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ISOLATION AND IDENTIFICATION OF EFFECTIVE MICROBIAL STRAIN FOR ACCELERATED BIODEGRADATION OF LEATHER INDUSTRY WASTE

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Abstract:

Ecological biotechnology reduces the negative impact of industrial activities to the environment, by different bioremediation processes. Such biotechnological measures are non-polluting and environmentally safe. The use of specific microorganisms for the degradation of leather residues is an ecological alternative and an ecological tool for bioremediation. The aim of this study was to isolate some microbial strain capable to accelerate the degradation of leather waste, reducing the pollution that causes environmental damage. Three isolated bacterial strains, DA7, DA10 and DA13, were selected for their ability to produce extracellular proteases. These strains were identified with the Biolog-Microbial Identification System as Brevundimonas diminuta, Bacillus cereus/thuringiensis and Bacillus cereus, respectively. Studies revealed that the best enzymatic activity is higher after 120 hours cultivation, at 35°C temperature, 135 rpm and pH 7.0 units.

Key words*: bacterial, isolation, identification, protease, leather, degradation, waste, pollution.*

INTRODUCTION

Accelerated and disorganized industrial development has generated several environmental hazards. Therefore, it is demanding to promote sustainable development of rationally used natural resources to maintain existing ecosystems or to restore contaminated environments. This could be achieved by reducing, degrading, collecting or recycling the polluting materials as well as by improving industrialization processes, diminishing the side effects on humans, animals and on the environment.

Proteolytic enzymes catalyze the hydrolysis of the peptide bonds between amino-acids residues of protein (Dhillon et al., 2016). Such enzymes are produced by a wide range of microorganisms, like bacteria, including actinomycetes, molds, yeast etc. (Akcana & Uyar, 2011).

Selected microorganisms have the capacity to produce proteolytic enzymes using leather as unique source of carbon and nitrogen. In bacteria, this enzyme is produced mainly by strains belonging to *Bacillus* genus, especially, *B. licheniformis*, *B. horikoshii, B. sphaericus, B. furmis, B. alcalophilus, B. subtilis* (Ellaiah et al., 2011). *Bacillus* species are the main producers of extracellular proteases and industrial sectors frequently use *B. subtilis* for the production of various enzymes (Dubal et al., 2008).

The aim of the present study was to evaluate the proteases producing potential of three selected bacterial strains isolated from leather waste.

MATERIALS AND METHODS

Bacterial isolation

The microorganisms were obtained from leather waste decomposition for 90 days in the soil.

Samples of this substrate was introduced into sterile medium with pH 7.0 containing 0.5 g/l NaCl, 0.5 g/l $CaCO_3$, 0.35g/l K_2HPO_4.

After 48 h of incubation at 35°C and 135 rpm, the culture was diluted ten times and plated on agar Petri dishes.

Bacterial characterization

Newly isolated bacteria (90 colonies) were tested on three specific media: PCA containing 0.5% tryptone, 0.1% glucose, 0.25% yeast

extract and 2% agar, pH 7; Starch-Agar, containing 0.5% tryptone, 1% starch, 0.25% yeast extract, 2% agar, pH 7.2 and CMC-Agar containing 0.5% tryptone, 0.1% carboxymethyl cellulose, 0.25% yeast extract, 2% agar, pH 7. Cultures were incubated for 48h at 35°C in order to select the highest enzyme producing strains (Habib et al., 2012).
Protease production was evaluated on Slim Milk Agar.

Biomass production of leather degrading bacteria
The isolates were grown in minimal media containing 1.0 g/l NaCl, 0.05 g/l $CaCl_2$, 0.7 g/l KH_2PO_4, 0.9g/l $MgSO_4$, 2.38 g/l K_2HPO_4, 3.0 g/l sucrose, 0.6 g/l leather and incubated 120 h at 135 rpm, 35°C.
Biomass growth was evaluated by measuring the optical density (O.D.) at 600 nm using Biomate 3 spectrophotometer.
Proteolytic activity was spectrophotometric measured at 578 nm, according to Anson method (1938). The reaction mixture contained 0.5 mL of enzymatic solution and 1 mL of 1% casein in 0.2M phosphate buffer (pH 7). Samples were incubated at 37°C for 10 min. The enzymatic reaction was stopped with 2 mL of 5% tricloracetic acid. The reaction mix was kept 30 min at room's temperature and then it was filtrated through 0.22 µm membrane. For every 0.5 mL filtrate was added 0.5 mL HCl 0.2N, 2 mL NaOH 0.5N and 0.6 mL Folin-Ciocâlteu 1:2. After 30 min. at room's temperature the extinction was measured.

Identification of the selected strains using Biolog-Microbial Identification System - GEN III Bacterial identification
The selected bacterial isolated were biochemically identified using the GEN III Biolog-Microbial Identification System.
Selected microbial strains were grown over night, in isolated colonies, on BUG (Biolog Universal Growth) media at 33°C. Fresh biomass was resuspended in Biolog specific inoculation fluid, type A or B (depending on bacterial multiplication rate), at 84 to 88% turbidity (at 590nm according to the standard protocol). Specific 96 well GEN III microplates were inoculated with freshly prepared bacterial suspension, 100 µl/well. Plates were then incubated at 33°C for 22-24 hours. After 22 to 24 hours of incubations, the optical densities were measured at 590nm and 750nm using the semi-automated Biolog Spectrophotometer. The phenotypic pattern was analyzed with MicroLog3 software compared with 2650 different microbial species included in the Biolog database.

RESULTS AND DISCUSSIONS

Isolation of leather degradation bacteria
A total number of 90 isolates was harvested from the leather wastes after 90 days of decomposition in soil. These bacterial strains were tested for different enzymes production like cellulase and amylase (Figure 1).

Figure 1. Amylase producing bacterial stains

In order to reveal the CMC-ase activity, the culture plated were treated with 0.1% Congo red solution and washed with 1% acetic acid. After these two step treatment the bacterial strains having cellulose activity presented a clear halo around their colonies. To detect amylase producing bacteria, the microbial cultures grown on starch containing medium were flooded with iodine solution. The enzyme producing strains revealed a clear halo around their colony. However, the remaining non-degraded starch formed an indigo-colored compound in the presence of iodine.

Protease-producing bacteria
Around the proteases-producing bacteria it was observed an opalescent halo, having 0.2-1.1 cm diameter, after first 24 hours of incubation at 35°C. Most of the tested isolates were milky white colored, dotted, with a diameter between 0.1-1 cm, with regular shape. Other colonies were yellow colored punctiform with irregular edges, having a diameter between 0.1-0.5cm (Table 1).
Oliveira et al. (2016) found, on Slim Milk Agar (SMA), hydrolysis zone ranging from 2.5 to 1

mm, while Barros et al. (2013) observed clearance zones ranging from 2.5 to 10.0 mm for *B. subtilis* stains grow on SMA plates at 30°C for 24 h. Singh et al. (2010) reported that among 70 proteolytic bacteria isolated from soil, among which 40% were considered good protease producers, exhibit clearing zones higher than 3 mm on milk agar plates after their incubation for 20 to 30 h at 37°C.

Those that presented the best activity following tests were selected for further experiments.

Bacterial biomass productivity

The maximum biomass production (0.590 U/ml) was obtained in the minimal media supplemented with 3 g of sucrose and leather substrate, after incubation at 35°C (Table 2).

Table 1. Results of bacterial- and halo-diameter measurements after 24 h of incubation at 35°C on Slim Milk Agar for protease detection

Sample	Bact. diam. (cm)	Halo diam. (cm)	Sample	Bact. diam. (cm)	Halo diam. (cm)	Sample	Bact. diam. (cm)	Halo diam. (cm)	Sample	Bact. diam. (cm)	Halo diam. (cm)
DA1	0.3	0.3	DA25	0.9	0.9	DA49	0.2	0.4	DA73	0.3	0.7
DA2	0.9	0.8	DA26	0.3	0.6	DA50	0.3	0.4	DA74	0.2	0.3
DA3	0.2	0.3	DA27	0.7	0.9	DA51	0.1	0.5	DA75	0.2	0.5
DA4	0.7	0.7	DA28	0.2	0.3	DA52	0.5	0.9	DA76	0.7	0.9
DA5	0.7	0.8	DA29	0.4	0.4	DA53	0.1	0.3	DA77	0.9	0.7
DA6	0.8	0.8	DA30	0.1	0.3	DA54	0.2	0.6	DA78	0.1	0.5
DA7	**0.8**	**1.0**	DA31	0.8	0.7	DA55	0.7	0.7	DA79	0.1	0.3
DA8	0.2	0.3	DA32	0.9	0.9	DA56	0.8	0.8	DA80	0.3	0.5
DA9	0.5	0.7	DA33	0.7	0.8	DA57	0.3	0.7	DA81	0.1	0.5
DA10	**1.0**	**1.1**	DA34	0.1	0.6	DA58	0.6	0.9	DA82	0.4	0.8
DA11	0.1	0.2	DA35	0.1	0.3	DA59	0.7	0.7	DA83	0.7	0.7
DA12	0.3	0.5	DA36	0.2	0.3	DA60	0.7	0.8	DA84	0.8	0.7
DA13	**0.9**	**0.9**	DA37	0.2	0.5	DA61	0.9	0.6	DA85	0.8	0.5
DA14	0.7	0.9	DA38	0.2	0.3	DA62	0.8	0.9	DA86	0.5	0.9
DA15	0.8	0.9	DA39	0.3	0.4	DA63	0.7	0.9	DA87	0.2	0.5
DA16	0.3	0.7	DA40	0.6	0.8	DA64	0.5	0.9	DA88	0.2	0.6
DA17	0.4	0.7	DA41	0.9	0.9	DA65	0.3	0.6	DA89	0.7	0.8
DA18	0.1	0.4	DA42	0.7	0.9	DA66	0.2	0.5	DA90	0.1	0.4
DA19	0.5	0.6	DA43	0.7	0.8	DA67	0.8	0.8			
DA20	1.0	0.9	DA44	0.5	0.7	DA68	0.9	1.0			
DA21	0.9	0.9	DA45	0.9	0.9	DA69	1.0	0.9			
DA22	1.0	0.8	DA46	0.9	0.9	DA70	0.5	0.7			
DA23	0.1	0.4	DA47	0.3	0.7	DA71	0.5	0.8			
DA24	0.7	0.8	DA48	0.7	0.8	DA72	0.9	0.8			

Table 2. Bacterial biomass production after 48 h

Sample	D.O 600 nm	Sample	D.O 600 nm	Sample	D.O 600 nm	Sample	D.O 600 nm
DA1	0.187	DA24	0.197	DA47	0.210	DA70	0.187
DA2	0.198	DA25	0.191	DA48	0.282	DA71	0.266
DA3	0.183	DA26	0.207	DA49	0.297	DA72	0.278
DA4	0.180	DA27	0.189	DA50	0.202	DA73	0.295
DA5	0.188	DA28	0.203	DA51	0.271	DA74	0.266
DA6	0.191	DA29	0.297	DA52	0.189	DA75	0.263
DA7	**0.299**	DA30	0.239	DA53	0.186	DA76	0.251
DA8	0.189	DA31	0.215	DA54	0.205	DA77	0.280
DA9	0.197	DA32	0.199	DA55	0.201	DA78	0.222
DA10	**0.590**	DA33	0.219	DA56	0.180	DA79	0.278
DA11	0.201	DA34	0.281	DA57	0.191	DA80	0.235
DA12	0.203	DA35	0.263	DA58	0.207	DA81	0.290
DA13	**0.347**	DA36	0.254	DA59	0.219	DA82	0.244
DA14	0.219	DA37	0.233	DA60	0.189	DA83	0.188
DA15	0.193	DA38	0.279	DA61	0.231	DA84	0.207
DA16	0.204	DA39	0.289	DA62	0.276	DA85	0.183
DA17	0.289	DA40	0.255	DA63	0.203	DA86	0.189
DA18	0.277	DA41	0.199	DA64	0.218	DA87	0.199
DA19	0.180	DA42	0.277	DA65	0.297	DA88	0.272
DA20	0.287	DA43	0.264	DA66	0.246	DA89	0.263
DA21	0.288	DA44	0.200	DA67	0.239	DA90	0.209
DA22	0.182	DA45	0.185	DA68	0.222		
DA23	0.181	DA46	0.191	DA69	0.214		

Spectrophotometric quantification of proteolytic activity

Bacterial growth and protein digestion were evaluated for the three selected bacterial strains (DA7, DA10 and DA13). Results showed that DA10 was the most efficient strain, reaching the highest OD values at 578nm. Enzyme activity was evaluated as 0.556 U/ml after 24 h, and 0.987 U/ml after 120h of incubation (Table 3).

Identification of selected microorganisms

Based on the Biolog GEN III fingerprints of each strain, the selected bacteria were identified as *Brevundimonas diminua* (strain DA7) – Figure 2, *Bacillus cereus/thuringiensis* (strain DA10), and *Bacillus cereus* (strain DA13).

The Biolog identification revealed a similarity index higher than 0.5 for each analysed strain, and distance value of at least 2 points between the most similar microbial profiles, which reveals a clear species-specific identification for DA7 and DA13.

However, this identification system cannot reveal all de differences between *B. cereus* and *B. thuringiensis*.

Table 3. Proteolytic activity evaluated by spectrophotometric quantification at 578 nm

	Incubation time					
Strain	24 h	48 h	72 h	96 h	120 h	Average
DA 13	0.231	0.415	0.586	0.938	0.993	0.633
DA 10	0.556	0.630	0.815	0.906	0.987	0.779
DA 7	0.183	0.139	0.426	0.506	0.616	0.374

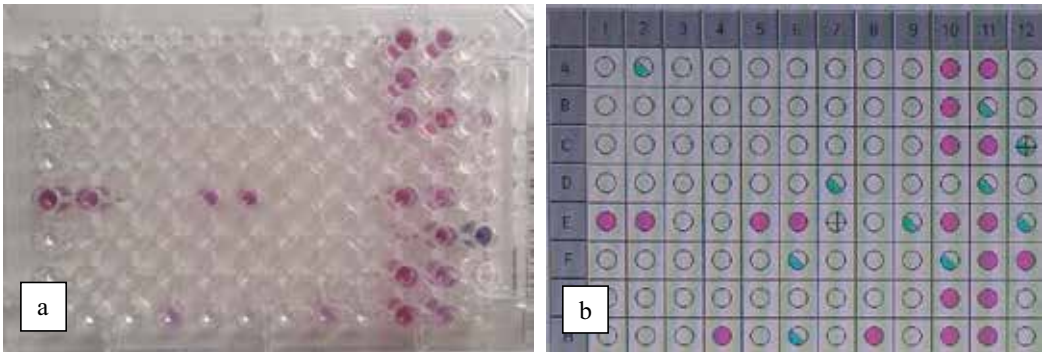

Figure 2. *Brevundimonas diminua* strain DA7:
a. Inoculated GEN III plate after 22 h of incubation at 33°C; b. MicroLog3 scanned fingerprint of the microplate

CONCLUSIONS

Three bacterial strains were isolated from leather degrading wastes.

The selected bacterial strains exhibit the highest proteolytic activity of 0.987 U/ml after 120 hours of cultivation, at 35°C.

Upon identification, it was revealed that the microbial strains were: *Brevundimonas diminuta* (DA7), *Bacillus cereus/thuringiensis* (DA10) and *Bacillus cereus* (DA13).

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REFERENCES

Akcana, N., Uyar, F. (2011). Production of extracellular alkaline protease from Bacillus subtilis RSKK96 with solid fermentation. *EurAsian Journal of BioScience*, *5*, 64–72.

Anson, M. L. (1938). The estimation of pepsin, trypsin, papain, and cathepsin with haemoglobin. *J. Gen. Physiol*., *22*, 79–89.

Barros, F. F., Simiqueli, A. P., de Andrade, C. J., Pastore, G. M. (2013). Production of enzyme from agroindustrial waste by biosurfactant-producing strain of Bacillus subtilis. *Biotechnology Research International.*

Dhillon, A., Sharma, K., Rajulapati, V., Goyal, A. (2017). Proteolytic enzyme. In *Current developments in biotechnology and bioengineering. Production, isolation and purification of industrial products*, Ed. 1, Chapter 7, 149–173.

Dubal, S. A., Tilkari, Y. P., Momin, S. A., Borkar, I. V. (2008). Biotechnological routes in Flavour industries. *Advanced Biotech*., 20–31.

Ellaiah, P., Srinivasulu, B., Adinarayana, K. (2002). A review on microbial alkaline proteases. *Journal of Scientific & Industrial Research*, *61*, 690–04.

Habib, S. M. A., Fakhruddin, A. N. M., Begum, S., Ahmed, M. M. (2012). Isolation and screening of thermostable extracellular alkaline protease producing bacteria from tannery effluents. *J. Sci. Res*., *4*, 515–522.

Oliveira, C. T., Pellenz, L. J. Q., Bradelli, A., Daroit, D. J. (2016). Screening of bacteria for proteinase production and feather degradation. *Waste and biomass valorization*, *7*(3), 447–53.

Singh, S. K., Tripathi, V. R., Jain, R. K., Vikam, S., Garg, S. K. (2010). An antibiotic, heavy metal resistant and halotolerant Bacillus cereus SIU1 and its thermoalkaline protease. *Microbial Cell Factories*, *9*(1), 59.