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Abstract

Soil is the most complicated biomaterial on the planet .It is a natural source for microorganisms and is a natural laboratory to do experiments. Soil, which arises from the weathering of parent rock materials, is by definition capable of acting as a habitat for microorganisms. Microbially, the most active soil is the upper 16- to 17.2-cm–thick plow layer. As with any other material, the physical habitat is of prime importance in determining and regulating biological activity. However, until recently, the opaque nature of soil has meant that any interrogation of its interior architecture has been relatively rudimentary, restricted to simple qualitative expressions of the physical heterogeneity that fail to relate to any specific function. However, new techniques and insights into the biophysical and biochemical processes of this inner space are leading to the developments of theoretical frameworks and experimental approaches that will allow us to sustainably manage earth's most important resource. We introduce the concept that the soil-microbe system is self-organized and acts as a potential source for soil enzymes. It also suggests new priorities for research based on an integrative approach that combines biochemistry and biophysics. Microbial exploitation for the production of soil enzymes like keratinases, pectinases, xylanases, and lipases is highly attractive for applications in fruit, detergent, textile, tanning, meat paper industries, and waste water treatment.

Chapter 14
Actinomycetes: Sources for Soil Enzymes

V. Suneetha and Zaved Ahmed Khan

14.1 Introduction

The classification of the Prokaryotes is a complex issue. Their major subdivisions are the bacteria (Schizophyta), the blue green algae (Cyanophyta), and the Actinomycetales. The latter are sometimes called the "higher bacteria," organisms possessing properties intermediate between the fungi and the bacteria. The Actinomycetes are gram-positive organisms that tend to grow slowly as branching elements. They are prokaryotes, sporulated, powdery growth organisms, and show similarity to fungi in the formation of branched aerial mycelium, which profusely sporulate. But these Actinomycetes differ from fungi in the composition of cell wall; they do not have chitin and cellulose that are commonly found in the cell wall of fungi. The number of Actinomycetes increases in the presence of decomposing organic matter. Depending on the abundance in the soil, the common genera of Actinomycetes are *Streptomyces* (70%), *Nocardia*, and *Micromonospora* although *Actinomyces*, *Actinoplanes*, and *Streptosporangium* have been encountered. The term Actinomycetes is used to indicate organisms belonging to the Actinomycetales, a major subdivision of the Prokaryotae, the kingdom that comprises all organisms with a prokaryotic cell. They were long regarded as fungi, as is reflected in their name: aktino (gr) = ray, mykes (gr) = mushroom (=fungus).

Actinomycetes are divided into several families:

- The family Actinomycetaceae comprises two genera: *Actinomyces* and *Nocardia*. A few species are pathogenic. *A. israelii* causes actinomycosis in man and *A. bovis* in cattle.

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- The family *Mycobacteriaceae* has a single genus, Mycobacterium, which contains several pathogenic species causing diseases such as leprosy and tuberculosis.
- The family *Streptomyceteae* comprises several organisms found in the soil. They are rarely pathogenic. In contrast, several species of the genus *Streptomyces* produce antibiotics. Erythromycin is produced by *Streptomyces erythreus*.

The characteristic earthy smell of compost is caused by Actinomycetes. Actinomycetes are a form of fungi-like bacteria that form long, thread-like branched filaments that look like gray spider web stretching through the compost. Different species of Actinomycetes predominate during each phase of the composting process (the mesophillic, thermophillic, and maturation phases) but are most easily seen during the early stages of the composting process in the outer 10–15 cm of the pile. Actinomycetes are the primary decomposers of tough plant materials like bark, newspaper, and woody stems. They are especially effective at attacking tough, raw plant tissues (cellulose, chitin, and lignin), softening them up for their less enterprising relatives. The morphology of an Actinomycete growing on agar can provide useful clues to its identity but viewing isolated colonies can give little worthwhile information. Examine the organism streaked in a cross-hatched pattern on the surface of the agar, first with a stereomicroscope and then with a transmitted light microscope with a 40× long-working distance objective to avoid water condensation on the front lens. The appearance of hyphae within the agar and the nature of the spores on the aerial hyphae are important but can only be observed when growth is thin and the medium promotes differentiation.

Sometimes the name "Actinomycetes" is used restrictively for members of the genus Actinomyces only, instead of all members of the Actinomycetales. The industrial enzyme sector in India is growing fast for meeting the need of pharmaceutical, food processing, leather, detergents, pulp, paper, and textile industries. In view of vigorously growing demand, the enzyme industry and sector is poised for high growth rates, necessitating increase in the production or import. The product range and services are growing rapidly as the use of enzymes is gaining widespread acceptance. In addition to the Indian market, export opportunities are also there for the manufacturers. Studies on specific enzyme producing Actinomycetes other than fungi are rather fragmentary and limited in our country. In view of the importance of soil enzymes producing Actinomycetes and scarce information available in our country, it was interesting to carry out studies in this direction (Suneetha 2004). AU1

The use of enzyme-mediated processes can be traced back to ancient civilizations. Today nearly 4,000 enzymes are known, of which many are commercially produced. The majority of the industrial enzymes are of microbial origin. Until the 1960s, the total sales of enzymes were only a few million dollars annually, but the market has since grown spectacularly. Because of improved understanding of production biochemistry, the fermentation processes, and recovery methods, an increasing number of enzymes can be produced affordably. Also, advances in the methods of using enzymes have greatly expanded the demand. Furthermore, because of the many different transformations that enzymes can catalyze, the

number of enzymes used commercially continues to multiply. Enzyme-mediated reactions are attractive alternatives to tedious and expensive chemical methods, and they find great use in a large number of fields such as food, dairy, pharmaceutical, detergent, textile, and cosmetic industries. In the above scenario, enzymes such as proteases and amylases have dominated the world market owing to their hydrolytic reactions for proteins and carbohydrates. ~~However,~~ with the realization of the biocatalytic potential of microbial lipases in both aqueous and nonaqueous media in the last one and a half decades, industrial fronts have shifted toward utilizing these enzymes for a variety of reactions of immense importance. AU2

For commercialization of any valuable product, emphasis is laid on the use of relatively low-cost raw materials that are converted into commercially important and useful products or services. This process requires coordinated coupling of several units and operations. The efficiency depends on adequate upstream operations and the process of recovery of the product, which includes a series of careful and meticulous steps collectively referred to as downstream processing. Important organisms and their natural products come into light mainly when new screening systems are utilized or when samples from different sources are examined. Further the isolated native organisms are improved upon by conventional techniques or by modern techniques involving rDNA technology. Due to the availability of powerful expression systems in various microbes, the large-scale commercial production of new and useful enzymes is becoming increasingly attractive. The focus of enzyme technology is thus the microorganism, the Actinomycetes itself, that has the capacity to produce the soil enzymes. Though there are many advantages and applications of microbial enzymes, these are still not being exploited as biocatalysts to their optimum capacity for various reasons.

14.2 Keratinases

The microbes like *Actinomycetes* hold prominent position in microbial world because of their diversity and proven ability to produce soil enzymes. They are also potential sources of proteases with typical substrate specificities such as Keratinases that attack normally unreactive protein called keratin. Microbial Keratinases reported till date are produced from organisms like *Streptomycetes*, *Bacillus* species, and fungal members. The studies on isolation, characterization, and strain improvement of keratinase producing organisms have both economic and ecological value but have not been fully exploited yet. The limiting aspect in the wide scale usage of Keratinases is mainly an efficient and cost-effective method for fermentative production of keratinase. Though in recent years patents are being obtained internationally for strains and process of fermentation of Keratinases, there is very little effort in this direction (Table 14.1).

To develop indigenous product technology in India, as great economical and ecological value is associated with the production of Keratinases, any effort to develop the process will be of great potential.

t1.1 **Table 14.1** Some of the reported keratinase producing Actinomycetes

t1.2 Strains	References
t1.3 Actinomycetes: *Doratomyces microsporus*	Vignardet et al. (1999), Gradisar et al. (2000)
Streptomyces spp.	Noval and Nickerson (1959), Nickerson et al. (1963),
S. fradiae, S raminofaciens	Tatsushi et al. (1967), Elmayergi and Smith (1971),
S. pactum, *Streptomyces* spp. A_{11}, *1349, 1382, BA7, etc.*	Young and Smith (1975), Kunert and Stransky (1988), Kunert (1989), Mukhopadhyay and Chandra (1990), Galas and Kaluzewska (1992), Bockle et al. (1995), Letourneau et al. (1998), Szabo et al. (2000), Ichida et al. (2001), Ivanko et al. (2002), Korkmaz et al. (2003),
t1.4	Suneetha (2003)
t1.5 *Thermoactinomyces* spp. *Thermoactinomyces candidus*	Ignatova et al. (1999)

AU3 AU4 AU5 AU6 AU7 AU8

111 Tirumala hills is a famous pilgrim center in Andhra Pradesh. It is located in
112 Eastern Ghats on Seshachalam hill range, with North latitude of 13–14′ and East
113 longitude of 70–21′. It is 2,820 feet above the sea level and 100 square miles in
114 extent. Tirumala is visited by thousands of pilgrims every day from all over India
115 as well as the world, throughout year to worship Lord Venkateswara (Hindu
116 mythology). The place has relatively high deposits of keratin as most of the
117 pilgrims coming to Tirumala consider head tonsuring as the most sacred offering
118 to God. Further the prevailing climatic conditions also facilitate the growth of
119 thermo-tolerant organisms. Thus, this place was presumed to provide good enrich-
120 ment for potential keratinophilic and keratinolytic organisms, and soil samples
121 collected from various locations in Tirumala hills and Tirupati were analyzed to
122 isolate potential keratinolytic organisms. It is expected that the present emphasis
123 on keratinase producing organisms may lead to a step forward in the process of
124 development of indigenous kerazyme technology (Suneetha 2004).

125 14.3 Pectinases

126 The structural unit of pectin has a complex structure. Preparations consist of substruc-
127 tural entities that depend on their source and extraction methodology. Commercial
128 extraction causes extensive degradation of the neutral sugar-containing side chains.
129 The majority of the structure consists of homopolymeric partially methylated poly-α-
130 (1 → 4)-D-galacturonic acid residues (Fig.14.1), but there are substantial "hairy" non-
131 gelling areas (Fig.14.2) of alternating α-(1 → 2)-L-rhamnosyl-α-(1 → 4)-D-galactur-
132 onosyl sections containing branch points with mostly neutral side chains (1–20
133 residues) of mainly L-arabinose and D-galactose (rhamnogalacturonan I). Pectins
134 may also contain rhamnogalacturonan II side chains containing other residues such
135 as D-xylose, L-fucose, D-electronic acid, D-apipose, 3-deoxy-D-manno-2-octulosonic
136 acid (Kdo), and 3-deoxy-D-lyxo-2-heptulosonic acid (Dha) attached to poly-α-

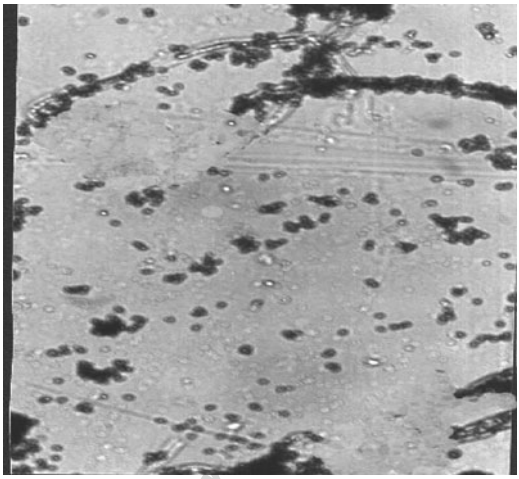

Fig 14.1 Micromorphology of Actinomycetes

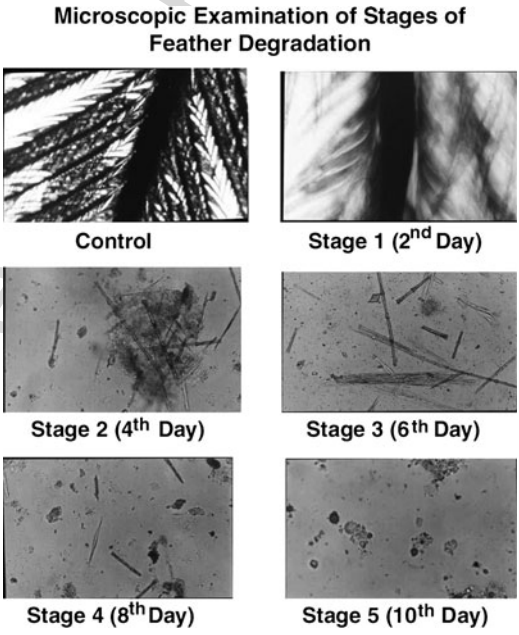

Fig 14.2 Keratin (feather) degradation

(1 → 4)-D-galacturonic acid regions. Enzymes are bio-catalysts. They speed up the rate of chemical reactions taking place in living cells. The reactants of enzyme-catalyzed reactions are termed as substrates and each enzyme is specific in character, acting on a particular substrate(s) to produce particular product or products. Enzyme usage in the fruit industry is a recent innovation. Application of biotechnology to

industrial operations is no longer an academic or potentially useful alternative proposition for the future. Enzymes have become big business, with a wide range of industries using commercial enzymes, in addition to the feed industry. The world annual sales of industrial enzymes were recently valued at $1 billion. Three-quarters of the market is for enzymes involved in the hydrolysis of natural polymers. Food-processing enzymes including pectinases account for about 45% of enzyme usage (Fig 14.3). AU9

Pectinase was found to be the most efficient commercialized enzyme in degrading the fruit waste. Pectinolytic enzymes or pectinases is a group of enzymes that hydrolyze the pectic substances, present mostly in plants. Enzymes are usually offered as "cocktails" of several activities rather than a single enzymatic activity. However, in many cases, the different enzyme activities can still act on the same composition, as the composition can have a complex chemical structure having various types of chemical bounds, requiring different enzyme activities for break down. An example of this is the enzyme cocktails offered as "pectinase." Such pectinase composition often contains one or more of the following activities: polygalacturonase, pectin lysase, pectin methyl esterase. Pectinase preparations are often used in fruit juice processing. It is preferred in the present invention that the enzyme preparation used contains at least one of these three activities mentioned, preferably two, more preferably all three.

Pectinases are a group of enzymes that catalyze the degradation of pectic polymers present in plants. Pectinolytic enzymes are widely distributed in higher plants and microorganisms. Based on their catalytic action, pectin-degrading enzymes have been classified into two major groups: the first group is represented by pectin esterase (PE) and the second by polygalacturonase (PG) and pectin lyase (PL) (Table 14.2).

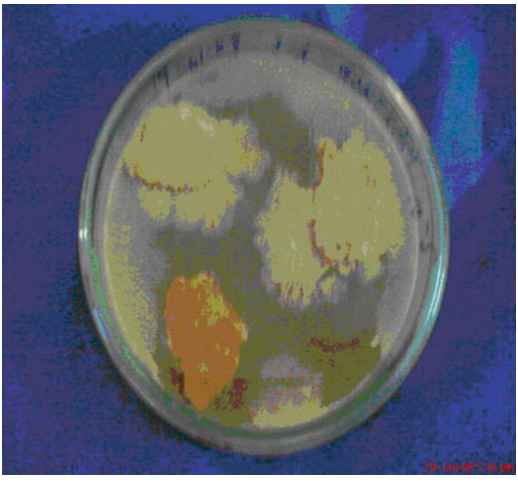

Fig 14.3 Pectin degradation AU17

Table 14.2 Some of the reported petinase producing microorganism t2.1

Source	Organism	References	
Actinomycetes	*Streptomyces* spp.	Suneetha et al. (2003)	t2.3
	Streptomyces spp.	Suneetha et al. (2003)	t2.4
	Streptomyces lydicus	Jacob and Prema (2006)	t2.5
	Streptomyces sp. *RCK-SC*	Chander Kuhad et al. (2004)	t2.6
	Streptomyces sp. *QG-11-3*	Qasim Khalil et al. (2000)	t2.7
	Thermomonospora flisca	Stutzenberger (1987)	t2.8
	Streptomyces viridochromogenes	Agate et al. (1962)	t2.9

14.4 Xylanases 168

Xylans are hydrolyzed mainly by ß-1,4-endoxylanases (1,4- ß-D-xylan xylanohydrolases EC 3.2.1.8.) and xylosidases (1,4-ß-D-xylan xylohydrolases EC 3.2.1.37). The interests in xylan-degrading enzyme and its application in the pulp and paper industries have advanced significantly over the past few years (Agate et al. 1962). Mainly, cellulose-free xylanase is of great importance in the paper industry. The cost of carbon source plays another major role in the economics of xylanase production. Hence, an approach to reduce the cost of xylanase production is by the use of lignocellulosic materials as substrates such as wheat bran, rice straw, corn cob, agricultural wastes, sugar cane baggase rather than opting for the expensive pure xylans. For the development of suitable xylanase as a pre-bleaching agent, the stability of enzyme at higher optimum pH and temperature is desirable (Bockle et al. 1995). Alkaline xylanase also finds a number of applications. For example, because of high solubility of xylan at alkaline pH, alkaline xylanases may have good potential in the conversion of lignocellulosic wastes to fermentable sugars.

Many xylanases producing alkaliphilic microbial strains have been reported from different laboratories. However, the xylanase from many of the alkaliphilic strains have their optimum pH around neutrality. Naturally occurring habitats for the isolation of alkaliphilic microbial strain are scattered in different parts of the world (Bockle and Muller 1997). Most of the reported literature on xylanase has concentrated on the characterization of the enzyme and its application as a pre-bleaching agent (Nickerson 1947). Relatively less attention was paid to the optimization of xylanase production. In the present study, an attempt was made to describe the optimization studies related to xylanase production from Actinomycetes, which is isolated from soil near paper industry (Fig. 14.4, Table 14.3).

14.5 Lipases 193

Although bacteria have the highest total population in soil followed by Actinomycetes and fungi, the frequencies of lipase producing bacteria, Actinomycetes, and fungi were 8.5, 55.9, and 23.3%, respectively. Although little research has been published

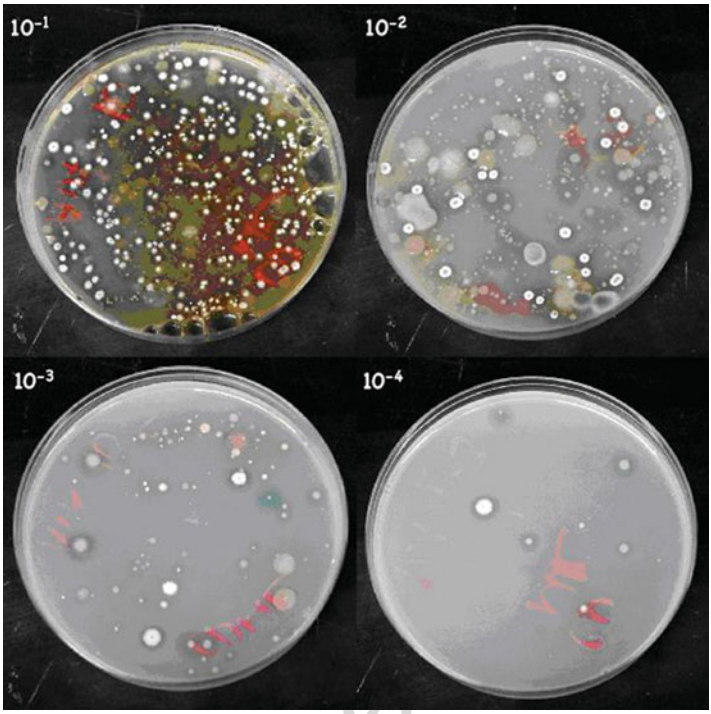

Fig 14.4 Xylan degradation by Actinomycetes

t3.1 **Table 14.3** Some reported Actinomycetes producing xylanase

t3.2 Actinomycetes	References	
t3.3 *Actinomyces* from soil	Suneetha (2003), Suneetha et al. (2006)	AU10
t3.4 *Streptomyces olivaceoviridis* E-86	Ding (2004)	AU11
t3.5 *Streptomyces* sp.	Rawashdeh (2005), *Streptomyces* sp. (strain Ib 24D)	AU12

197 on lipolytic activity in Streptomyces considering their widespread use within antibi-
198 otic production, lipase activity has been detected. Lipase production in a wide range of
199 *Streptomycetes* has been investigated. However, lipolytic activity was found in only a
200 few strains tested (15). Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3)
201 are ubiquitous enzymes of considerable physiological significance and industrial
202 potential. Lipases catalyze the hydrolysis of triacylglycerols to glycerol and free
203 fatty acids. In contrast to esterases, lipases are activated only when adsorbed to an
204 oil–water interface and do not hydrolyze dissolved substrates in the bulk fluid. A true
205 lipase will split emulsified esters of glycerin and long-chain fatty acids such as triolein
206 and tripalmitin. Lipases are serine hydrolases and display little activity in aqueous
207 solutions containing soluble substrates. In eukaryotes, lipases are involved in various
208 stages of lipid metabolism including fat digestion, absorption, reconstitution, and

lipoprotein metabolism. In plants, lipases are found in energy-reserve tissues. How lipases and lipids interact at the interface is still not entirely clear and is a subject of intense investigation. The natural substrates of lipases are triacylglycerols, having very low solubility in water. Under natural conditions, they catalyze the hydrolysis of ester bonds at the interface between an insoluble substrate phase and the aqueous phase in which the enzyme is dissolved. Under certain experimental conditions, such as in the absence of water, they are capable of reversing the reaction. The reverse reaction leads to esterification and formation of glycerides from fatty acids and glycerol. The occurrence of the lipase reaction at an interface between the substrate and the aqueous phase causes difficulties in the assay and kinetic analysis of the reaction. The usual industrial lipases are special classes of esterase enzymes that act on fats and oils, and hydrolyze them initially into the substituted glycerides and fatty acids, and finally on total hydrolysis into glycerol and fatty acids (Table 14.4).

The focus of soil is thus the habitat for microorganisms, especially Actinomycetes, the wonderful organisms that have the capacity to produce the enzymes. Though there are many advantages and applications of microbial enzymes, these have still not being exploited as biocatalysts to their optimum capacity (Fig. 14.5).

14.6 Conclusion

Table 14.4 Reported lipase producing Actinomycetes

Source	Genus	Species	References
Actinomycetes	Streptomyces	*Streptomyces fradiae NCIB* 8233	Sztajer et al. (1988)
		Streptomyces sp. *PCB27*	Sztajer et al. (1988)
		Streptomyces sp. *CCM 33*	Sztajer et al. (1988)
		S. Coelicolor	Hou (1994)

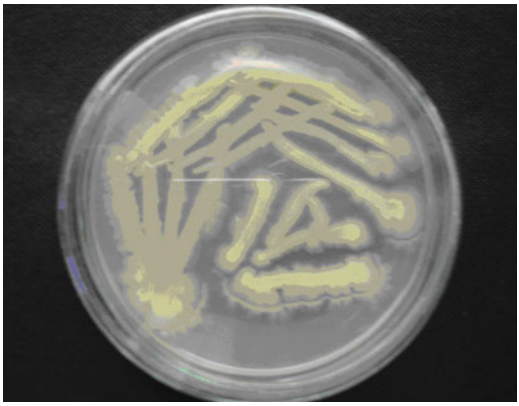

Fig 14.5 Lipid degradation by Actinomycetes

The use of Actinomycetes for industrial purposes has a long history, which is long before the realization of activities of other microorganisms. We have discussed the production of enzymes like keratinases, pectinases, xylanases, and lipases for commercial applications and screening methods. With the improvement of screening methods, we will be able to produce other commercial products from Actinomycetes in near future. It also suggests new priorities for research based on an integrative approach that combines biochemistry and biophysics, and microbial exploitation for the production of soil enzymes is highly attractive for applications in fruit, detergent, textile, tanning, meat paper industries, waste water treatment, and other bioremediation technology.

References

Agate AD, Bilimoria MH, Bhat JV (1962) Pectin transeliminase activity in *Streptomyces viridochromogenes*. Curr Sci 31:462–463

Bockle B, Muller R (1997) Reduction of disulfide bonds by *Streptomyces pactum* during growth on chicken feathers. Appl Environ Microbiol 63:790–792

Bockle B, Galunsky B, Muller R (1995) Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. Appl Environ Microbiol 61:3705–3710

Burtt EH Jr, Ichida JM (1999) Occurrence of feather-degrading *Bacilli* in the plumage of birds. Auk 116:364–372

Chander Kuhad R, Kapoor M, Rustagi R (2004) Enhanced production of an alkaline pectinase from *Streptomyces* sp RCK-SC by whole-cell immobilization and solid-state cultivation. World J Microbiol Biotechnol 20:257–263

Galas E, Kaluzewska M (1992) Proteinases of *Streptomyces fradiae*: catalytic and some physicochemical properties of keratinolytic proteinase. Acta Microbiologia Pol 41:169–177

Ivanko OV, Varbanet's LD, Valahurova OV, Nahorna SS, Redchyts TI, Zhdanova NM (2002) Screening of collagenase and keratinase producers. Mikrobiol Z 64:31–36

Jacob N, Prema P (2006) Influence of mode of fermentation on production of polygalacturonase by a novel strain of *Streptomyces lydicus*. Food Technol Biotechnol 44:263–267

Khalil Beg Q, Bhushan B, Kapoor M, Hoondal GS (2000) Effect of amino acids on production of xylanase and pectinase from *Streptomyces* sp. QG-11-3. World J Microbiol Biotechnol 16:211–213

Korkmaz H, Unaldi MN, Asian B, Coral G, Arikan B, Dincer S, Colak O (2003) Keratinolytic activity of *Streptomyces strain* BA-7, a new isolate from Turkey. Ann Microbiol 53:85–93

Kunert J, Stransky Z (1988) Thiosulfate production from cystine by the keratinolytic prokaryote *Streptomyces fradiae*. Arch Microbiol 15:600–601

Letourneau F, Soussote V, Bressollier P, Branland P, Verneuil B (1998) Keratinolytic activity of *Streptomyces sp.S.KL-02:* A new isolated strain. Lett Appl Microbiol 26:77–80

Mukhopadhyay RP, Chandra AL (1990) Keratinase of a *Streptomycete*. Indian J Exp Biol 28:575–577

Mukhopadhyay RP, Chandra AL (1993) Protease of a keratinolytic *Streptomycete* to unhair goat skin. Indian J Exp Biol 31:557–558

Nickerson WJ (1947) Biology of pathogenic fungi. Mycologia. 14. Waltham, MA, pp 10–28

Nickerson WJ, Durand SC (1963) Keratinase-II. Properties of the crystalline enzyme. Biochemical and Biophysical Acta 77:87–99

Nickerson WJ, Noval JJ, Robison RS (1963) Properties of the enzyme conjugate elaborated by *Streptomyces fradiae*. Biochem Biophys Acta 77:73–86

Pace GW, Smith IH (1981) Recovery of microbial polysaccharides. In: Second European Congress of Biotechnology, Eastbourne. Society of Chemical Industry, London, pp 36–37

Stutzenberger FJ (1987) Inducible thermoalkalophilic polygalacturonate lyase from *Thermornonospora fusca*. J Bacteriol 169:2774–2780

Suneetha V, Lakshmi VV (2004) Fermentative production of kerazyme with isolates from Tirumala hills. In: Deshmukh AM (ed) Biotechnology in agriculture industry and environment. Microbiology Society, Karad, pp 198–203

Szabo I, Benedek A, Szabo IM, Barabas GY (2000) Feather degradation with a thermotolerant *Streptomyces graminofaciens* strain. World J Microbiol Biotechnol 16:253–255

Tatsushi M, Hiroshige T, Tsuzuki T (1967) Multiple proteolytic enzymes of *Streptomyces fraidae:* production, isolation and preliminary characterization. Biochem Biophys Acta 139:382–397

Vignardet C, Guillaume YC, Friedrich J, Millet J (1999) A first order experimental design to assess soluble proteins released by a new keratinase from *Doratomyces microsporus* on human substrates. Int J Pharm (Amsterdam) 191:95–102

Vignardet C, Guillaume YC, Michel L, Friedrich J, Millet J (2001) Comparison of two hard keratinous substrates submitted to the action of a keratinase using an experimental design. Int J Pharm 224:115–122

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