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Screening of leather deteriorating Microflora

Microbiology

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ABSTRACT

Leather is deteriorable because of its main constituent i.e. proteinous material. The main protein in skin is collagen, which also is a main component in bone, cartilage and teeth. Microorganisms with these capacities are proteolytic bacteria and fungi that possess specific enzymes like collagenases and keratinase capable of cleaving the proteins of the parchment by hydrolysis. In present research, ninety one bacteria and seventy three fungi were isolated from 50 deteriorated leather samples.27 bacterial isolates and 11 fungi showed collagenase, keratinase, gelatinase and caseinase production. The most efficient four bacterial leather deteriorates were identified and was found to be belonging to genus Bacillus. 16s r RNA sequencing were done and the sequence were deposited to GenBank for accession number. Most efficient leather deteriorating fungi belongs to Aspergillus Sp., Hence this isolate can be proficiently used to treat leather industry waste and recycling of these organic materials.

Introduction:

Leather making is an important socio-economic activity for several countries throughout the world and used everywhere in daily life (Nadia Zara Jaouadi et al., 2013). The leather is an organic material that contains many nutrients for microorganisms. It is made up of 96.5% fibrous proteins and 3.5% are albumin and globulin. The fibrous protein present in leather are collagen (98%), elastin (1%) and keratin (1%). Leather Tanning is a general term use for numerous processing steps involve in converting animal hide and skins in to final leather. (Tissier and Chensais, 2000).Though in the process of making leather it is sought to remove as much of any other protein than collagen as possible. Leather is a stabilized collagen product Therefore, leather is composed almost solely of collagen. Collagen is a major fibrous element of skin, bones, tendons, cartilage, blood vessels, and teeth found in all multicellular organisms. Collagenases are endopeptidase that digests native collagen in the triple helix region. Unlike animal collagenases that split collagen in its native triple-helical conformation, bacterial collagenase is unique because it can degrade both water-insoluble native collagens and water-soluble denatured ones. It can attack almost all collagen types, and is able to make multiple cleavages within triple helical regions (Wanderley et al., 2015).

The waste generated from a leather industry includes fleshing, chrome shaving, chrome splits, buffing dust skin trimmings and hair. Out of 1000 kg of raw hide used for leather making only 150 kg is converted into processed leather whereas remaining 850 kg remain as solid waste. The leather industry has commonly been associated with high pollution. Microorganisms can be used in order to degrade natural fibers like fur, leather wastes because they synthesized extracellular enzymes that can break chemical bonds in these materials. Some proteolytic enzymes, like collagenases, registered an increasing use for industrial applications in fur and leather industry because they are nontoxic and eco-friendly (Khandelwal S.R. et al., 2014).The aim of the present research work was to isolate and identify efficient leather deteriorating bacteria and fungi from deterioted leather samples.

MATERIALS AND METHODS:

Materials:

Collagen peptide type I (TC343-10G), Nutrient broth (M002-100G).All other reagents used were of analytical grade.

Isolation, screening and identification of leather deteriorates:

Leather deteriorates (bacteria and Fungi) were isolated from fifty deterioted leathers samples collected from leather factory (Kedar leather product, Aurangabad) and various types of naturally deteriorated finished leather's articles proceed further for isolation, screening and identification of leather deteriorates.

Primary screening:

The leather samples were incubated in 5 g/L peptone solution at 37°C for 24 h. Initial bacterial and fungal isolation was carried out on nutrient agar and potato dextrose agar respectively. The plates were incubated at 37°C for 48 h. and 30°C for 5-7 days respectively. Bacterial as well as fungal colonies with different morphology, size and colour were picked and purified proceed further for secondary screening. (Mozotto et al., 2011 and Awasthi et al., 2011).

Secondary screening

Amongst bacterial and fungal isolates, a well isolated colony was used for screening for their ability to hydrolyzed collagen, on collagen agar plates. The plates were incubated at 37°C for 48 h. and 30°C for 48-72h. After incubation to each individual colony, a drop of mercuric chloride precipitation reagent was added. Bacteria and fungi showing collagenolytic activity were screened with larger transparent circle around the bacterial and fungal colony (Lili Liu, 2010). At the same time caseinase, keratinase and gelatinase activity was also detected on in feather meal (1%), gelatin agar (1%) plates and casein on milk agar plate (10%) incubated at 37°Cfor 24h.(Mozotto, 2011).

Among these isolated strains, the most efficient bacterial isolate were identified by morphological, biochemical characteristics as described in the Bergey's manual of systematic bacteriology and by 16s rDNA sequencing and the sequence were deposited to GenBank with accession number. Fungal isolates were identified by morphology, pigment produced and wet mount and confirmed by recognized botany lab, Aurangabad.

Collagenase production by isolates:

For Bacteria and fungi:

For collagenase production, triplicate set of flasks were inoculated by selected bacterial strain (2% inoculum) and 6mm disc of 5-7 days old fungi culture in a fermentation medium of composition glucose 20g/L, collagen10g/L, $CaCl_2$ 0.05g/L, $NaH_2PO_4$0.5g/L, $K_2HPO_4$0.5g/L. The flasks were incubated at 37°C for 48h and 30°C for 72h in an orbital shaker at 180 rpm respectively. Afterward fermentation medium was centrifuged at 4°C and 4000rpm for 10min, the supernatants were collected as a source of crude collagenase (Sayyed et al., 2012) and subjected to assay.

Collagenase assay:

Collagenolytic activity was measured according to the Hamdy (2008),

method. 5mg collagen type I used as substrate, absorbance was measured at 575nm.One unit (U) of enzyme activity equals to one micromole of L-leucine equivalents released from collagen under specified conditions.

Result and Discussion:

Isolation, screening and identification of leather deteriorates:

Primary screening

In primary screening 91 bacteria and 73 fungi were isolated on nutrient and potato dextrose agar, respectively from 50 deteriorated leather samples after incubation of 24h and 72h at 37°C and 28°C± 2 respectively. Well isolated colonies were used for screening of their ability to hydrolyze keratin, collagen, (leather major protein) gelatin and casein on feather meal, collagen, and gelatin and milk agar plates respectively.

Out of 91isolates obtained in primary screening, 27 bacterial isolates and out of 73 fungal isolates 11 were found to produced keratinase, collagenase, gelatinase and caseinase. These isolates were subjected for secondary screening in terms of quantitative estimation of the amount of collagenase under study. Preliminary identification of the selected bacterial isolates was carried out by morphological and biochemical characteristics as per Bergey's Mannual of systemic Bacteriology, 2nd Edition as shown in Table 1 and fungal were identified by wet mount and confirmed by recognized botany lab, Aurangabad.

Table 1. Morphological and Biochemical Characteristics of Bacterial leather isolates:

Bacteria	Size	Shape	Color	Margin	Elevation	Consistency	Opacity	Gram Characte	Motility
1	4mm	circular	white	irregular	flat	Moist	Opaque	+ve	Motile
2	2mm	circular	Off white	irregular	flat	Butrous	Opaque	+ve	Motile
3	2mm	circular	orange	entire	convex	Butrous	Opaque	-ve	Nonmotile
4	3mm	circular	Off white	Eros(serrate	flat	Butrous	Translucent	+ve	Motile
5	2mm	circular	Off white	entire	flat	Butrous	Opaque	+	Motile
6	4mm	circular	Off white	entire	raised	Moist	Opaque	-	Motile
7	1mm	circular	yellowish	entire	flat	Butrous	Translucent	+	Motile
8	3mm	irregular	white	irregular	flat	Butrous	Opaque	+	Motile
9	4mm	circular	Off white	entire	flat	Mucoid	Opaque	+	Motile
10	1mm	circular	Golden Y	entire	convex	Butrous	Opaque	-	Nonmotile
11	1mm	circular	yellow	entire	convex	Butrous	Opaque	-	Nonmotile
12	3mm	circular	orange	entire	flat	Butrous	Opaque	+	Nonmotile
13	1.5m	circular	orange	entire	raised	Butrous	Opaque	+	Motile
14	1mm	circular	Lt yellow	entire	flat	Butrous	Opaque	-	Nonmotile
15	2mm	circular	Light peach	serrate	flat	Mucoid	Translucent	+	Nonmotile
16	1mm	circular	Lt Y	entire	flat	Butrous	Opaque	-	Sluggishly motile
17	1mm	circular	Golden Y	entire	raised	Butrous	Opaque	-	Sluggishly motile
18	2mm	circular	Off W	entire	flat	Butrous	Opaque	-	Motile
19	1mm	circular	Ivorywhite	entire	convex	Butrous	Opaque	-	Sluggishly motile
20	2mm	circular	Lt peach	entire	flat	Mucoid	Opaque	+	Nonmotile
21	2mm	circular	G yellow	entire	Convex	Butrous		-	Motile
22	3mm	serrate	Off white watery	entire	pulvinate	Mucoid	Translucent	+	Motile
23	3mm	circular	white	entire	flat	Butrous	Opaque	+	Motile
24	2mm	circular	Off white	entire	Umbonat	Butrous	Opaque	+	Motile
25	1mm	circular	Lemon Yellow	entire	convex	Mucoid	Opaque	-	Sluggishly motile
26	1mm	circular	white	entire	flat	Butrous	Opaque	+	Motile
27	2mm	circular	Off white	entire	Raised-convex	Mucoid	Opaque	+	Motile

Secondary screening:

Most efficient collagenase producing bacteria and fungi were screened by quantitative assay. Four proficient bacteria were proceed for identification by16srDNA analysis and nucleotide sequences were submitted to GenBank with an accession No. as *Bacillus megaterium KM369985 strain SAK, Bacillus cereus KP015746 (Lr3/2), Bacillus pumilus KP015747 (H4.9/8), Bacillus amyloliquefaciens KP015745 (strain JS518)* and *Aspergillus niger*, *Aspergillus nidulans, Aspergillus flavus, Penicllium sp., Paecilomyces varioti* were found to be most efficient leather deteriorates. Sanchez-Navarro et al. (2013), isolated bacteria such as *Staphylococcus sp.* and *Brevibacterium sp*. *Pseudomonas sp.* and *Bacillus sp*. from some deteriorated footwear samples in their research work. *B. licheniformis PWD-1*, *Brevibacillus brevis* isolated by Jaouadi et al., (2013) from different parts of the tanned deteriorated leathers. Rathore et al., (2013) reported various finished leather deterioration by large group of fungi such as *Aspergillus flavus, A. parasiticus*, *A. oryzae, A. chevalieri, A. nidulans, A. fumigatus, A. conicus, A. humicola, A. terreus, Penicillium stipitatu, P. camemberti, Alternaria geophila, Fusarium neoceras, Rhizopus nigricans*. *R. oryzae* isolated in their study. *Bacillus* spp. and *Aspergillus Sp.* were the most prevalent and found in nearly all deteriorated leather samples.

Collagenase assay:

All selected bacterial and fungal isolates showed collagenase production in a range of 600-949 U/ml after three days and five days respectively. Maximum collagenase production 600U/ml, *KP015745Bacillus amyloliquefaciens* 949±15 by using collagen peptide type I as a substrate after 3 days of incubation. All *Aspergillus Sp.* produced collagenase 720±31-778±14U/ml only after 5 days of incubation, *Penicillium Sp.* showed 781±9.54U/ml, *P. varioti* showed 730±30 after 120h. 379.80 Us /ml collagenase activity by *Penicillium Sp.* after 126 h of incubation by Wanderley et al., (2015). *Bacillus amyloliquefaciens* (Israel-Roming et al.,2014) showed collagenase activity 23.7U/ml after 10 days using sheep fur as a substrate. *R. saloni* showed collagenase activity 147.77U/ml after 7 days of incubation period reported by Hamdy et al., (2008) in their study. Collagenase production by bacterial and fungal leather isolates were found to be most efficient as compared to the cited reports.

Conclusion: *Bacillus* spp. and *Aspergillus Sp.* were the most prevalent and found in nearly all deteriorated leather samples. All selected bacterial and fungal isolates showed collagenase production in a range of 600-949 U/ml after three days and five days respectively. Hence this isolate can be proficiently used to treat leather industry waste and recycling of these organic materials.

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