, optimization is a key issue in the agenda of industrial fermentation processes. Carrying out an optimization step aims to seek for a low cost medium that supports the growth of the employed producer microbial strain along with achieving optimal levels of the desired end products while taking as little time as possible. Generally, the first step in the optimization of fermentation processes involves applying the one variable at a time (OVAT) approach. This approach does not consider the interactions among independent variables. It varies one factor and keeps the other factors at constant levels each time. This step is crucial in the optimization since, it highlights the key determinants involved in the process. However, the optimal level of each key determinant along with the corresponding response (the optimal level of the desired products) could not be reached by this approach unless the response surface methodology is applied (Gokhade et al. 1991).

The objective of the present study is to highlight the most important key determinants required for an efficient sheep wool biodegradation directed by the *B. subtilis* recombinant cells. The aim is extended to search for a simple low cost medium supporting the growth of recombinant cells along with possibly achieving high levels of released end products of sheep wool hydrolysis.

Materials and methods

Chemical and reagents

Yeast extract, peptone and bacteriological agar used in this study were Oxoid type. Ninhydrin, hydrindantin, Coomassiae brilliant blue G250 (CBB G250), acrylamide, bis-acrylamide, temed, ammounium persulfate, methylcellosolve and ethyl alcohol were purchased from Sigma. Silver nitrate, bovine serum albumin and glycine were obtained from Winlab. Molecular weight protein marker was purchased from Fermentas.

Bacterial strains and plasmid

B. subtilis DB100 *his*$^-$ *met*$^-$(p5.2) was used in this study to direct the biodegradation of sheep wool waste. The strain harbors the multicopy recombinant plasmid (p5.2) that carries a complete alkaline protease (*apr E*) gene (Oulad Haddar et al. 2009). Moreover, the wild type keratinolytic bacterium, *B. subtilis* DB100 *his*$^-$ *met*$^-$, was used in this study as well.

Media and OVAT experiments

Peptone yeast extract (PY) medium (Bernhardt et al. 1978) [10 g bactopeptone, 5 g yeast extract, and 5 g NaCl per liter] was used to activate the bacterial strain. PA medium is PY supplemented with 1.5% agar agar. Generally, kanamycin was added to the growth media at a final concentration of 10 μg/ml. Basal medium II (Williams et al. 1990) [0.5 g NH_4Cl, 0.5 g NaCl, 0.3 g K_2HPO_4, 0.4 g KH_2PO_4, 0.1 g $MgCl_2$ and 0.1 g yeast extract per liter] was used in the process of sheep wool biodegradation. All experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml of basal medium II. Different levels of yeast extract [0.0, 0.01, 0.05% (w/v)] and NH_4Cl [0.0, 0.05, 0.1% (w/v)] were added to basal medium II supplemented with 3% (w/v) sheep wool unless otherwise stated. Three concentrations of sheep wool [1%, 2% and 3% (w/v)] were tested as well. Sheep wool-based basal medium II was adjusted to have initial pH of 6, 7, 8 and 9, separately was inoculated by activated *B. subtilis* recombinant cells. Different inoculum sizes [(2, 3, 5% (v/v)], were used to inoculate basal medium II supplemented with 3% (w/v) sheep wool. Generally, each ml of the activated *B. subtilis* recombinant cells culture

contained 3×10^8 CFU. Sheep wool cultures were incubated at three incubation temperatures (30°C, 37°C and 45°C). Moreover, sheep wool cultures were incubated with agitation at 150 and 250 rpm. Sheep wool culture incubated statically was conducted in parallel. The effect of kanamycin omission from the growth medium was tested as well by conducting sheep wool cultures with and without kanamycin in parallel. Cultures containing two times autoclaved sheep wool, acid, alkali treated sheep wool, defatted sheep wool and chopped sheep wool, separately were conducted. For each type of treatment, a control culture containing untreated sheep wool was conducted parallel to study the effect of this treatment on the efficacy of sheep wool biodegradation process. The effect of using distilled water and tap water instead of modified basal medium II was tested as well. All sheep wool cultures were incubated for 5 days. All experiments were carried out in triplicates. All experiments of optimization were directed with the *B. subtilis* DB 100 (p5.2) recombinant cells unless otherwise stated.

Determination of viable cells and soluble proteins

Viable bacterial cells were determined according to the method described before (Pelczar and Chan 1977). Soluble proteins resulted from sheep wool biodegradation were determined as described earlier by Bradford 1976. A standard curve for bovine serum albumin was established.

Determination of the keratinolytic activity of the alkaline protease enzyme

The keratinolytic activity of the alkaline protease enzyme was determined as described earlier by Oulad Haddar et al. 2009 based on the NH_2-free amino groups that were released as a result of the biodegradation of keratin (here sheep wool) by the bacterial cells. NH_2-free amino groups were determined using ninhydrin as described by Pearce et al. 1988. A standard curve for leucine was established.

Pretreatment of sheep wool

Sheep wool was pretreated by autoclaving one and two times at 121°C for 30 min each time. Chopping into small pieces, defatting (Folch et al. 1957) as well as treatment by acid and alkali were performed as reported before (Zaghloul et al. 2004). After, treatment of sheep wool with acid and alkali, the final pH of the medium was adjusted to 7.0. For each type of treatment, a control culture containing untreated sheep wool was conducted parallel to study the effect of this treatment on the efficacy of sheep wool biodegradation process.

Activation of the *B. subtilis* recombinant cells

Single fresh colonies of the *B. subtilis* recombinant cells were used to inoculate 50 ml of PY medium. The culture was grown at 37°C with agitation at 200 rpm for 2.5 h. Three milliliters of the growing culture, unless otherwise stated were centrifuged at 7,000 rpm for 3 min using a microcentrifuge. One milliter of the activated inoculum contained 3×10^8 CFU. The bacterial pellet was washed with saline [0.85% (w/v) NaCl] then used to inoculate 100 ml of sheep wool-based media.

Monitoring NH_2-free amino groups and soluble proteins

Inoculated sheep wool-based media were incubated at 37°C with agitation at 150 rpm for 5 days, unless otherwise stated. One mililiter samples were taken each day from the cultures and were centrifuged at 7,000 rpm for 3 min in a microcentrifuge. The supernatants were used to determine the level of NH_2-free amino groups and soluble proteins released from the fermentation process mentioned above.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE) was carried out as described earlier (Laemmli 1970). Protein bands were visualized by staining them with silver stain as described earlier (Morrissey 1981).

Analysis of amino acids

Free amino acids, except tryptophan, resulted from sheep wool biodegradation by the *B. subtilis* recombinant cells were determined using Beckman 119 CL amino acid analyzer (Speckman et al. 1958).

Statistical significance of the experimental data

Statistical significances of the obtained experimental data were determined based on the overlap rule for SE (standard error) bars as reported earlier by Cumming et al. 2007. Gap refers to the number of error bar arms that would fit between the bottom of the error bars on the controls (C) and the top of the bars on the experimental results (E); i.e., a gap of 2 refers to the distance between the C and E error bars is equal to twice the average of the SEs for the two samples (C and E). When $n = 3$, and double the length of the SE error bars just touch (i.e., the gap is 2 SEs), P-value is ≈ 0.05.

Results and discussion

Effect of using the *B. subtilis* recombinant cells on the efficacy of sheep wool biodegradation process

To address the high efficacy of the *B. subtilis* recombinant cells to direct the biodegradation of sheep wool, two cultures of sheep wool (3% w/v) were directed by *B. subtilis* recombinant cells and *B. subtilis* host cells, separately. The net levels of released soluble proteins and NH_2-free amino groups were enhanced by 7.9 and 5.7, respectively in cultures directed by *B. subtilis* recombinant after 4 days of incubation when compared to those of cultures directed by *B. subtilis* host cells (Fig. 1). These differences in the levels of soluble proteins and NH_2-free amino groups are significant ($P < 0.05$) (Fig. 1). Moreover, physical appearance of sheep wool reveals its complete solubilization after 3 days of incubation in cultures directed by *B. subtilis* recombinant cells (Fig. 2). However, cultures directed by *B.subtilis* host cells demonstrate a high proportion of residual undegraded sheep wool (90% w/v) after 5 days of incubation. These obtained high levels of keratinolytic activity of the alkaline protease (a key enzyme in sheep wool biodegradation) in terms of NH_2-free amino groups in cultures directed by the *B. subtilis* recombinant cells were mainly attributed to the high copy number and the small size of the recombinant plasmid (p5.2) harbored by these cells as reported earlier by Oulad Haddar et al (2009). In the line of these results along with attaining optimized

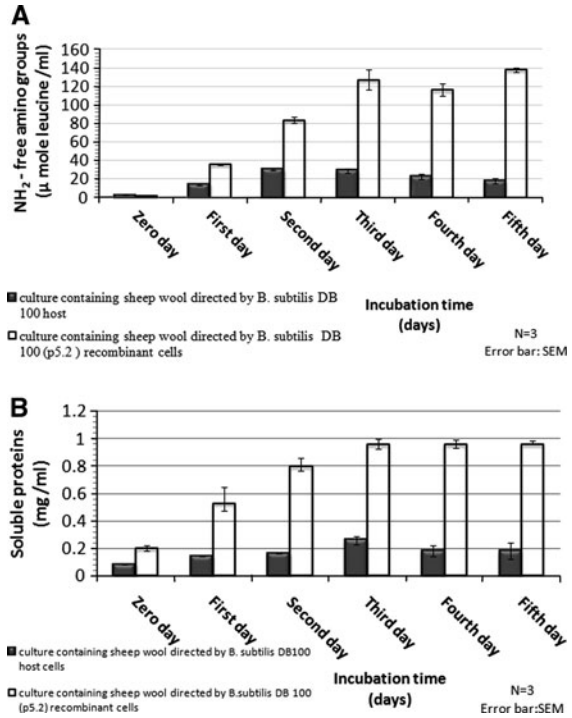


Fig. 1 Effect of using *B.subtilis* cells recombinant cells to direct the biodegradation of sheep wool. **a** Filled and opened bars represent the levels of NH_2-free amino groups released in cultures directed by *B.subtilis* host cells and *B.subtilis* recombinant cells, respectively. **b** Filled and empty bars represent the levels of soluble proteins in cultures directed by *B.subtilis* host cells and *B.subtilis* recombinant cells, respectively. Values are the average derived from three experiments. SEM: standard error of the mean of values. Significant mean values are those with P-value < 0.05 according to the overlap rule of SE bars

conditions for sheep wool biodegradation, *B.subtilis* recombinant cells were selected to direct further optimization experiments in this study.

Effect of sheep wool pretreatment

Effect of autoclaving times

The effect of changing the number of autoclaving times for the sheep wool on the efficiency of sheep wool biodegradation process was tested. Basal medium II supplemented with one and two times autoclaved sheep wool (3% w/v) was inoculated, separately, with the activated *B. subtilis* recombinant cells. The net levels of released NH_2-free amino groups and soluble proteins were greatly enhanced by 3.1 and 2.2 fold increase, respectively in cultures

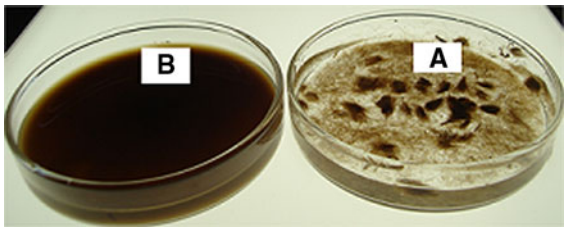


Fig. 2 Complete solubilzation of sheep wool waste by keratinase-producing *B. subtilis* recombinant cells. **a** physical appearance of sheep wool waste at day 0. **b** physical appearance of sheep wool waste after 3 days of incubation with keratinase-producing *B. subtilis* recombinant cells

containing two times autoclaved sheep wool by the third day of the incubation period when compared to those of cultures containing one time autoclaved sheep wool (Fig. 3). Generally, sheep wool cultures containing two times autoclaved sheep wool show significant higher levels ($P < 0.05$) of released end products when compared to those of sheep wool cultures containing one time autoclaved sheep wool as shown in Fig. 3. It seems that autoclaving results in sheep wool more vulnerable to the action of the keratinolytic alkaline protease enzyme. Autoclaving unfolds the polypeptide chains of keratin that are highly cross linked by S–S bonds, hydrophobic interactions and hydrogen bonds as well (Williams et al. 1990; Nam et al. 2002; Böckle et al. 1995). Our previous data concerning the biodegradation of feathers via the same cloned enzyme revealed the same observation (Zaghloul et al. 2004). On the other hand, the present finding is not in accordance with that reported about the behavior of keratinolytic serine protease of *Streptomyces pactum* DSM 40530 towards autoclaved feathers (Böckle et al. 1995).

Effect of chopping

Two cultures of the *B. subtilis* recombinant cells were supplemented with 3% (w/v) unchopped and chopped sheep wool, separately. A fold increase of 1.26 and 1.95 in the net levels of released soluble proteins and NH_2-free amino groups was recorded in cultures containing chopped sheep wool (Fig. 4) after 5 days of incubation, respectively. These differences in the net levels of released soluble proteins and NH_2-free amino groups are significant ($P < 0.05$) as indicated in Fig. 4. This could be attributed to the increase in surface area provided by chopping. Such increase in

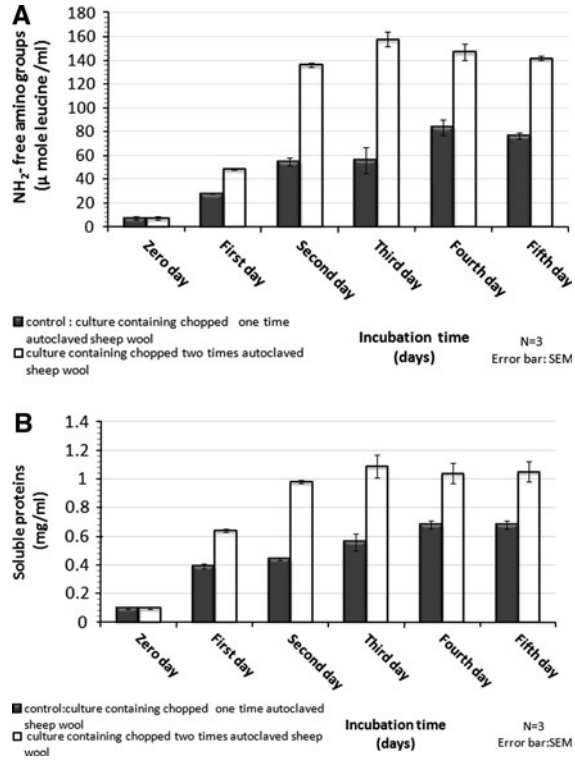


Fig. 3 Effect of autoclaving times. **a** Filled and empty bars represent the levels of NH_2-free amino groups released in cultures containing one and two times autoclaved sheep wool, respectively. **b** Filled and opened bars represent the levels of soluble proteins in cultures containing one and two times autoclaved sheep wool, respectively. Values are the average derived from three experiments ($N = 3$). SEM: standard error of the mean of values. Significant mean values are those with P-value < 0.05 according the overlap rule of SE bars

surface area would allow the cloned keratinolytic alkaline protease to bind to the surface of the substrate and consequently more released end products will be produced. This finding is in agreement with a previous report stating that, chopping of feathers greatly enhanced the efficiency of the biodegradation process by the same cloned enzyme (Zaghloul et al. 2004).

Effect of defatting of sheep wool

It has reported that, there is a layer of lipids on the surface of sheep wool. Chemical analysis of these lipids has shown that, they are a complex mixture of polar and non-polar lipids with long chain fatty acids (Hutchinson et al. 2007). The presence of lipids may hinder the binding of the keratinolytic enzymes on

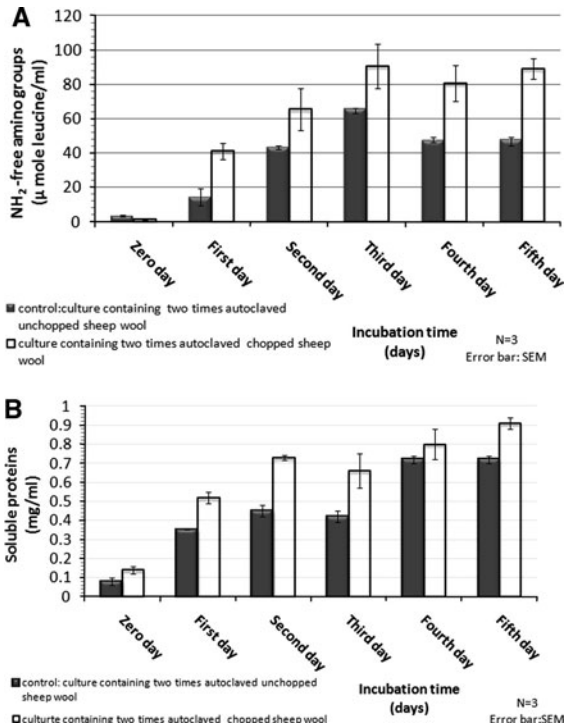


Fig. 4 Effect of sheep wool chopping. **a** Filled and empty bars represent the levels of NH_2-free amino groups released in cultures containing two times autoclaved unchopped and chopped sheep wool, respectively. **b** Filled and empty bars represent the levels of soluble proteins in cultures containing two times autoclaved unchopped and chopped sheep wool, respectively. Values are the average derived from three experiments ($N = 3$). SEM: standard error of the mean values. Significant mean values are those with P-value < 0.05 according the overlap rule of SE bars

the surface of sheep wool. To clarify the issue, basal medium II was supplemented with defatted and undefatted 3% (w/v) sheep wool, separately and was inoculated with the activated *B. subtilis* recombinant cells. The net levels of released NH_2-free amino groups were 140 and 150 µmol leucine/ml in cultures supplemented with undefatted and defatted sheep wool, respectively after 3 days of incubation. However, the net levels of released soluble proteins were 0.72 mg/ml in both cultures after 4 days of incubation. Data reveal that, there are no significance differences ($P > 0.05$) in the levels of released end products (NH_2-free amino groups and soluble proteins) in both cultures. It seems that, washing of sheep wool with tap water followed by distilled water, a process routinely carried out to prepare sheep wool prior to its addition to culture media, is a sufficient step to remove lanolin from sheep wool. In other words, the utilization of sheep wool does not require this step and consequently the process would be a cost effective one.

Effect of acid and alkali treatment

Alkali and acid treated sheep wool (3% w/v) were added separately to basal medium II which was inoculated as mentioned above. There are no significant differences ($P > 0.05$) in the net levels of released NH_2-free amino groups in cultures containing alkali treated sheep wool when compared to those of cultures containing untreated sheep wool from day 2 to day 4 (Fig. 5a). However, cultures containing alkali treated sheep wool showed a 2.6 fold increase in the net levels of released soluble proteins when compared to those of cultures containing untreated sheep wool after 4 days of incubation (Fig. 5b). These differences in the net levels of released soluble proteins from day 2 to day 4 are significant ($P < 0.05$) as shown in Fig. 5b. Alkali treatment of sheep wool results in converting sheep wool to a form more accessible to the action of the cloned keratinolytic alkaline protease enzyme via disruption of the S–S bonds and consequently more soluble proteins are released. The situation is completely different in cultures containing acid treated sheep wool. No release of NH_2-free amino groups or soluble proteins is detected in cultures supplemented with acid treated sheep wool before 4 days of incubation. It was reported that, sheep wool has some pigments such as melanins (Ozeki et al. 2007). It could be that, these pigments upon treatment with acid may be converted to secondary compounds that might have inhibitory effect on the growth of the recombinant bacterial cells. It is also possible that acid treatment would result in putting some charges on the surface of sheep wool that may hinder the attachment of the keratinolytic bacteria on the surface of sheep wool. It was reported that, the process of cell attachment of keratinolytic bacteria on the surface of keratin is a prerequisite for achieving keratinolysis event (Bressollier et al. 1999). On the other hand, data from a previous study on β-keratins with different physicochemical properties from that of sheep wool (α-keratins), are in disagreement with the present finding (Zaghloul et al. 2004). However, the decision to treat sheep wool with NaOH during the scaling up processes will depend mainly on the cost effectiveness

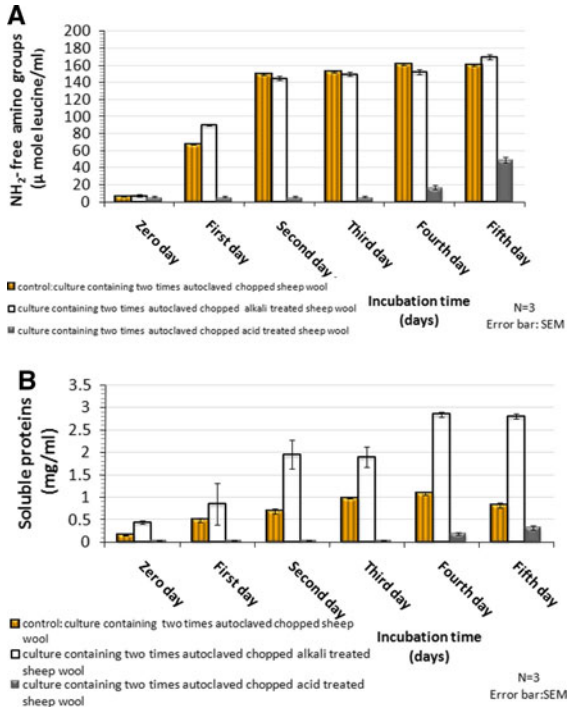


Fig. 5 Effect of acid-alkali treatment of sheep wool. **a** Semi filled, empty and filled bars represent the levels of NH_2-free amino groups released in cultures containing untreated, alkali treated and acid treated sheep wool, respectively. **b** Semi filled, empty and filled bars represent the levels of soluble proteins released in cultures containing untreated, alkali treated and acid treated sheep wool, respectively. Values are the average derived from three experiments ($N = 3$). SEM: standard error of the mean values. Significant mean values are those with P-value < 0.05 according the overlap rule of SE bars

of this step and the type of end products needing NH_2-free amino groups or soluble proteins. In other words, treatment of sheep wool with NaOH is highly recommended when one wants to obtain high levels of soluble proteins from this process.

Effect of physical factors

Effect of agitation speed

Agitation speed is one of the limiting factors in submerged state fermentation processes. Microbial enzymes are susceptible to mechanical force, thus the elaborate shape of these complex molecules may disturb to such degree that denaturation could occur (Elibol and Moreira 2005). To counter this, the effect of agitation speed on the efficiency of sheep wool biodegradation was tested in this study. Two bacterial sheep wool cultures were incubated as mentioned in Materials and Methods with two agitation speeds (150 rpm and 250 rpm) and a third culture was incubated without agitation. Generally, the net levels of released NH_2-free amino groups were greatly enhanced in cultures incubated with agitation when compared to those of cultures incubated statically (Fig. 6). A fold increase of 2.78 in the net levels of released NH_2-free amino groups after 5 days of incubation in agitated cultures at 150 rpm were obtained when compared to those of cultures incubated statically (Fig. 6a). Significant differences ($P < 0.05$) in the net levels of released NH_2-free amino groups in agitated and static cultures are

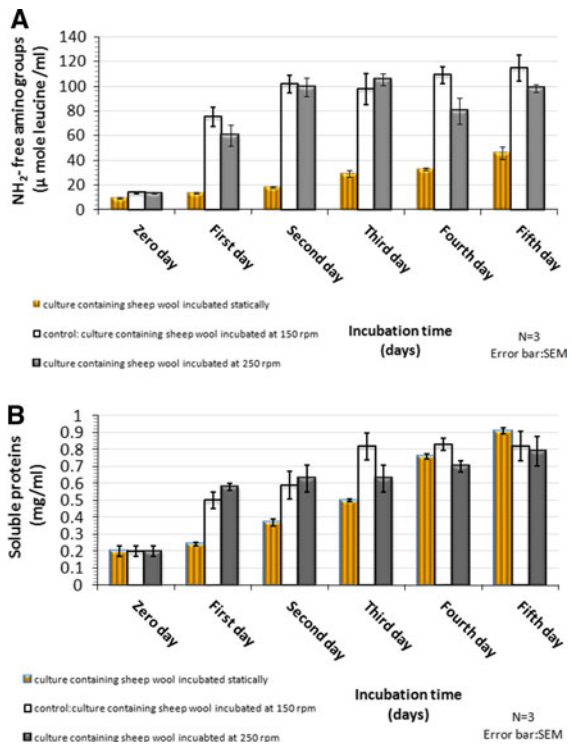


Fig. 6 Effect of agitation speed. **a** Semi filled, empty and filled bars represent the levels of NH_2-free amino groups released in cultures in incubated with agitation speed at 0.0, 150 and 250 rpm, respectively. **b** Semi filled, empty and filled bars represent the levels of soluble proteins released in cultures incubated with agitation speed at 0.0, 150 and 250 rpm, respectively. Values are the average derived from three experiments ($N = 3$). SEM: standard error of the mean values. Significant mean values are those with P-value < 0.05 according the overlap rule of SE bars

recorded along 5 days (Fig. 6a). Changing agitation speed to 250 rpm is not accompanied by an significant increase in the net levels of released end products in cultures incubated at 250 rpm from day 1 to day 5 (overlapped SEM bars or SEM bars with gaps <twice SEMs of both cases) as indicated in Fig. 6. Significant differences ($P < 0.05$) in the net levels of soluble proteins in agitated and static cultures were obtained only at the first and the third day of incubation (Fig. 6b). However, no significant differences were obtained in the net levels of soluble proteins in agitated and static cultures at the second, fourth and fifth day of incubation (Fig. 6b). As a matter of fact, higher agitation speed is required during the log phase of the bacterial growth to accelerate the rate of growth and consequently the number of cells needing to degrade sheep wool will increase. By the onset of the stationary phase bacterial cells start to lower their growth rate step wisely. So, there is no need to utilize higher agitation speed at stationary phase. Moreover, *B. subtilis* recombinant cells spend short log phase upon growing on a keratin-based medium (4–5 h) and prolonged stationary phase. Although, the cloned keratinolytic alkaline protease enzyme in these recombinant *B.subtilis* cells starts to be produced at late log phase, the great bulk of this enzyme is mainly produced at the stationary phase (Oulad Haddar et al. 2009). This keratinolytic alkaline protease is the key enzyme in the keratin biodegradation process. This highlights the issue of non-growth related product formation observed in many microbial fermentation processes (Genckal and Tari 2006). Although cultures incubated at 250 rpm showed higher levels of cells number 4.88×10^9 CFU/ml when compared to those of cultures incubated at 150 rpm (6.0×10^7 CFU/ml) after 3 days of incubation, comparable levels of the released end products were obtained in both cultures. This could be attributed to the presence of metabolically inactive cells in cultures containing high number of cells (grown at 250 rpm) since the available nutrients and oxygen supply will be limited upon overloading of cells. Present data highlight the use of lower agitation upon scaling up this fermentation process.

Effect of incubation temperature

Three cultures of the *B. subtilis* recombinant cells supplemented with 3% (w/v) sheep wool were grown at three different temperatures (30°C, 37°C and 45°C). Data reveal that, the net levels of released soluble proteins ($\approx$0.72 mg/ml) are almost quite similar in the three cultures after 3 days of incubation. However, cultures grown at 30°C and 37°C show closer net levels of NH_2-free amino groups (125.67 μmol leucine/ml) to that of cultures grown at 45°C (115.49 μmol leucine/ml) after 5 days of incubation. It seems that, the temperature required for optimal growth for these recombinant bacterial cells does not vary greatly from that required to achieve the optimal levels of the keratinolytic alkaline protease enzyme (a key enzyme in the biodegradation of sheep wool here). Data indicate that, biodegradation of sheep wool on large scale can be carried out at 30°C. On the other hand, using lower temperatures than 30°C could not be encouraged in the present study, since the bacterial strain employed in this study is a mesophilic one. In other words, lower temperatures than 30°C will not sustain satisfied bacterial growth. Unsatisfied levels of bacterial growth will result in lower levels of the keratinolytic alkaline protease enzyme. Present data are in disagreement with previous reports stating that, optimal temperatures for keratin solubilization by *B. subtilis* mutant cells and *B. licheniformis* cells were 42°C and 45°C, respectively (CAI et al. 2008; Balint et al. 2005). Conversely, present data are in agreement with a previous report stating that, optimal temperature for keratin solubilization by *Vibrio* sp. Kr2 was 30°C. (Grazziotin et al. 2007). Generally, utilizing low growth temperatures upon scaling up fermentation processes will reduce the overall cost of the process.

Effect of medium composition

Effect of initial pH of the growth medium

Four bacterial cultures containing 3% (w/v) sheep wool were directed by the recombinant cells at different initial pHs values of 6.0, 7.0, 8.0 and 9.0. The lowest net levels of released end products are obtained in cultures where the initial pH was 6.0. However, cultures directed at pHs 7.0, 8.0 and 9.0 display comparable values in the net levels of released NH_2-free amino groups ($\approx$130 μmol leucine/ml). On the other hand, the net levels of released soluble proteins are slightly increased with the initial medium alkalinity. This observation is in a good

agreement with other studies which stated that, measurable keratin hydrolysis directed by *Chryseobacterium* sp. kr6, *Bacillus* spp. and *Vibrio* sp. Kr2 was observed between pHs values of 6.0–9.0 (Riffel et al. 2007; Balint et al. 2005; El-Refai et al. 2005; Grazziotin et al. 2007). Conversely, present data disagree with a previous report stating that, the residual keratin hydrolysate derived from the action of a *B. subtilis* mutant strain decreased gradually with the initial medium alkalinity (CAI et al. 2008). These differences may be attributed to the strain difference.

Effect of sheep wool concentration

Different sheep wool percents [(1%, 2% and 3% (w/v)] were added to basal medium II to determine the best sheep wool percent required for an efficient sheep wool biodegradation. Cultures supplemented with 3% (w/v) sheep wool demonstrate the highest net levels of released end products. A fold increase 2.06 and 2.35 in the net levels of released NH_2-free amino groups and soluble proteins was achieved in cultures containing 3% (w/v) sheep wool when compared to those of cultures containing 2% (w/v) sheep wool after 3 days of incubation, respectively (Table 1). Generally, cultures containing 3% (w/v) sheep wool show higher significant levels ($P < 0.05$) of soluble proteins when compared to those of cultures containing 2% (w/v) sheep wool along 5 days of incubation (Table 1). On the other hand, cultures containing 3% (w/v) sheep wool show higher significant levels ($P < 0.05$) of NH_2-free amino groups when compared to those of cultures containing 2% (w/v) sheep wool from day 3 to day 5 (Table 1). Data are in agreement with those reported previously that, proteins and peptides in the growth medium increase the synthesis of proteases in numbers of microorganisms (El-Refai et al. 2005). However, a threshold region regarding the optimum sheep wool concentration needed to direct an efficient sheep wool biodegradation is not justified yet. This addresses the indispensable need to carry out future experiments directed with sheep wool concentrations higher than 3% (w/v). This finding is in disagreement with a previous report stating that, keratin hydrolysis is inversely proportional to the keratin concentration in the growth medium (El-Refai et al. 2005). However, this comparison is not being justified yet unless the rate of keratin hydrolysis is uniform in both cases. Present data confer that, the cloned keratinolytic alkaline protease enzyme shows higher keratinolytic activity towards α-keratins. Data reveal that, sheep wool concentration is a key determinant in the process of sheep wool biodegradation. Further studies are needed to define the exact optimal sheep wool concentration that is required to obtain higher levels of released end products. This could be achieved through a response surface methodology approach.

Effect of yeast extract concentration

To test the effect of yeast extract concentration (one of the components of basal medium II) on the process of sheep wool biodegradation, yeast extract was added in three concentrations [0.0%, 0.01% and 0.05% (w/v)] to basal medium II supplemented with 3% (w/v) sheep wool. A dramatic decrease in the net levels of released NH_2-free amino groups was observed in cultures containing 0.05% (w/v) yeast extract when compared to those of cultures containing either 0.01% (w/v) or no yeast extract. Moreover, no remarkable differences in the net levels of released end products (90–100 μmol leucine/ml NH_2-free amino groups and 0.7 mg/ml soluble proteins) were observed in cultures containing either 0.01% (w/v) yeast extract or no yeast extract after 4 days of incubation. On the other hand, the net levels of released soluble proteins increased (1.6 fold) in cultures containing no yeast extract when compared to those of cultures containing 0.05% (w/v) yeast extract after 4 days of incubation (Table 2). It seems that, using yeast extract in concentrations higher than 0.01% (w/v), the original concentration used in basal medium II, result in a delay in the consumption of the sheep wool as bacterial cells would logically prefer to utilize yeast extract. This finding is in agreement with those indicated that, the percent of keratin hydrolysis directed by *B. pumilus* and *B. cereus* decreased upon increasing the concentration of yeast extract in the growth medium (El-Refai et al. 2005; Sousa et al. 2007). On the other hand, the presented data are in disagreement with a previous report stating that, the amount of soluble proteins in *Vibrio* sp. Kr2 keratin hydrolysate increased upon adding yeast extract (0.5% w/v) to the keratin medium (Grazziotin et al. 2007). From the above results this study recommends that yeast extract is omitted from the growth medium and consequently, a scaling up of the valorization of sheep wool would be warranted concerning the cost effectiveness of the method.

Table 1 Effect of sheep wool concentration and inoculum size on the biodegradation of sheep wool directed by keratinase-producing *B. subtilis* recombinant cells

Sheep wool concentration (w/v)						
1%			2%		3%	
Days	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)
0	0.039 ± 0.00	1.45 ± 0.00	0.039 ± 0.00	7.2 ± 0.25	0.14 ± 0.020	8.50 ± 1.90
1	0.063 ± 0.002	32.27 ± 2.40	0.24 ± 0.005	47.27 ± 3.60	0.52 ± 0.023	57.09 ± 0.84
2	0.18 ± 0.009	43.64 ± 3.80	0.345 ± 0.006	70.78 ± 5.00	0.73 ± 0.038	87.27 ± 4.40
3	0.17 ± 0.003	33.94 ± 0.56	0.26 ± 0.009	60.60 ± 3.80	0.66 ± 0.018	118.55 ± 1.40
4	0.15 ± 0.010	54.55 ± 1.19	0.29 ± 0.010	72.72 ± 6.30	0.80 ± 0.010	112.36 ± 2.60
5	0.034 ± 0.009	33.81 ± 0.66	0.13 ± 0.010	70.55 ± 5.20	0.91 ± 0.016	133.09 ± 3.50
Inoculum size (v/v)[a]						
2%			3%		5%	
Days	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/log CFU)	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/log CFU)	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/log CFU)
0	0.04 ± 0.001	0.45 ± 0.012	0.04 ± 0.001	0.52 ± 0.04	0.04 ± 0.001	0.50 ± 0.043
1	0.58 ± 0.02	1.67 ± 0.30	0.65 ± 0.032	3.09 ± 0.19	0.61 ± 0.033	3.60 ± 0.08
2	0.70 ± 0.037	4.36 ± 0.70	0.78 ± 0.04	7.35 ± 1.00	0.80 ± 0.040	6.19 ± 0.40
3	0.63 ± 0.030	6.98 ± 1.00	0.72 ± 0.033	10.28 ± 1.30	0.70 ± 0.032	7.35 ± 0.76
4	0.69 ± 0.025	9.19 ± 1.20	0.70 ± 0.05	16.62 ± 1.90	0.70 ± 0.05	12.90 ± 1.30
5	0.73 ± 0.036	12.88 ± 2.50	0.80 ± 0.038	18.6 ± 2.20	0.73 ± 0.038	14.99 ± 1.86

Means with significant values were taken at $P < 0.05$ according to the overlap rule for SE bars as described before by Cumming et al. (2007)

N.B: All values are average of data derived from three experiments including the standard error: Mean values ± SEM

[a] Each ml of the mother culture contained 3×10^8 colony forming unit

Effect of NH_4Cl

In order to determine the effect of NH_4Cl (one of the components of basal medium II) on the biodegradation of sheep wool, three concentrations of NH_4Cl, [0.0%, 0.05% and 0.1% (w/v)] were separately added to basal medium II supplemented with 3% (w/v) sheep wool. Data reveal that, the net levels of released end products in the three cultures are almost quite similar (no significant differences). The net levels of released soluble proteins and NH_2-free amino groups were (about 0.66 mg/ml and 100 μmol leucine/ml) after 4 and 5 days of incubation, respectively (Table 2). The present finding is in disagreement with that of a report stating that, the percent of keratin hydrolysis directed by *B. pumilus* decreased upon increasing the concentration of NH_4Cl in the growth medium (El-Refai et al. 2005). Conversely, levels of soluble proteins in *Vibrio* sp. Kr2 keratin hydrolysate slightly increased upon adding NH_4Cl to keratin medium when compared to that of keratin hydrolysate without addition of NH_4Cl (Grazziotin et al. 2007). This study recommends the omission of NH_4Cl from the growth medium, basal medium II upon valorization of sheep wool on a large scale. The above finding concerning the ability of the recombinant bacterial cells to grow in a medium containing no yeast extract and no NH_4Cl reveals the ability of these recombinant bacterial cells to utilize sheep wool as a sole source of carbon and nitrogen.

Table 2 Effect of yeast extract and NH_4Cl concentrations on sheep wool biodegradation directed by keratinase-producing *B. subtilis* recombinant cells

Yeast extract (w/v)						
0.0%			0.01%[a]		0.05%	
Days	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)
0	0.04 ± 0.029	7.80 ± 0.49	0.04 ± 0.029	7.80 ± 1.50	0.04 ± 0.020	7.8 ± 0.48
1	0.30 ± 0.005	42.42 ± 4.89	0.39 ± 0.015	32.87 ± 0.69	0.24 ± 0.012	16.0 ± 0.98
2	0.42 ± 0.020	83.63 ± 0.97	0.40 ± 0.015	75.16 ± 1.80	0.41 ± 0.057	28.7 ± 1.80
3	0.45 ± 0.010	94.55 ± 2.16	0.48 ± 0.029	73.24 ± 6.15	0.29 ± 0.036	40.0 ± 0.57
4	0.73 ± 0.010	109.33 ± 1.15	0.72 ± 0.029	98.48 ± 6.65	0.46 ± 0.035	43.64 ± 5.00
5	0.70 ± 0.010	136.18 ± 3.7	0.73 ± 0.027	77.58 ± 1.66	0.5 ± 0.028	33.69 ± 2.00
NH_4Cl (w/v)						
0.0%			0.05%[a]		0.1%	
Days	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)	Soluble proteins (mg/ml)	NH_2-free amino groups (μmol leucine/ml)
0	0.21 ± 0.010	9.60 ± 0.60	0.20 ± 0.020	9.60 ± 0.11	0.20 ± 0.011	35.64 ± 0.25
1	0.49 ± 0.0018	60.0 ± 1.89	0.33 ± 0.030	69.49 ± 0.36	0.40 ± 0.038	89.45 ± 0.66
2	0.68 ± 0.013	116.4 ± 1.7	0.56 ± 0.090	118.78 ± 5.0	0.48 ± 0.010	129.45 ± 8.50
3	0.80 ± 0.079	105.0 ± 3.3	0.53 ± 0.078	116.36 ± 8.50	0.58 ± 0.010	152.00 ± 7.24
4	0.76 ± 0.070	94.0 ± 1.8	0.76 ± 0.030	104.55 ± 10.0	0.83 ± 0.040	126.00 ± 4.70
5	0.84 ± 0.070	107.6 ± 1.8	0.60 ± 0.035	118.55 ± 10.0	0.68 ± 0.060	145.45 ± 10.30

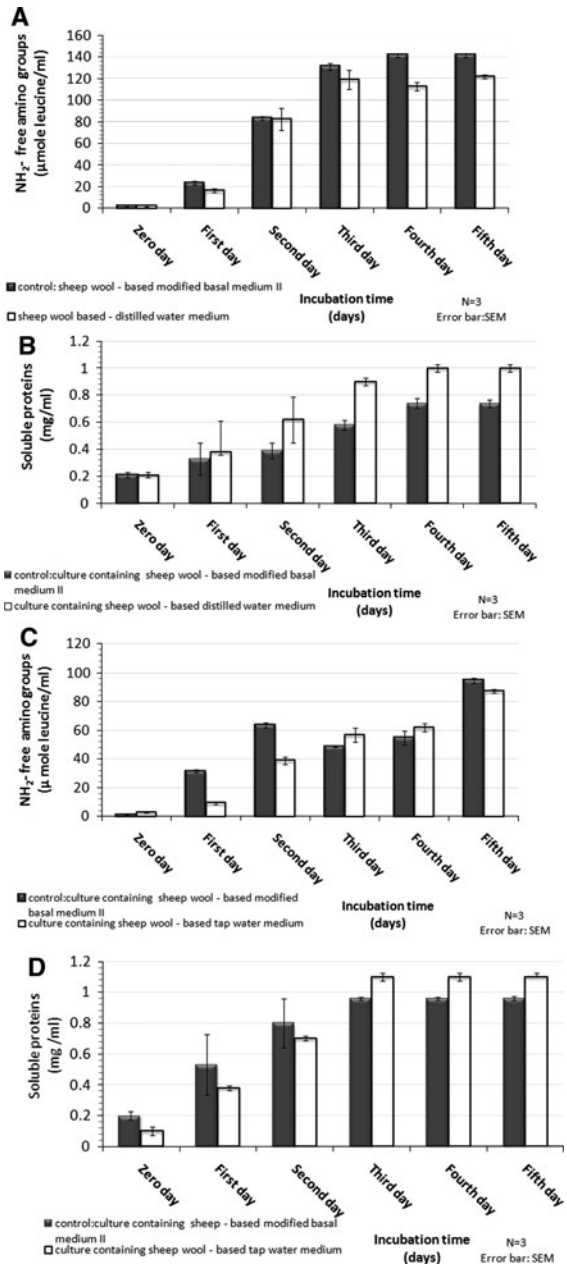
Means with significant values were taken at $P < 0.05$ according to the overlap rule for SE bars as described before by Cumming et al. (2007)

N.B: All values are average of data derived from three experiments including the standard error: Mean values ± SEM

[a] Original concentration of yeast extract and NH_4Cl in basal medium II

Effect of using distilled and tap water instead of modified basal medium II

The above findings concerning the ability of the recombinant bacterial strain to utilize sheep wool as a sole source of carbon and nitrogen addressed the urgent need to test the ability of the bacterial cells to grow in tap or distilled water instead of modified basal medium II. Three bacterial growth media, sheep wool-based modified basal medium II (basal medium II without yeast extract and NH_4Cl), sheep wool-based tap water medium and sheep wool-based distilled water medium, all supplemented with 3% (w/v) sheep wool were inoculated with activated bacterial cells. Data indicate no significant differences in the levels of released NH_2-free amino groups in cultures containing sheep wool-based modified basal medium II and those containing sheep wool-based distilled water medium during the first 3 days of incubation (Fig. 7a). However, significant differences ($P < 0.05$) in the levels of released NH_2-free amino groups in cultures containing sheep wool-based modified basal medium II and those containing sheep wool-based distilled water medium from day 4 to day 5 were obtained (Fig. 7a). Regarding the levels of released soluble proteins, significant differences ($P < 0.05$) were noticed from day 3 to day 5 between cultures containing sheep wool-based modified basal medium II and those containing sheep wool-based distilled water medium (Fig. 7b). On the other hand, no significant differences in the levels of released NH_2-free amino groups were obtained during the last 3 days of incubation in cultures containing sheep wool-based modified basal medium II and those containing sheep wool-based tap water medium



◀ **Fig. 7** Effect of using water instead of modified basal medium II. **a** Filled and empty bars represent the levels of NH_2-free amino groups released in cultures containing sheep-based modified basal medium II and sheep wool-based distilled water medium, respectively. **b** Filled and empty bars represent the levels of soluble proteins released in cultures containing sheep wool-based modified basal medium II and sheep wool-based distilled water medium, respectively. **c** Filled and empty bars represent the levels of NH_2-free amino groups released in cultures containing sheep wool-based modified basal medium II and sheep wool-based tap water medium, respectively. **d** Filled and empty bars represent the levels of soluble proteins released in cultures containing sheep-based modified basal medium II and sheep wool-based tap water medium, respectively. Values are the average derived from three experiments ($N = 3$). SEM: standard error of the mean values. Significant mean values are those with P-value < 0.05 according the overlap rule of SE bars

(Fig. 7c). However, significant differences ($P < 0.05$) were noticed in the levels of released soluble proteins during the last 3 days of incubation in cultures containing sheep wool-based modified basal medium II and those containing sheep wool-based tap water medium (Fig. 7d). This result is not surprising since sheep wool contains several elements such as Mg, Ca, K, Na, Cu, P, Mn, Fe, B, Al and Zn (Zheljazkov 2005). Although there are some noticeable significant differences in the levels of released end products in the above cultures, these obtained levels of are satisfactory to a great extent when we considered the cost effectiveness issue. The present study proposes three media; sheep wool-based modified basal medium II, sheep wool-based tap water medium and sheep wool-based distilled water medium to be used in the course of sheep wool biodegradation. Although, the present data indicate that, one can use tap water or distilled water instead of basal medium II or modified basal medium II and this would be cheaper, even though the quality of tap water undergoes seasonal variations concerning tap water treatments.

Effect of kanamycin omission from the growth medium

Since the recombinant plasmid (p5.2) carries the complete alkaline protease gene as well as a kanamycin resistance gene, it is a routine to include kanamycin in the cultures for maintaining high levels of plasmid stability. However, antibiotic addition to the growth medium in large scale fermentations comes at a high cost. Consequently, testing the effect of kanamycin omission from growth medium has been thoroughly addressed here. Cultures of basal medium II were supplemented with 3% (w/v) sheep wool and inoculated with *B. subtilis* DB 100 (p5.2) in the absence and the presence of kanamycin (10 µg/ml medium). Data show that, the net levels of released end products in both cultures were greatly comparable. The net levels of released soluble proteins and NH_2-free amino groups were 0.83 mg/ml and 90 µmol leucine/ml after 4 days of incubation in both cultures,

respectively. Data indicate that, there is no need to add kanamycin and consequently the cost will be cheaper.

Effect of inoculum size

Determination of the optimum inoculum size required to direct the biodegradation process is an important aspect to consider when optimizing bioprocesses. Basal medium II supplemented with 3% (w/v) sheep wool was inoculated separately with three inocula sizes; [2%, 3%, and 5% (v/v)]. Each ml contained 3×10^8 colony forming units (CFU). Data reveal that, no significant differences ($P > 0.05$) are noticed in the net levels of released end products in the three mentioned above cultures (Table 1). A possible explanation of this finding would be that, increasing the inoculum size beyond 2% result in a gradual overload of cells facing nutrients or oxygen limitations. So only a certain number of cells would be allowed to grow even if there is a considerable increase in the initial number of bacterial cells. This highlights the phenomenon stating that, the bacterial cells just discussed prefer end products formation to biomass formation. Again, this finding is highly correlated with the issue of non-growth associated product formation observed in many fermentation processes (Genckal and Tari 2006). The present finding is in disagreement with a previous report stating that, a decrease in the proportion of keratin hydrolysis directed by a *B. subtilis* mutant strain was noticed upon increasing the initial inoculum size from 2–10% (v/v) (Cai et al. 2008). In fact, the comparison may not be justified well as the number of cells in each ml added to the cultures should be equal in all cases. The three tested inocula sizes localized in the threshold region, however, there is an indispensable need to use combination sets of lower/higher ratios of both inoculum sizes and sheep wool concentration in future experiments. Present data indicate that, inoculums size is a key determinant issue in the fermentation process discussed in this study. Reaching the optimal level of inoculum size will be achieved through a response surface methodology in future experiments.

Effect of incubation time

Determining the precise time required to end up the fermentation process is a key issue. Complete solubilzation of sheep wool was achieved on the third day as observed from the physical appearance of sheep wool (Fig. 2). An overview of the above experiments show that, the highest or near the highest net levels of released end products are achieved after approximately 3 days of incubation. However, elongation of incubation time beyond 3 days sometimes imposes a slight increase in the net levels of released end products. Upon scaling up, these slight increases might be negligible or had a considerable weight from the standpoint of cost effectiveness. However, other experiences reveal that, shorter fermentations time would be much more profitable in industrial scales when compared to the extra end products yields (Genckal and Tari 2006). It seems that, the optimal time required to achieve the highest levels of released end products derived from sheep wool hydrolysis may be 3 days or less. Present data indicate that, incubation time is a key determinant in the fermentation process here. Identifying the exact incubation time required for obtaining the highest possible levels of released end products will be reached through a response surface methodology approach.

Analysis of soluble proteins in sheep wool hydrolysate

Soluble proteins produced from the hydrolysis of sheep wool are considered to be one of the important end products derived from the biodegradation process. Released soluble proteins were monitored throughout the degradation process using SDS-PAGE (Fig. 8). Data demonstrate that, no soluble proteins could be detected on gels at day 0. However, as the incubation time is increased, more bands of soluble proteins could be detected (from day 1 to day 5). Soluble proteins of high and intermediate molecular weights are present in the sheep wool hydrolysate. Moreover, soluble proteins of low molecular weights (15–20 KDa) could be detected only on the last day of incubation.

Analysis of amino acids in sheep wool hydrolysate

Amino acids play an important role in living organisms. There has been an increase demand for the use of amino acids in many areas such as food, feed additives and drug pharmaceutical industries. Keratin, the main component of sheep wool, represents nearly 90% of sheep wool weight. Biotechnological applications

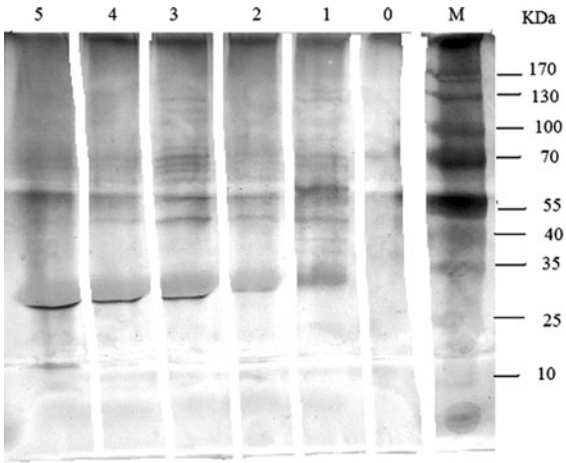


Fig. 8 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE) showing soluble proteins resulted from sheep wool waste biodegradation. Lane M: represents molecular weight protein marker. Lanes (0, 1, 2, 3, 4 and 5) represent 30 μg soluble proteins of the culture from day 0 to day 5, respectively

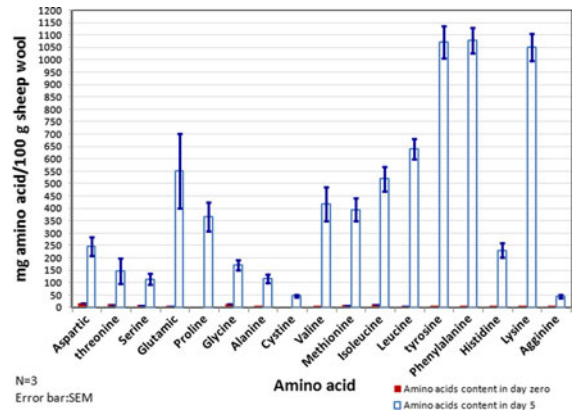


Fig. 9 Amino acids profile of the recombinant *B. subtilis* sheep wool hydrolysate. Filled and empty bars represent the levels of amino acids released in day 0 and day 5. Values are the average derived from three experiments ($N = 3$). SEM: standard error of the mean values. Significant mean values are those with P-value < 0.05 according the overlap rule of SE bars

consider the use of keratin-degrading microorganisms or keratinolytic enzymes in the production of amino acids and peptides. Moreover, the amino acids resulting from the utilization of keratin-containing materials are considered to be one important product that can promote several new industries.

Amino acids produced from sheep wool biodegradation are shown in Fig. 9. Data indicate that, the amino acids phenylalanine, tyrosine and lysine are the most abundant amino acids in sheep wool hydrolysate, respectively. However, other amino acids such as leucine, glutamic acid, isoleucine, valine, methionine, proline and histidine show moderate levels in the sheep wool hydrolysate. The high levels of aromatic amino acids in sheep wool hydrolysate as shown in Fig. 8 reflect the high affinity of the cloned keratinolytic alkaline protease enzyme for cutting beside the aromatic amino acids residues. On the other hand, the levels of all amino acids in the resulting sheep wool hydrolysate at day 5 were greatly enhanced if compared to those at day 0 ($P < 0.01$) as shown in Fig. 8.

This finding is in disagreement with a previous report stating that, *B. cereus* sheep wool hydrolysate showed high levels of the amino acids; glutamic, serine, proline, leucine, arginine, threonine and aspartic acid (Sousa et al. 2007). This difference in the amino acid pattern could be attributed to a strain difference and the specificity of the produced keratinolytic protease enzyme. As a result of this study, it is highly recommended that *B. subtilis* DB 100 (p5.2) sheep wool hydrolysate is used for feed additives since it has a high content of free amino acids and essentially amino acids such as methionine, lysine and histidine. On the other hand, *B. subtilis* DB 100 (p5.2) sheep wool hydrolysate shows higher content of free amino acids and soluble proteins when compared to the content of more expensive alternatives (blood and casein hydrolysates) which are routinely used in bacteriological media (Vasileva-Tonkova et al. 2007). Generally, the number of amino acids residues per 100 g of casein and blood hydrolysates ranges from 0.92 to 17.04 (Vasileva-Tonkova et al. 2007), while the number of each amino acid residues in sheep wool hydrolysate of B.*subtilis* DB 100 (p5.2) derived from 100 g sheep wool ranges from 1.0×10^{21} to 7.8×10^{21}. As can be seen, sheep wool hydrolystae of *B.subtilis* DB100 (p5.2) is rich in nutritionally essential amino acids such as arginine, lysine and phenylalanine (1.55×10^{20}, 4.3×10^{21} and 3.9×10^{21} residues per 100 g sheep wool, respectively) when compared to those of Eladin hydrolystae (arginine 6.37 residue per 100 g hydrolysate) and blood hydrolystae (phenylalanine 11.2 and 5.54 residues per 100 g hydrolysate, respectively) (Vasileva-Tonkova et al. 2007).

Conclusion

OVAT approach was used in this study to define the most important key determinants with a significant weight that affect sheep wool biodegradation directed by the *B. subtilis* recombinant cells. The highlighted key determinants are sheep wool concentration, incubation time and inoculum size. These key determinants will be subjected to a further study through a response surface methodology to identify the optimal level for each. Two steps are highlighted which are required to render sheep wool waste in an acceptable form to bacterial cells, washing of sheep wool by tap water followed by chopping into small pieces. A compromise among three aspects, the yield of released soluble proteins, the cost and the time, must be carried out before making a decision concerning the use of alkali treated sheep wool. Fortunately, the whole process of sheep wool biodegradation could be successfully conducted in the absence of kanamycin. Consequently, the process will be visible considering cost effectiveness. This study has succeeded to present three simple cost effective sheep wool-based media for the process of sheep wool biodegradation. Levels of released end products in sheep wool hydrolysates are very comparable when growing these recombinant bacterial cells on the aforementioned three proposed media. The overall cost of sheep wool biodegradation will be greatly reduced, especially when one uses a large scale process. This study supports and encourages the recycling of sheep wool waste by *B. subtilis* recombinant cells. A better yield of liberated end products could be achieved through employing a response surface methodology concerning sheep wool biodegradation to define the exact optimal levels of the aforementioned key determinants. On the other hand, end products resulting from sheep wool biodegradation could promote several industries such as amino acids, enzymes and soluble proteins-based industries.

Acknowledgements The authors are very grateful to Chemist/Heba-Allah S. Marey, Assistant lecturer of the Institute of Graduate Studies and Research (IGSR), Environmental Studies Department, Alexandria University, Egypt for her help concerning data analysis. The authors are also very grateful to the Scientific Academy for Research and Technology for its financial support.

References

Anbu P, Gopinatha SCB, Hilda A, Lakshmi PL, Annaduraic G (2005) Purification of keratinase from poultry farm isolate-*Scopulariopsis brevicaulis* and statistical optimization of enzyme activity. Enzyme Microb Technol 369:639–647

Balint B, Zottan B, Toth A, Rakhey G, Perei K, Kovacs KL (2005) Utilization of keratin containing biowastes to produce hydrogen. Appl Microbiol Biotechnol 69:404–410

Bernal C, Diaz I, Coello N (2006) Response surface methodology for the optimization of keratinase production in culture medium containing feathers produced by *Kocuria rosea*. Can J Microbiol 52:445–450

Bernhardt K, Schrempf H, Goebel W (1978) Bacteriocin and antibiotic resistance plasmids in *Bacillus cereus* and *Bacillus subtilis*. J Bacteriol 133:897–903

Böckle B, Galunsky B, Müller R (1995) Characterization of keratinolytic serine protease from *Streptomyces pactum* DSM 40530. Appl Environ Microbiol 61:3705–3710

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye bindings. Anal Biochem 72:248–254

Brandelli A, Riffel A (2005) Production of an extracellular keratinase from *Chyrseobacterium* sp. growing on raw feathers. Electron J Biotechnol 8(1):35–42

Brandelli A, Daroit DJ, Riffel A (2010) Biochemical features of microbial keratinases and their production and applications. Appl Microbiol Biotechnol 85(6):1735–1750

Bressollier P, Letourneau F, Urdaei M, Verneuil B (1999) Purification and characterization of keratinolytic serine proetaese from *Streptomyces albiodflavus*. Appl Environ Microbiol 65:2570–2576

Cai C-g, Lou B-g, Zheng X-d (2008) Keratinase production and keratinase degradation by a mutant strain of *Bacillus subtilis*. J Zhejiang Univ Sci B 9(1):60–67

Cao ZJ, Zhang Q, Wei DK, Chen L, Wang XQ, Zhou MH (2009) Characterization of a novel *Stenotrophomomnas* isolate with high keratinase activity and purification of the enzyme. J Ind Microbiol Biotechnol 36(2):181–188

Carolin G, Kerstin S, Stan JJB, Leon DK, John van der O, Willem Mde Vos, Garabed A (2005) Cloning and expression of islandicum, a novel thermostable subtilisin from *Fervidobacterium islandicum,* in *E.coli*.71. Appl Environ Microbiol 71(7):3951–3958

Cumming G, Fidler F, Vaux DL (2007) Error bars in experimental biology. J Cell Biol 177(1):7–11

De Azeredo LAI, De Lin MB, Coelho RR, Freire DMG (2006) Thermophilic protease production by *Streptomyces* sp. 594. In submerged and solid state fermentation using feather meal. J Appl Microbiol 100:641–647

Deivasigamani B, Alagappan KM (2008) Industrial application of keratinase and soluble proteins from feather keratins. J Environ Biol 29(6):933–936

Elibol M, Moreira AR (2005) Optimizing some factors affecting alkaline protease production by a marine bacterium *Teredinobacter turniae* under solid state fermentation. Process Biochem 409:1951–1956

El-Refai HA, AbdelNaby MA, Gaballa A, El-Araby MH, Abdel Fattah AF (2005) Improvement of the newly isolated *Bacillus pumilus* FH9 keratinolytic activity. Process Biochem 40:2325–2332

Evans KL, Crowder J, Miller ES (2000) Subtilisins of *Bacillus* spp. hydrolyze keratin and allow growth on feathers. Can J Microbiol 46:1004–1011

Fakhfakh N, Kanoun S, Manni L, Nasri M (2009) Production and biochemical and molecular characterization of a keratinolytic serine protease from chicken feather-degrading *Bacillus licheniformis* RPk. Can J Microbiol 55:427–436

Folch J, Lees M, Stanley G (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497

Friedrich NB, Antranikian G (1996) Keratin degradation by *Fervidobacterium pennavorans*, a novel thermophilic anaerobic species of the order thermotogales. Appl Environ Microbiol 62(8):2875–2882

Genckal H, Tari C (2006) Alkaline protease production from alkalophilic *Bacillus* sp. isolated from natural habitats. Enzyme Microb Technol 39:703–710

Gessesse A, Hatti-Kaul R, Berhanu A, Gashe BA, Mattiasson B (2003) Novel alkaline proteases from alkalophilic bacteria grown on chicken feather. Enzyme Microb Technol 329:519–524

Gokhade DV, Patil SG, Batawde KB (1991) Optimization of cellulose production by *Aspergillus niger* NCIM. Appl Biochem Biotechnol 30(2):99–109

Gousterova A, Braikova D, Geshov I, Christov P, Tishinov K, Tonkova TE, Haertle T, Nedkov P (2005) Degradation of keratin and collagen wastes by newly isolated *Thermoactinomycetes* or by alkaline hydrolysis. Lett Appl Microbiol 40:335–340

Gradisar H, Friedrich J (2000) Keratinase of *Doratomyces microspous*. Appl Microbiol Biotechnol 53:196–200

Gradisar H, Freiedrich J, Krizaj I, Jerala R (2005) Similarities and specificities of fungal keratinolytic proteases: comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some new known proteases. Appl Environ Microbiol 71(7):3420–3426

Grazziotin A, Pimentel FA, Sangali S, de Jong EV, Brandelli A (2007) Production of feather protein hydrolysate by keratinolytic bacterium *Vibrio* sp. Kr2. Bioresour Technol 98:3172–3175

Greasham RL (1983) Biotechnology. In: Rehm HJ, read G, Puhler A, Stagler P (eds) Bioprocessing. VCH publishers, New York, USA, pp 128–139

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

Hossain MS, Azad AK, Abu Sayem SM, Mostafa G, Hoq MM (2007) Production and partial characterization of feather-degrading keratinolytic serine protease from Bacillus licheniformis MZK-3. J Biol Sci 7(4):599–606

Hutchinson S, Evans D, Carion G, Kattenbett J (2007) An evaluation of the action of thioestrases on the surface of wool. Enzyme Microb Tehcnol 40:1794–1800

Ignatova Z, Gousterova G, Spassov G, Nedkov P (1999) Isolation and partial characterization of extracellular keratinase from a wool degrading thermophlic actinomycete strain *Thermoactinomycetes candidus*. Can J Microbiol 45:217–222

Ionata E, Cangangella F, Bionconi G, Benna Y, Sakamoto M, Capsaao A, Rossi M, La Cara F (2008) A novel keratinase from *Clostridium sporogenes* bv. Pennavorans bv. Nov., a thermotolerant organism isolated from solfataric muds. Microbiol Res 163:105–112

Khardenavis AA, Kapley A, Purohit HJ (2009) Processing of poultry feathers by alkaline keratin-hydrolyzing enzymes from *Serratia* sp. HPC1383. Waste Manag 29: 1409–1415

Kluskens LD, Voorhorst WG, Siezen RJ, Schwerdtfeger RM, Antranikian G, van der Oost J, de Vos WM (2002) Molecular characterization of fervidolysin, a subtilisin-like serine protease from the thermophilic bacterium *Fervidobacterium pennavorans*. Extermophiles 6:185–194

Laemmli UK (1970) Cleavage of the structural proteins during the assembly of the head of the bacteriophage T4. Nature 227:680–685

Lahl WJ, Braun SD (1994) Enzymatic production of protein hydrolysates for food use. Food Technol 48:68–71

Lin X, Kelemen DW, Miller ES, Shih JCH (1995) Nucleotide sequence and expression of *ker A*, the gene encoding for a keratinolytic protease of *Bacillus licheniformis* PWD-1. Appl Environ Microbiol 61:1469–1474

Lin HH, Yin LJ, Jiang ST (2009a) Cloning, expression and purification of *Pseudomonas aeruginosa* keratinase in *Escherichia coli* AD949 (DE3) pLys S expression system. J Agric Food Chem 57(9):3506–3511

Lin HH, Yin LJ, Jiang ST (2009b) Expression and purification of *Pseudomonas aeruginosa* keratinase in *Bacillus subtilis* DB104 expression system. J Agric Food Chem 57(17): 7779–7784

Mazotto AM, Cedrola SM, Lins U, Rosado AS, Silva KT, Chares JQ, Rabinovitch L, Zingali RB, Vermelho AB (2010) Keratinolytic activity of *Bacillus subtilis* AMR using human hair. Lett Appl Microbial 50(1):89–96

McNeil SJ, Sunderland MR, Zaitseva LI (2007) Closed-looped wool carpet recycling. Res Conserv Recycl 51: 220–224

Mitsuiki S, Sakai M, Moriyam Y, Goto M, Furukawa K (2002) Purification and some properties of a keratinolytic enzyme from an alkaophilic *Nocardiopsis* sp. TOA-1. Biosci Biotechnol Biochem 66(1):164–167

Mitsuiki S, Ichikawa M, Oka T, Sakai M, Moriyama Y, Sameshima Y, Goto M, Furukawa K (2004) Molecular characterization of a keratinolytic enzyme from an alkalophilc *Nocardiopsis* sp.TOA-1. Enzyme Microb Technol 34:482–489

Moran J, Summers JD, Slinger SJ (1996) Amino acid imbalance as the cause of inferior performance of feather meal and the implication of disulfide bonding in raw feathers as the reason for poor digestibility. Poult Sci 45:1257–1266

Morrissey JH (1981) Silver stain for proteins in polyacrylamide gels. A modified procedure with enhanced uniform sensitivity. Anal Biochem 117:307–310

Mortiz JS, Latshaw JD (2001) Indicators of nutritional value of hydrolyzed feather meal. Poult Sci 80:79–86

Nam GW, Lee DW, Lee HS, Lee NJ, Kim BC, Choe EA, Hwang JK, Suhartono MT, Pyum YR (2002) Native-feather degradation by *Fervidobacterium islandicum*

Aw-1, a newly isolated keratinase producing thermophilic anaerobe. Arch Microbiol 178(6):538–544

Odetallah NH, Wang JJ, Garlich JD, Shih JCH (2003) Keratinase in starter diets improves growth of broilers chicks. Poult Sci 82:1–7

Onifade AA, Al-Sane NA, Al-Mussalam AA, 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. Biores Technol 66:1–11

Oulad Haddar H, Zaghloul TI, Saeed HM (2009) Biodegradation of native feather keratin by *Bacillus subtilis* recombinant strains. Biodegradation 20:687–694

Ozeki H, Ito S, Wakamatsu K (2007) Chemical characterization of melanin in sheep wool. Pigment Cell Res 9(2):51–57

Papadopoulos MC, El Boushy AR, Roodbeen AE, Ketelaars EH (1986) Effects of processing time and moisture content on amino acid composition and nitrogen characteristics of feather meal. Anim Feed Sci Technol 14:279–290

Pearce KN, Karahalios D, Friedman M (1988) Ninhydrin assay for proteolysis in ripening cheese. J Food Sci 53(2): 432–435

Pelczar MJ, Chan EC (1977) Laboratory exercises in microbiology, 4th edn. McGraw-Hill Book Company, New York

Puri S, Beg QK, Gupta R (2002) Optimization of alkaline protease production from *Bacillus* sp. by response surface methodology. Curr Microbiol 44:286–290

Radha S, Gunasekaran P (2008) Sustained expression of keratinase gene under *PxylA* and *Pamyl* promoters in the recombinant *Bacillus megaterium* MS 941. Biores Technol 99:5528–5537

Ramnani P, Gupta R (2004) Optimization of medium composition for keratinase production from feather by *Bacillus licheniformis* RG1 using statistical methods involved response surface methodology. Biotechnol Appl Biochem 40:191–196

Ramnani P, Singh R, Gupta R (2005) Keratinolytic potentials of *Bacillus licheniformis* RG1: structural and biochemical mechanism of feather degradation. Can J Microbiol 51:191–196

Riessen S, Antranikian G (2001) Isolation of *Thermoanerobacter keratinophilus* sp. Nov, a novel thermophilic anaerobic bacterium with keratinolytic activity. Extremophiles 5:399–408

Riffel A, Brandelli A (2002) Isolation and characterization of a feather degrading bacterium from the poultry processing industry. J Ind Microbiol Biotechnol 29:255–258

Riffel A, Bandelli A, Bellato C de MB, Souza GHMFS, Eberlin MN, Marcos NE, Tavares FCA (2007) Purification and characterization of a keratinolytic metalloprotease from *Chryseobacterium* sp. kr6. J Biotechnol 128:693–703

Sangali S, Brandelli A (2000a) Isolation and characterization of a novel feather-degrading bacterial strain. Appl Biochem Biotechnol 87:17–24

Sangali S, Brandelli A (2000b) Feather hydrolysis of a *Vibrio* sp. strain kr2. J Appl Microbiol 89:735–743

Shih JCH (1993) Recent development in poultry waste digestion and feather utilization—a review. Poult Sci 72: 1617–1620

Sousa F, Jus S, Erbel A, Kokol V, Cavco-Paulo A, Gubitz GM (2007) A novel metalloprotease from *Bacillus cereus* for protein fibre processing. Enzyme Microb Technol 40:1772–1781

Speckman DH, Stein WH, Moore S (1958) Automatic ecording apparatus for use in the chromatography of amino acids. Anal Chem 30:1190–1205

Suntornsuk W, Tongjun J, Onnim P, Oyama H, Ratanakanokchai K, Kusamran T, Oda K (2005) Purification and characterization of keratinase from a thermotolerant feather-degrading bacterium. World J Microbial Biotechnol 21:1111–1117

Suzuki Y, Tsujimoto Y, Matsu H, Watanabe K (2006) Decomposition of extremely hard to degrade animal proteins by thermophilic bacteria. J Biosci Bioeng 102(2):73–81

Syed DG, Lee JC, Li WJ, Kim CJ, Agasar D (2009) Production, characterization and application of keratinase from *Streptomyces gulbargensis*. Biores Technol 100: 1868–1871

Vasileva-Tonkova E, Nustorova M, Gushterova A (2007) New protein hydrolysates from collagen wastes used as peptone as bacterial growth. Curr Microbiol 54:54–57

Wang X, Parson CM (1997) Effects of processing systems on protein quality of feather meal and hog hair meals. Poult Sci 76:491–496

Williams CM, Richter CS, MacKenzie JM, Shih JCH (1990) Isolation, identification, and characterization of a feather degrading bacterium. Appl Environ Microbiol 56: 1509–1515

Williams CM, Lee CG, Garlich JD, Shih JCH (1991) Evaluation of a bacterial feather fermentation product, feather hydrolysate, as a feed protein. Poult Sci 70:85–94

Xie F, Chao Y, yang X, Yang J, Zue Z, Luo Y, Qian S (2010) Purification and characterization of four keratinases produced by *Streptomyces* sp. strain 16. in native human foot skin medium. Biores Technol 101(1):344–350

Zaghloul TI (1998) Cloned *Bacillus subtilis* protease (*aprA*) gene showing high level of keratinolytic activity. Appl Biochem Bioetchnol 70–72:199–205

Zaghloul TI, Abdelaziz A, Mostafa MH (1994) High level of expression and stability of the cloned alkaline protease (*aprA*) gene in *Bacillus subtilis*. Enzyme Microb Technol 16:534–537

Zaghloul TI, Al-Bahra M, Al-Azmeh H (1998) Isolation, identification and keratinolytic activity of several feather degrading bacterial isolates. Appl Biochem Biotechnol 70–72:207–213

Zaghloul TI, Haroun MA, El-Gayar K, Abdelal A (2004) Degradation of chicken feather waste through a *Bacillus subtilis* recombinant strain. Polym Plast Trend Eng 43(6):1589–1599

Zheljazkov VD (2005) Assessment of wool waste and hair waste as soil amendment and nutrient source. J Environ Qual 34:2310–2317

Zheljazkov VD, Stratton GW, Pincock J, Butler S, Jeliazkova EA, Nedkov NK, Gerard PD (2009) Wool waste as organic nutrient source for container-grown plants. Waste Manag 29(7):2160–2164