Construction of simplified microbial consortia to degrade recalcitrant materials based on enrichment and dilution-to-extinction cultures

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Running title: Simplifying microbial consortia for biodegradation

Keywords: simplified microbial consortia, biodegradation, enrichment cultivation, dilution-to-extinction, recalcitrant materials

Abstract

The capacity of microbes degrading recalcitrant materials has been extensively explored from environmental remediation to industrial applications. Although significant achievements were obtained with single strains, focus is now going toward the use of microbial consortia because of advantages in terms of functional stability and efficiency. While consortia assembly attempts were made from several known single strains, another approach consists in obtaining consortia from complex environmental microbial communities in search for novel microbial species, genes and functions. However, assembling efficient microbial consortia from complex environmental communities is far from trivial due to large diversity and biotic interactions at play. Here we propose a strategy containing enrichment and dilution-to-extinction cultures to construct simplified microbial consortia (SMC) for keratinous waste management, from complex environmental communities. Gradual dilutions were performed from a keratinolytic microbial consortium, and dilution 10^{-9} was selected to construct a SMC library. Further compositional analysis and keratinolytic activity assays demonstrated that microbial consortia were successfully simplified, without impacting their biodegradation capabilities. These SMC possess promising potential for efficient keratinous valorization. More importantly, this reasoning and methodology could be transferred to other topics involving screening for simplified communities for biodegradation, thus considerably broadening its application scope.

Importance

Microbial consortia have got more and more attention and extensive applications due to their potential advantages. However, a high diversity of microbes is likely to hide uncontrollable risks in practice specific to novel strains and complicated interaction networks. Exploring a convenient and efficient way to construct simplified microbial consortia is able to broaden the applied scope of microbes. This study presents the approach based on enrichment and dilution-to-extinction cultures, which gain abundance microbial consortia including some without losing efficiency from the enriched functional microbial community. The microbial interactions at the strain level were evaluated by using compositional identification and correlation analysis, which contribute to revealing the roles of microbes in the degradation process of recalcitrant materials. Our findings provide a systematic scheme to achieve optimizing microbial consortia for biodegradation from an environmental sample, could be readily applied to a range of recalcitrant materials management from environmental remediation to industrial applications.

Introduction

Microbial degradation aims to harness the potential of enzymatic processes of naturally occurring microorganisms to break down complex, and usually environmentally recalcitrant materials (1). Biodegradation is widely used in agricultural and industrial fields, especially when dealing with substances, which are difficult to dissolve and/or resistant to decomposition under mild conditions (2, 3). Large number of microorganisms have been isolated and characterized

for their efficient capacity to degrade recalcitrant molecules such as organophosphorus compounds, being the most commercially favored group of pesticides, also posing a risk to human health (4, 5). Another example is the utilization of microbes to metabolize lignocellulose materials which represent a renewable carbon source with potential application as biofuel (6). To that end, microbial consortia gained from complex environmental communities harboring unknown genes, metabolic activities and species, hold a colossal application potential for enhancing the efficiency of bioprocesses, particularly when dealing with substances that are resistant to decomposition (7, 8). They have received increasing attention due to their promising advantages on handling environmental problems (9).

Microbial consortia consist of several species working together, providing both robustness and broad metabolic capacities (9). They may be assembled synthetically from scratch by combining several isolated strains (10), or conversely yielded from complex environmental microbial communities, like soil (11). Mixed populations are not only able to perform some difficult tasks that would not even be possible for individual strains, but offer more stability and resilience against environmental fluctuations (12). They are superior compared to single strains with respect to degradative efficiency in many cases (13). Unlike single strains isolation, enrichment process involving directed artificial selection is often used to obtain desired microbial consortia from the environment (14, 15). In general, composition of consortia gradually changes over time, including a richness reduction along with emergence of dominant microbes due to competitive exclusion (16). Nevertheless, many species are likely to benefit from the enrichment due to complex microbial interactions conferring functional stability and redundancy (17, 18). For instance, a total of 17 bacterial strains and 13 methanogens were identified from established

93 microbial consortia depolymerizing lignin (19). Therefore, this intrinsic high diversity level of
94 enriched microbial consortia represents a bottleneck in our attempts to move forward with
95 potential industrial applications due to several aspects, like i) potential negative correlation with
96 efficiency (20), ii) species with undesired function, iii) security threat posed by pathogens
97 presence, and iv) risks of losing the properties of interest if hold by keystone species. Utilization
98 of microbial consortia with less complexity, but equal efficiency, lead to more controlled and
99 optimized industrial processes (10). Hence, it is crucial to find reliable strategies to narrow
100 down the diversity towards optimum simplified microbial consortia. A reductive-screening
101 approach was applied to construct effective minimal microbial consortia for lignocellulose
102 degradation based on different metabolic functional groups (10). Additionally, artificial
103 selection approaches (dilution, toxicity, and heat) have been employed to obtain desired
104 bacterial consortia (21). A minimal effective hydrogen-producing microbial consortium was
105 constructed *via* dilution-to-extinction culture from cow rumen communities (22). Dilution-to-
106 extinction culture is expected to provide more advantages compared to conventional isolation
107 and assembly as it i) generates many microbial combinations ready to be screened, ii) includes
108 all strains from the initial microbial pool that might be lost due to cultivation/isolation biases
109 and iii) ensure that all microbes are physically present and interacting spontaneously (23).
110
111 Keratins are insoluble fibrous proteins with cross-linked components, representing the most
112 abundant proteins in epithelial cells (24). Keratinous materials are classified as one of the waste
113 materials categories according to European parliament regulations (25). They could cause an
114 environmental imbalance and potential pollution to soil or water because of their recalcitrant
115 form in municipal waste (26). Hence, seeking the effective approach to keratinous waste

116 management will contribute to eliminate their environmental risks. In fact, keratinous materials
117 are estimated to have considerable economic value after biodegradation (27). A microbial
118 consortium (KMCG6) was enriched from an environmental sample culturing in α-keratin based
119 medium, which possesses an efficient keratinolytic activity (11). Despite successful diversity
120 reduction during enrichment, it still includes plenty of microbes affiliating to distinct genera
121 (11). In this work, we applied the concept of dilution-to-extinction culture to KMCG6 as a case
122 study (Fig. 1), resulting in efficient simplified microbial consortia (SMC).
123
124 **Results and Discussion**
125
126 **Phylogenetic analysis of microbial consortium KMCG6.** Microbial consortia have shown
127 numerous advantages for the processing of recalcitrant molecules and materials which are hard
128 to degrade or convert (9), and may even represent environmental and threats (26).
129 Microorganisms represent a colossal, yet still a poorly tapped resource for applied microbiology
130 and biotechnology. Many efforts attempting to learn from nature have used microbial consortia
131 for bioconversion. Microbial consortium KMCG6, displaying efficient keratinolytic activity,
132 was enriched from a soil sample as previously described (11). Taxonomic composition analysis
133 at phylogenetic levels (class, genus, and OTU) along the enrichment process was done over time
134 at different generation batches (KMCG1, KMCG3, and KMCG6), showing a progressive
135 decrease in consortia complexity (richness) over generation time (Fig. 2a). Ten, nine and five
136 different classes were observed from KMCG1, KMCG3, and KMCG6, respectively. At the
137 genus level, there were 44 and 31 genera classified from KMCG1 and KMCG3, and 14 from
138 KMCG6. Only seven known genera (*Pseudochrobactrum, Chryseobacterium, Lysinibacillus,*

Acinetobacter, Buttiauxella, Stenotrophomonas, and Comamonas) and one unclassified were detected with a relative abundance > 0.1 % from KMCG6 (Fig. 2b). A similar trend was observed at the most sensitive OTU level, as the observed richness decreased from 162 (KMCG1) to 85 (KMCG6), implicating that several OTUs were affiliated per genera. Seven OTUs were classified to *Pseudochrobactrum,* which was the most diverse genera, followed by *Chryseobacterium and Stenotrophomonas,* both containing four OTUs. Notably, 61.61% of the total sequences were clustered to OTU_457, representing the *Chryseobacterium sp. KMC2* (*CKMC2*) in the KMCG6. *Pseudochrobactrum sp. KMC2* (*PKCM2*) and *Acinetobacter sp. KMC2* (*AKMC2*) accounted for 11.28 % and 5.04 %, respectively. These results show that selection reduced diversity and specific strains were enriched in the consortia.

Note that it is preferable to obtain microbial consortia with desired functions prior to practical implementation, which can be obtained from naturally occurring microbial communities. But in most cases, this is still not sufficient to reach a desired effective consortium with a low diversity, which can be further used for downstream industrial applications (28, 29). For instance, more than 85 potential strains were detected from KMCG6, although the actual amount may be lower because multi-OTUs could originate from a same single strain due to intrinsic variability amongst multiple copies of 16S rRNA gene (30, 31). KMCG6 still had a high diversity with 14 genera and more than 21 dominating OTUs (> 0.1 %) with distinct relative abundance, which likely corresponded to strains with different functionality towards keratin degradation. It indicates that the community diversity was still too high for understanding the underlying mechanisms. Therefore, simplification of this system is required to obtain more controllable consortia for downstream keratinous waste management, and with potentially enhanced activity.

Optimal dilution for construction of a library of SMC. The dilution-to-extinction strategy has already proven its efficiency for obtaining functional isolates and microbial consortia from various initial environmental inoculums such as seawater and rumen liquor (32-34), which was adapted here to our pre-enriched KMCG6. In order to determine the optimal dilution to KMCG6, we performed gradient dilutions with 24 replicates in keratin medium (KM). Cell density, protease, and keratinase activities measuring from individuals were used to evaluate the dissimilarity between distinct dilutions. Approximately 21 % (5) and 92 % (22) of the diluted replicates displayed no cell growth from dilution 10^{-9} and 10^{-10}, respectively. Dilution 10^{-10} was excluded based on the observed low growth and lack of sufficient degradation efficiency. Comparison of different dilutions was calculated according to their characteristics of degrading capacities (cell growth and enzyme activities) with Euclidean distance (Fig. 3a). It suggests that the profiles of dilution 10^{-2} to 10^{-8} had high similarity. Notably, dilution 10^{-9} shows a clear and expected higher variability compared to lower dilutions (PERMANOVA, FDR < 0.1), such as e.g. 10^{-8}, which has a good potential for assembling effective SMC.

Once an efficient pre-enriched consortium is secured, determining optimal dilution is as a critical step to obtain good heterogeneity in the subsequent SMC. Here, serial dilutions were carried out to simplify KMCG6. A comprehensive comparison was done for all dilutions, including 24 replicates per dilution. Concerning the number of replicates included, a sufficient quantity provides more reliable and statistically sound results. Previous studies showed that few replicates cultured from distinct dilutions resulted in a limited heterogeneity of functional microbial consortia (33, 35). The higher the better, as it will give greater chance to reproduce

and/or even improve the efficiency observed. However, a parsimonious approach would be to rely on an adjusted number of replicates, estimated based on prior evaluation of the taxonomic compositions and abundance of the constituting microbial members in the pre-enriched community.

In addition, we performed CFU counting on LB agar to verify the number of viable cells between dilution 10^{-8} (mean = 33 CFU) and dilution 10^{-9} (mean = 3 CFU), showing an expected decrease of 10-fold (Fig. 3b). More importantly, they possessed various degradative capacities, including high performance in KM. Dilution 10^{-9} hereby was selected as an optimal dilution to further construct a library of SMC consisting of 96 replicates (Fig. S1, for reviewers only). Additionally, 18 SMC were selected for further functional assessment with additional three SMC from dilution 10^{-8}.

Diversity and structure of SMC during keratin degradation. The taxonomic classification of these selected SMC was observed at OTU level using 16S rRNA gene amplicon sequencing (Fig. 4a). In total, 15 OTUs were found in the SMC with a relative abundance above 0.1 %, including 12 observed in KMCG6. Three new detected members were *Chryseobacterium sp. KMC5*, *Pseudomonas sp. KMC1* and *Stenotrophomonas sp. KMC4*. SMC were clustered into seven groups according to their taxonomic composition (weighted UniFrac). Three SMC from dilution 10^{-8}: SMC1, SMC2, and SMC3 were clustered as group 1 (Gr1), all featuring the dominant *CKMC2* (> 62.9%). The relative abundance of *Stenotrophomonas sp. KMC3* and *PKMC2* were more than 5 %. As expected, the composition of SMC in dilution 10^{-9} was set apart from 10^{-8}, showing a substantial difference between these two dilutions. 18 SMC were divided into six

groups (Gr2 – Gr7), which had heterogeneous profiles in terms of OTU diversity (OUTs = 2-12). 14 SMC (SMC4 – SMC17) from dilution 10^{-9} still featured the dominant *CKMC2*, which initially constituted the majority of KMCG6. Four SMC (SMC18 – SMC21) contained little to none of *CKMC2* and they were classified into two different groups (Gr6 and Gr7).

It is a remarkable fact that several phylogenetically-related strains from the same species co-exist in the KMCG6 (Fig. 2b), which is consistent with another similar enrichment study (28). The functions of individual strains are likely to be diverse within species and it is an unresolved challenge to identify the intraspecific variablility (36). Here taxonomic classification of most individual SMC showed that only a few strains belonging to same species remained, which demonstrates that the method conveniently selected a simplified microbial consortium with specific functional strains, avoiding the ones with undesired traits. Besides, strains with low abundance emerged when compared to the initial microbial consortium (KMCG6), suggesting the approach is also efficient to acquire potentially crucial rare species into the SMC.

Comparative analysis of degradative capacities. The initial microbial community is likely to be divided into different functional groups along with the gradual dilution process due to random reassembly of microbes caused by extinction and sampling effects (23). Cell density was measured at day three for all SMC along with protease and keratinase activities. This timeframe is optimized to achieve the highest cell densities and enzyme activities according to our previous work (11). The keratinolytic characteristics of SMC were measured individually, and displayed on the basis of the defined groups by consortia composition (Fig. 4b, 4c and 4d). Additionally, KMCG6 and the *CKMC2* isolate were compared to these groups. These results clearly show two distinct performance categories present amongst the groups in term of cell

density, enzyme activity, and residue ratio. The first category includes mostly groups from dilution 10^{-9}: Gr2, Gr3, Gr4, and Gr5, together with Gr1 (10^{-8}), all displaying good performance similar to KMCG6, while Gr6 and Gr7 have low performance with weak keratinolytic activity. SMC from 10^{-8} (SMC1, SMC2, and SMC3) show good growth rates, with OD_{600nm} reaching up to (1.15 – 1.3). The OD_{600} of the 18 SMC from dilution 10^{-9} ranges from 0.1 to 1.2. No visible difference in cell growth is observed between the good performers and KMCG6. It is worth noticing that the protease activity from Gr5 was significantly higher than *CKMC2*. On the other hand, the residue ratio of SMC from dilution 10^{-9} was very divergent, indicating that the dilution-to-extinction cultures have succeeded in generating heterogeneous functional SMC.

Potential biotic interactions. To enhance the association between strains and degradative capacity, a Pearson's correlation based network was adopted (Fig. 5). As expected, the cell density and enzyme activities had a significant positive correlation with degradation efficiency. Seven strains (*CKMC2, PKMC2, Psedochrobacterum sp. KMC5, Psedochrobacterum sp. KMC6, Psedochrobacterum sp. KMC7, Stenotrophomonas sp. KMC2* (*SKMC2*) *and Stenotrophomonas sp. KMC4*) were connected with keratinolytic activity, which are likely to be the key players in the keratin degradation. Among them, *CKMC2* was the only strain that had positive correlations with all of the keratinolytic characteristics, suggesting that *CKMC2* is the keystone strain in the degradative process. Interestingly, all of the other strains in this correlated network have negative correlations with *CKMC2*. However, *PKMC2* has also a positive correlation with the degradation efficiency while having a negative one with *CKMC2,* indicating that these two strains likely undergo competitive exclusion, leading to the dominance of *CKMC2* (Fig. 4a). *PKMC2* also has numerous positive correlations with other strains such as *SKMC2* and

Pseudochrobactrum sp. KMC7, indicating that *PKMC2* may play a significant role to maintain the structure of the microbial consortia. However, *SKMC2* is associated to a decrease in degradation efficiency, indicating that it may be a cheater strategist benefiting from *PKMC2*. Additional four strains (*Acinetobacter sp. KMC1*, *AKMC2*, *Stenotrophomonas sp. KMC3*, and *Pseudomonas sp. KMC1*) only had satellite correlations, which probably have no effect on keratin degradation and could therefore be removed from the community. The network analysis based on the SMC library can be used to identify actual microbial interaction at the strain level, which may help to further optimize functional microbial consortia.

The microbial diversity in the so-obtained SMC decreased remarkably while maintaining an equivalent keratinolytic capacity. It supports that dilution-to-extinction represents a simple and practical strategy, simplifying microbial consortia without losing efficiency. Currently, microbial consortia are not only applied on biodegradation. They have been applied extensively in other fields such as biomining, bioremediation, biofilm formation, or biosynthesis (37-39). We show that dilution-to-extinction culture is efficient to simplify microbial consortia, without impairing keratinolytic activity. Overall, a strategy combining enrichment and dilution-to-extinction cultures was applied successfully to assemble several SMC, which had heterogeneous capacities for keratinous waste management. Meanwhile, it provides a view of potential interactions among strains, which can be used for further designing and engineering of microbial consortia. This approach promotes the efficient selection of simplified functional consortia from high diversity environmental habitats and raises the possibility to obtain satisfactory microbial consortia for practical applications.

Materials and Methods

Substrate and medium preparation. Mixed α-keratin materials (raw bristles and hooves) were collected from a Danish Crown slaughterhouse (Bragesvej, Denmark), washed with tap water thoroughly, and cut to about 2 mm in diameter mechanically by Daka Sarval (Løsning, Denmark). Keratin medium (KM) was prepared with 1 % keratinous materials with mineral salt medium (0.5 g/L of NH_4Cl, 0.5 g/L of NaCl, 0.3 g/L of K_2HPO_4, 0.4 g/L of KH_2PO_4, 0.1 g/L of $MgCl_2 \cdot 6H_2O$) (40), with keratins being the sole carbon source. KM was sterilized by autoclaving (21 min, 120 °C).

Gradual dilutions of the microbial community. Pre-enriched microbial consortium "KMCG6" from Kang et al. (11) was obtained after serial enrichments over time in successive generation batches, namely Keratin Microbial Consortia Generation batch number 6: "KMCG6". KMCG6 was inoculated into the LB liquid medium for overnight cultivation with shaking until the optical density (OD_{600}) reached 0.7 - 0.8 (250 rpm, 24 °C). The cell suspension was gradually diluted in LB liquid medium with six dilutions (10^{-2}, 10^{-4}, 10^{-6}, 10^{-8}, 10^{-9} and 10^{-10}). For each dilution 24 replicates were prepared by transferring 200 µL into individual wells of a 96-well plate. Plates were incubated overnight (250 rpm, 24 °C). Subsequently, all replicates from different dilutions were inoculated into 24-well plates with 1.5 mL KM at 1:100 ratio, and grown for three days (250 rpm, 24 °C) before functional assessment *via* cell density and protease acitivity assays.

Cell density measurement and cell number count. (i) Cell density measurement. Cultures

were left standing for 10 min. to precipitate large suspended keratin particles after cultivation. Thereafter, 200 µL cell suspension from all dilutions were transferred to 96-well plates. Cell density was measured (OD_{600}) using a microplate reader (Biotek, ELx808). **(ii) Cell number count.** 200 µL cell suspension from 24 replicates of dilutions (10^{-8} and 10^{-9}) was plate spread on LB agar plates. Cell numbers were counted according to observable colony-forming units (CFU) on the plates after 48 h.

Construction of various microbial consortia library. A total of 96 SMC were obtained based on optimal dilution condition (10^{-9}) and 18 SMC were further selected from the library after the functional assessments. In addition, three SMC from dilution 10^{-8} were as the control. In order to evaluate the degradation capacities accurately, the cultivation of 21 SMC was scaled up to 100 mL KM by 1:100 ratio for 5 days (200 rpm, 24 °C).

Enzyme activity assays. (i) Protease activity assay. Protease activity of microbial consortia was assessed with azocasein (Sigma-Aldrich, St. Louis, MO, USA) as described previously (11). 100 µL supernatant with 50 µL 1% (w/v) azocasein were incubated at 30 °C for 30 min. with shaking at 200 rpm, then stopped by adding 150 µL 10% (w/v) trichloroacetic acid and incubated at 4 °C for 15 min. 100 µL mixture was mixed with 100 µL of 0.5 M NaOH after centrifugation. Absorbance was recorded in 96-well plates at 415 nm and one unit (U) of protease activity was defined as 0.01 increase in absorbance. **(ii) Keratinolytic activity assay**. Preparation procedure for azokeratin and associated keratinolytic activity assay were described previously (11). The prepared keratin materials were coupled with a diazotized aryl amine to produce a chromophoric derivative, sulfanilic acid azokeratin. Keratinase activity of microbial

consortia was assayed using azokeratin as a substrate. The reaction mixture of supernatant and azokeratin were incubated for 1 h at 30 °C with shaking at 200 rpm, then cooled to room temperature for 5 min. 200 µL supernatant without azokeratin was transferred to 96-well plates and absorbance was measured as above. One unit (U) of keratinase activity was defined as the amount of enzyme required for 0.01 increasing in absorbance.

Residual keratin substrate weight. Keratin residue was collected from all of the microbial consortia after five days of cultivation in KM. The residual substrate was washed with deionized water using vacuum filtration until the flow-through was colourless to remove all of the microbial biomass and then dried at 50 °C for 48 h. The residual substrate was weighed and reported as the percentage (w/w %) of initial keratin substrate.

Composition analysis of the microbial consortia. A total of 5 mL cell suspension was collected from each microbial consortium after three days of cultivation. DNA extraction was done using the FAST Soil DNA Kit (MP Biomedicals, U.S.A), following the manufacturer's instructions. PCR amplification and sequencing preparation were performed as previously described (41), using the primers Uni341F (5'-CCTACGGGRBGCASCAG-3') and Uni806R (5'-GGACTACHVGGGTWTCTAAT-3') flanking the V3 and V4 regions of the 16S rRNA gene (42, 43). Purification of PCR products was done with Agencourt AMPure XP Beads (Beckman Coulter Genomics, MA, USA) according to the manufacturer's instructions. They were further quantified using Quant-iT™ High-Sensitivity DNA Assay Kit (Life Technologies) and pooled in equimolar concentrations using SequalPrep™ Normalization Plate (Thermo Fisher Scientific) before concentration using the DNA Clean and Concentrator™-5 kit (Zymo Research, Irvine,

CA, USA). Finally, a 20 pM pooled library was subjected to paired-end (2x250 bp) high-throughput sequencing on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) using MiSeq reagent kit v2. Raw sequencing data were handled as previously described (44), which included quality control, trimming and clustering of sequences into OTU using a 97 % pairwise sequence similarity threshold. A representative sequence from each OTU cluster was given taxonomical annotation using RDP database (45). A read contingency table with OTU information including enrichment process was exported at the species level. The comparison of KCCG1, KMCG3, and KMCG6 was performed at class, genus and OTU levels. Phylogenetic tree of KMCG6 was constructed based on OTU sequences using MEGA7 (46) by maximum likelihood method. Only OTU with a relative abundance above 0.1 % were considered. The raw sequencing data is being prepared for deposition at the NIH short read archive.

Strain identification. SMC from dilution 10^{-9} with high keratinolytic activity were plated on LB plates and incubated at 24 °C for 48 h. In order to secure strain purity for accurate identification and isolation, single colony was picked and inoculated into LB liquid medium for overnight cultivation until OD_{600} reached 0.7 - 0.8 (250 rpm at 24 °C), then plated on new LB plates. This procedure was repeated three times until all colonies on the LB plates had the same morphological characteristics. Afterward, one single colony was picked, cultured in LB liquid medium overnight. DNA was extracted from 2 mL culture with FAST Soil DNA Kit (MP Biomedicals, U.S.A), following the manufacturer's instructions. The DNA was used as the template with primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′) to amplify the full 16S rRNA gene, followed by BLAST identification with assembled OTUs.

Statistical analysis. Euclidean distance was calculated to determine the functional dissimilarity of SMC from different dilutions in terms of cell density, enzymes activity, and residual ratio. The multivariate homogeneity of group dispersion was analysed with "betadisper" (R-package "vegan") and assessed using PERMANOVA. Benjamini–Hochberg correction was applied to control false discovery rate (FDR) for multiple testing (47). The significance level within dilutions was defined as the FDR < 0.1. For the selected 21 SMC, grouping was performed according to the compositional similarity using weighted UniFrac distance metric (48). Statistical differences of degrading capacities among SMC groups, KMCG6, and single strains were performed by one-way ANOVA using post-hoc Tukey's HSD test ($p < 0.05$). Relationships between microbes and degrading capacities were evaluated by using Pearson's correlation, and visualized with Gephi (49). All of the statistical analysis in this study was caculated by using R (version 3.5.0).

Acknowledgments

This research was funded by the Danish Innovation Fund (grant number 1308-00015B, Keratin2Protein) and also under the support of the Chinese Scholarship Council (CSC) Scholarship Program.

References

1. Vert M, Doi Y, Hellwich K-H, Hess M, Hodge P, Kubisa P, Rinaudo M, Schué F. 2012. Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). Pure and Applied Chemistry 84:377-410.
2. Chen M, Qin X, Zeng G. 2017. Biodegradation of carbon nanotubes, graphene, and their derivatives. Trends in biotechnology 35:836-846.
3. Huang J, Ling J, Kuang C, Chen J, Xu Y, Li Y. 2018. Microbial biodegradation of aniline at low concentrations by Pigmentiphaga daeguensis isolated from textile dyeing sludge. International Biodeterioration & Biodegradation 129:117-122.
4. Ghosal D, Ghosh S, Dutta TK, Ahn Y. 2016. Current state of knowledge in microbial degradation of polycyclic aromatic hydrocarbons (PAHs): a review. Frontiers in microbiology 7:1369.
5. Kumar S, Kaushik G, Dar MA, Nimesh S, Lopez-Chuken UJ, Villarreal-Chiu JF. 2018. Microbial Degradation of Organophosphate Pesticides: A Review. Pedosphere 28:190-208.
6. Brown ME, Chang MC. 2014. Exploring bacterial lignin degradation. Current opinion in chemical biology 19:1-7.
7. Shong J, Diaz MRJ, Collins CH. 2012. Towards synthetic microbial consortia for bioprocessing. Current Opinion in Biotechnology 23:798-802.
8. Subashchandrabose SR, Ramakrishnan B, Megharaj M, Venkateswarlu K, Naidu R. 2011. Consortia of cyanobacteria/microalgae and bacteria: biotechnological potential. Biotechnology advances 29:896-907.
9. Hays SG, Patrick WG, Ziesack M, Oxman N, Silver PA. 2015. Better together: engineering and application of microbial symbioses. Current opinion in biotechnology 36:40-49.
10. Puentes-Téllez PE, Salles JF. 2018. Construction of effective minimal active microbial consortia for lignocellulose degradation. Microbial ecology:1-11.
11. Kang D, Herschend J, Al-Soud WA, Mortensen MS, Gonzalo M, Jacquiod S, Sørensen SJ. 2018. Enrichment and characterization of an environmental microbial consortium displaying efficient keratinolytic activity. Bioresource technology 270:303-310.
12. Brenner K, You L, Arnold FH. 2008. Engineering microbial consortia: a new frontier in synthetic biology. Trends in biotechnology 26:483-489.
13. Mikesková H, Novotný Č, Svobodová K. 2012. Interspecific interactions in mixed microbial cultures in a biodegradation perspective. Applied microbiology and biotechnology 95:861-870.
14. Luo F, Devine CE, Edwards EA. 2016. Cultivating microbial dark matter in benzene□ degrading methanogenic consortia. Environmental microbiology 18:2923-2936.
15. Burniol-Figols A, Varrone C, Le SB, Daugaard AE, Skiadas IV, Gavala HN. 2018. Combined polyhydroxyalkanoates (PHA) and 1, 3-propanediol production from crude glycerol: Selective conversion of volatile fatty acids into PHA by mixed microbial consortia. Water research 136:180-191.
16. Jiménez DJ, Dini-Andreote F, DeAngelis KM, Singer SW, Salles JF, van Elsas JD. 2017. Ecological insights into the dynamics of plant biomass-degrading microbial consortia. Trends in microbiology 25:788-796.
17. Shade A, Peter H, Allison SD, Baho D, Berga M, Bürgmann H, Huber DH, Langenheder S, Lennon JT, Martiny JB. 2012. Fundamentals of microbial community resistance and resilience. Frontiers in microbiology 3:417.

18. Awasthi A, Singh M, Soni SK, Singh R, Kalra A. 2014. Biodiversity acts as insurance of productivity of bacterial communities under abiotic perturbations. The ISME journal 8:2445.
19. Wu Y-R, He J. 2013. Characterization of anaerobic consortia coupled lignin depolymerization with biomethane generation. Bioresource technology 139:5-12.
20. Banerjee S, Kirkby CA, Schmutter D, Bissett A, Kirkegaard JA, Richardson AE. 2016. Network analysis reveals functional redundancy and keystone taxa amongst bacterial and fungal communities during organic matter decomposition in an arable soil. Soil Biology and Biochemistry 97:188-198.
21. Lee D-J, Show K-Y, Wang A. 2013. Unconventional approaches to isolation and enrichment of functional microbial consortium–a review. Bioresource technology 136:697-706.
22. Wang A, Gao L, Ren N, Xu J, Liu C, Lee D-J. 2010. Enrichment strategy to select functional consortium from mixed cultures: consortium from rumen liquor for simultaneous cellulose degradation and hydrogen production. International Journal of Hydrogen Energy 35:13413-13418.
23. Roger F, Bertilsson S, Langenheder S, Osman OA, Gamfeldt L. 2016. Effects of multiple dimensions of bacterial diversity on functioning, stability and multifunctionality. Ecology 97:2716-2728.
24. Coulombe PA, Omary MB. 2002. 'Hard'and 'soft'principles defining the structure, function and regulation of keratin intermediate filaments. Current opinion in cell biology 14:110-122.
25. Korniłłowicz-Kowalska T, Bohacz J. 2011. Biodegradation of keratin waste: theory and practical aspects. Waste management 31:1689-1701.
26. Verma A, Singh H, Anwar S, Chattopadhyay A, Tiwari KK, Kaur S, Dhilon GS. 2017. Microbial keratinases: industrial enzymes with waste management potential. Critical reviews in biotechnology 37:476-491.
27. Mothé MG, Viana LM, Mothé CG. 2018. Thermal property study of keratin from industrial residue by extraction, processing and application. Journal of Thermal Analysis and Calorimetry 131:417-426.
28. Himmelberg AM, Brüls T, Farmani Z, Weyrauch P, Barthel G, Schrader W, Meckenstock RU. 2018. Anaerobic degradation of phenanthrene by a sulfate□reducing enrichment culture. Environmental microbiology 20:3589-3600.
29. Guerra AB, Oliveira JS, Silva-Portela RC, Araujo W, Carlos AC, Vasconcelos ATR, Freitas AT, Domingos YS, de Farias MF, Fernandes GJT. 2018. Metagenome enrichment approach used for selection of oil-degrading bacteria consortia for drill cutting residue bioremediation. Environmental Pollution 235:869-880.
30. Hakovirta JR, Prezioso S, Hodge D, Pillai SP, Weigel LM. 2016. Identification and analysis of informative single nucleotide polymorphisms in 16S rRNA gene sequences of the Bacillus cereus group. Journal of clinical microbiology:JCM. 01267-16.
31. Ninet B, Monod M, Emler S, Pawlowski J, Metral C, Rohner P, Auckenthaler R, Hirschel B. 1996. Two different 16S rRNA genes in a mycobacterial strain. Journal of Clinical Microbiology 34:2531-2536.
32. Hoefman S, van der Ha D, De Vos P, Boon N, Heylen K. 2012. Miniaturized extinction culturing is the preferred strategy for rapid isolation of fast□growing methane□oxidizing bacteria. Microbial biotechnology 5:368-378.
33. Ho K-L, Lee D-J, Su A, Chang J-S. 2012. Biohydrogen from lignocellulosic feedstock via one-step process. International Journal of Hydrogen Energy 37:15569-15574.

34. Sosa OA, Gifford SM, Repeta DJ, DeLong EF. 2015. High molecular weight dissolved organic matter enrichment selects for methylotrophs in dilution to extinction cultures. The ISME journal 9:2725.
35. Ho K-L, Lee D-J, Su A, Chang J-S. 2012. Biohydrogen from cellulosic feedstock: dilution-to-stimulation approach. International Journal of Hydrogen Energy 37:15582-15587.
36. Cianciaruso MV, Batalha MA, Gaston KJ, Petchey OL. 2009. Including intraspecific variability in functional diversity. Ecology 90:81-89.
37. Brune KD, Bayer T. 2012. Engineering microbial consortia to enhance biomining and bioremediation. Frontiers in microbiology 3:203.
38. Ren D, Madsen JS, Sørensen SJ, Burmølle M. 2015. High prevalence of biofilm synergy among bacterial soil isolates in cocultures indicates bacterial interspecific cooperation. The ISME journal 9:81.
39. Zhou K, Qiao K, Edgar S, Stephanopoulos G. 2015. Distributing a metabolic pathway among a microbial consortium enhances production of natural products. Nature biotechnology 33:377.
40. Bertsch A, Coello N. 2005. A biotechnological process for treatment and recycling poultry feathers as a feed ingredient. Bioresource technology 96:1703-1708.
41. Nunes I, Jacquiod S, Brejnrod A, Holm PE, Johansen A, Brandt KK, Priemé A, Sørensen SJ. 2016. Coping with copper: legacy effect of copper on potential activity of soil bacteria following a century of exposure. FEMS microbiology ecology 92:fiw175.
42. Takai K, Horikoshi K. 2000. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Applied and Environmental Microbiology 66:5066-5072.
43. Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic acids research 41:e1-e1.
44. Jacquiod S, Cyriaque V, Riber L, Al-Soud WA, Gillan DC, Wattiez R, Sørensen SJ. 2018. Long-term industrial metal contamination unexpectedly shaped diversity and activity response of sediment microbiome. Journal of hazardous materials 344:299-307.
45. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, Brown CT, Porras-Alfaro A, Kuske CR, Tiedje JM. 2013. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic acids research 42:D633-D642.
46. Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution 33:1870-1874.
47. Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the royal statistical society Series B (Methodological):289-300.
48. Lozupone C, Lladser ME, Knights D, Stombaugh J, Knight R. 2011. UniFrac: an effective distance metric for microbial community comparison. The ISME journal 5:169.
49. Bastian M, Heymann S, Jacomy M. Gephi: an open source software for exploring and manipulating networks, Third international AAAI conference on weblogs and social media. 2009.

Figures 1-5

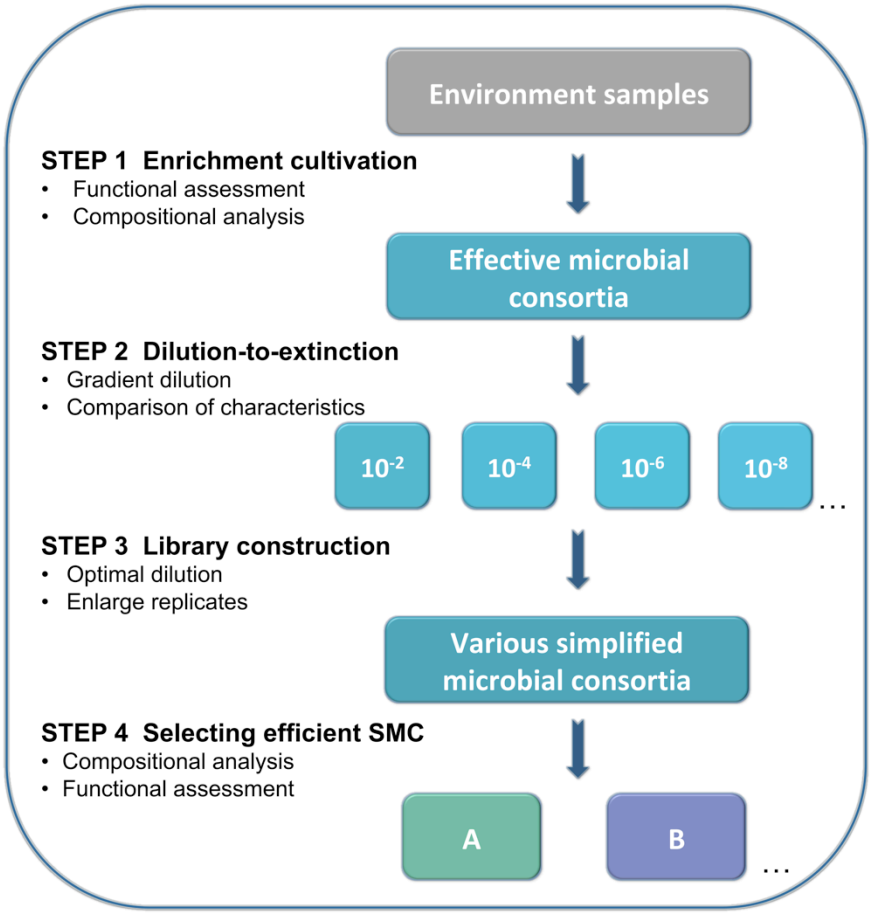

Fig. 1. Workflow of enrichment and dilution-to-extinction cultures to select simplified microbial consortia (SMC) for keratin degradation. This workflow includes four steps: 1) Enrichment for the desired traits e.g. keratinolytic activity by selection in keratin medium, where keratin is the sole carbon source. Several serial transfers were performed to obtain effective microbial consortia. This process was evaluated by functional assessment (cell density, enzymes activity, and ratio of the residual substrate) and compositional analysis. 2) Gradient dilution was conducted to the enriched effective microbial consortia. Six gradual dilutions were prepared, from dilution 10^{-2} to 10^{-10} with 24 replicates. The dissimilarity between dilutions was

539 evaluated by Euclidean distance calculation based on functional assessment criteria. 3) Library
540 construction was done from the dilution offering the optimal dissimilarity among replicates.
541 Dilution 10^{-9} was selected to construct the SMC library in this case. 4) Selection of the most
542 promising SMC is based on functional and compositional characterization.
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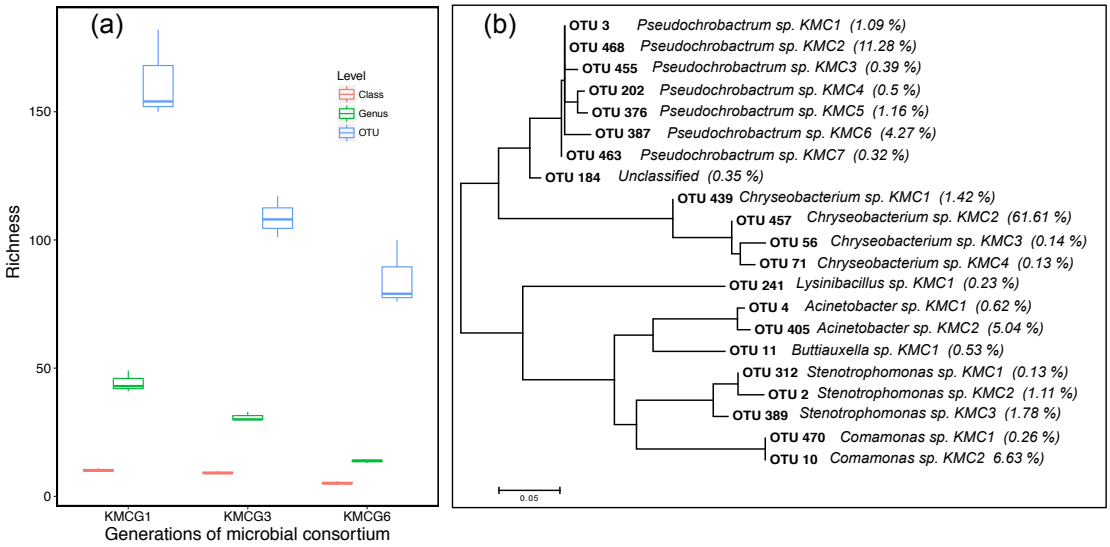
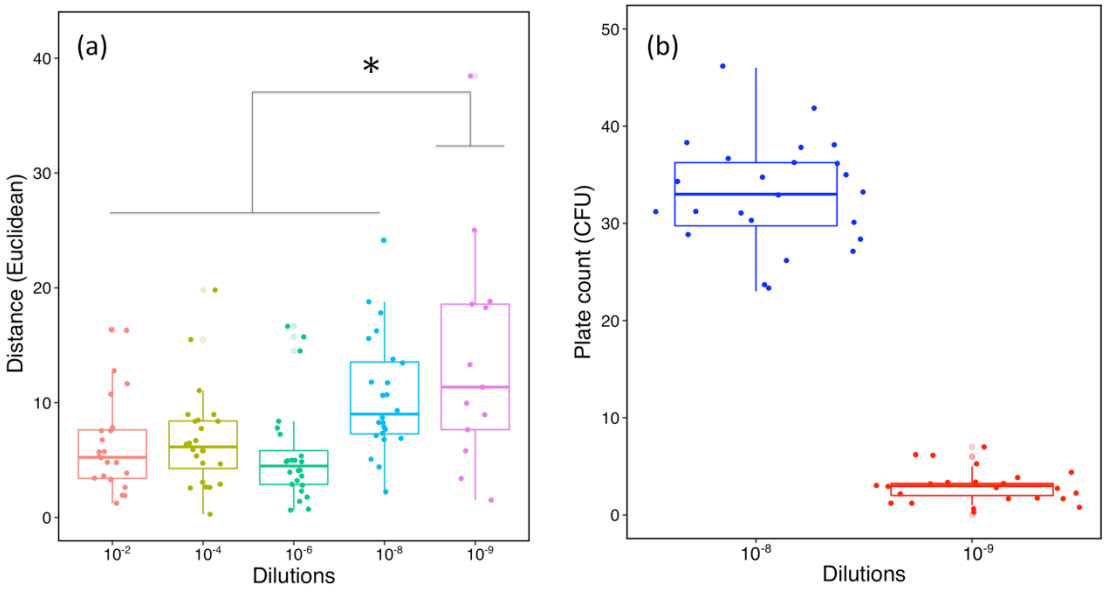


Fig. 2. Microbial diversity from the enrichment process. (a). The observed values of class, genus, and OTU from different generations in the enrichment process (KMCG1, KMCG3, and KMCG6). (b). Phylogenetic tree of dominant OTUs from KMCG6 (relative abundance > 0.1 %).



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553 **Fig. 3. Characteristic comparison of SMC from the dilution-to-extinction culture.** (a).

554 Distance comparison based on the characteristics of OD and protease and keratinase activity,

555 with Euclidean dissimilarity index (n=24). Star indicates significant statistical difference

556 (PERMANOVA, FDR < 0.1). (b). Numbers of CFU from dilution 10^{-8} and 10^{-9} by plate

557 counting (n=24).

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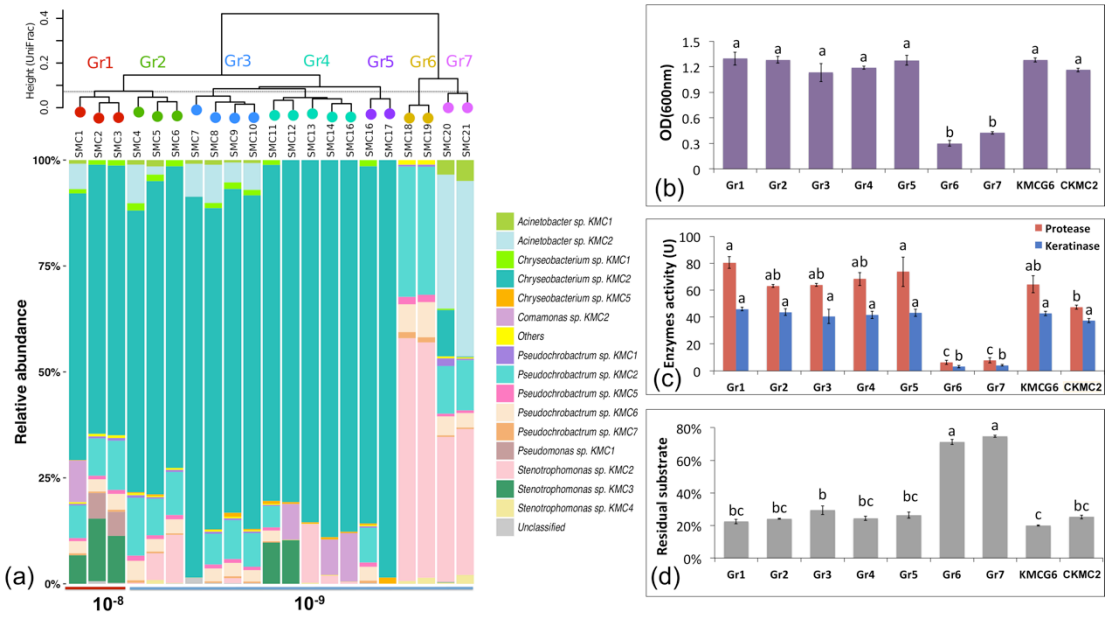

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561 **Fig. 4. Integrated comparison of selected simplified microbial consortia.** (a). 21 SMC

562 divided into seven groups based on their community similarities from 16s rRNA gene analysis

563 (weighted UniFrac distance). Group 1 (Gr1) includes SMC1, SMC2, and SMC3 from dilution

564 10^{-8}. 18 selected SMC (SMC4 – SMC21) were clustered as six groups (Gr2 – Gr7) from dilution

565 10^{-9}. (b). Cell density (OD_{600nm}) comparison of group 1-7, KMCG6 and the single strain

566 *Chryseobacterium sp. KMC2* (*CKMC2*). (c) Comparison of enzymes (protease and keratinase)

567 activity. (d) Comparison of the residual substrate ratio. Lowercase letters (e.g. a, b and c) in 4b,

568 4c and 4d refer to significant differences between groups with one-way ANOVA using post-hoc

569 Tukey's HSD test ($p < 0.05$).

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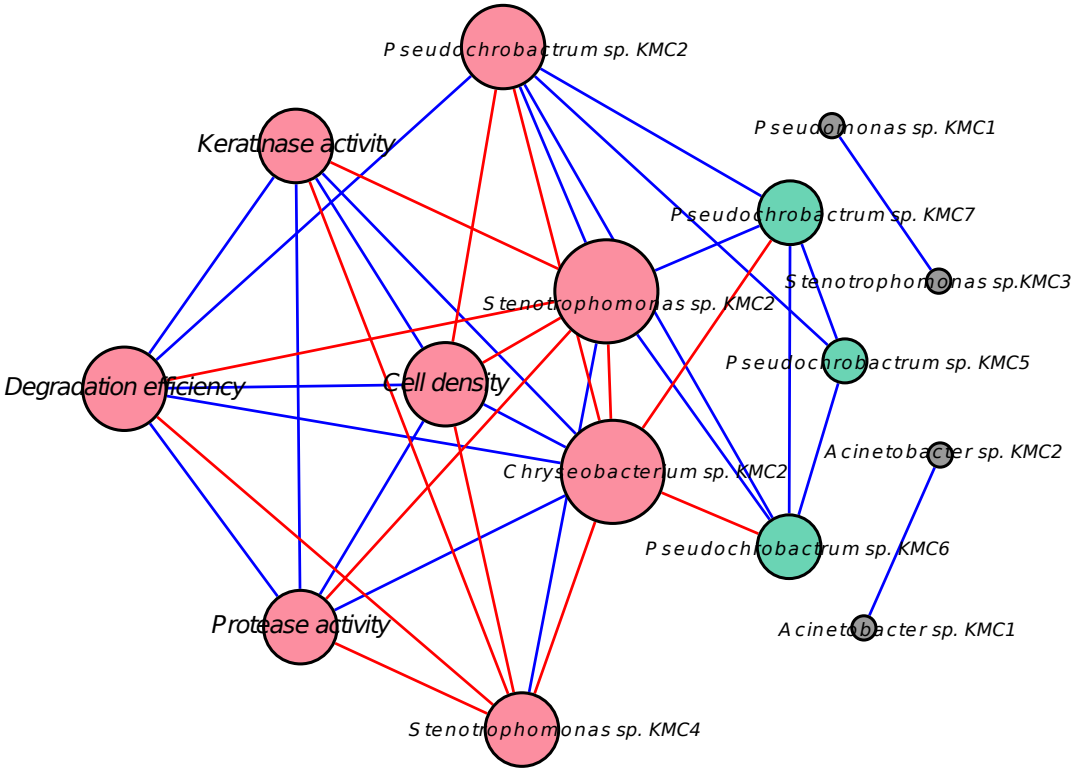

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573 **Fig. 5. Network showing relationships between strains and degradative capacity using**

574 **Pearson's correlation.** The nodes represent either OTUs or characteristics of keratin

575 degradation, e.g. degracation efficiency and keratinase activity. Red nodes indicate the direct

576 correlation with degradation efficiency (DE). Green nodes had an indirect correlation with DE

577 through other strains. Grey nodes had no significant correlation with DE. Size of the nodes

578 reflects the number of connections (degree); larger nodes have more significant connections.

579 Blue lines represent positive correlations and red lines represent negative correlation.