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Characterization of Keratin Degrading Alkaliphiles

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Abstract

Feather which mainly contains Keratin is one of the main poultry industry wastes and at the same time with high nutritional value if converted to soluble form. In this study, we isolated and characterized four alkaliphilic bacteria for their ability to degrade feather under alkaline conditions. Two of the isolates have been identified as *Lysinibacillus* spp. and both of them are growing optimally at 30 ℃ and pH 9. The others were identified as *Micrococcus* sp. and *Nocardiopsis* sp. and both of them showed optimum growth at 37 ℃ and pH 10.5. All the isolates are promising and potential tools that could be used for keratin degradation.

Keywords: Alkaliphiles, Feather, Keratin, *Lysinibacillus*, *Micrococcus* and *Nocardiopsis*

1. Introduction

Alkaliphiles are microorganisms that grow optimally at pH values above 9, but cannot grow or grow slowly near-neutral pH values. Alkaliphiles contain two main physiological groups of microorganisms; alkaliphiles and halo-alkaliphiles. Alkaliphiles grow only when pH is 9 or more and have an optimal growth pH around 10, whereas halo-alkaliphiles grow when pH is 9 or more as well as salinity is up to 33% (wt./vol.) (Horikoshi, 1999). Alkaliphiles can be isolated from neutral environments, sometimes even from acidic soil samples and feces. Halo-alkaliphiles have been mainly found in extremely alkaline-saline environments, such as the Rift Valley lakes of East Africa and the western Soda lakes of the United States

(Horikoshi, 1999).

Many valuable industrial products have been derived from alkaliphiles such as antibiotics and carotenoids(Horikoshi, 1999). They also play an important role in bio-geocycling of important inorganic compounds (Sarethy et al., 2011). Additionally, many enzymes from these organisms are widely used in many industrial applications, such as protease used as a an effective component of detergents (Kumar & Takagi, 1999). Many industries and household activities give various forms of proteinaceous wastes which may not be easily degraded and accumulated in the environment, keratinous wastes are an obvious example of these wastes (Mukhopadhyay & Chandra, 1992).

Keratin is a structural protein forming the mammalian outer most tissues like wool, nails and horns, and it could be considered as the main component of birds feather (Friedrich, Gradišar, Mandin, & Chaumont, 1999). Keratins are two types, α and β types, whereas, α keratins are two types; the soft α keratin found in epidermis and other epithelial tissues and the hard α keratins found in nail, hair, wool , claw, hoof and the horns of cattle, goats and sheep (Marshall, Orwin, & Gillespie, 1991). β-Keratins are exclusively found in reptiles and birds and it represents 90% of the barbs and barbules of the mature feather (Haake, König, & Sawyer, 1984; Kowata et al., 2014; WALKER & ROGERS, 1976). Keratin is described chemically as densely packed polypeptide strongly stabilized by several hydrogen bonds, disulfide bonds and hydrophobic interactions (Bradbury, 1973). This chemical nature gives keratin its strong stability against chemical reagents including acids, alkalis and common proteolytic enzymes such as trypsin, papain and pepsin (Mabrouk, 2008), and that led to a dramatic accumulation of keratinaceous wastes in the environment (Saha, Dhanasekaran, Shanmugapriya, & Latha, 2013). Many physical and chemical treatments could be performed for keratin degradation but this may cause environmental pollution. On the other hand, the biological treatment of such wastes provides an effective and eco-friendly tool for the removal and efficient use of these wastes.

From the north western coast of Egypt which is characterized by calcareous soil with alkaline pH and CaCO3 is dominated (Hassan, 2012), four keratin degrading bacteria have been isolated, identified and characterized morphologically and biochemically in order to use them in feather degradation.

2. Materials and Methods

2.1 Isolation of Alkaliphiles

Alkaline soil samples were collected from the north coast of Egypt on the Mediterranean Sea; the soil samples were collected in sterile bags and immediately transferred to the laboratory. A soil solution (10 %) was used as a solvent of the medium containing 10 g yeast extract and 10 g peptone. The initial pH was adjusted to 10 by 1N NaOH in order to create a selective medium for the growth of alkaliphiles only. After autoclaving, 1 ml of soil suspension (10%) was inoculated as the source of alkaliphiles and incubated at 37℃ for 48 h with 170 rpm orbital shaking.

After the incubation period, a loopful of the culture was streaked by compound streaking on a

solid medium containing the same aforementioned components. The inoculated plates were incubated at 37 ℃ for 24-48 h then the obtained colonies were purified by further streaking procedures. After all, the obtained pure colonies were harvested in glycerol stocks for further experiments.

2.2 Screening for Feather Degradation

- Feather preparation: The added feather was firstly defatted by soaking in chloroform–methanol (1:1 v/v) for 4 days with changing this solvent once a day then washed by water which subsequently air drying until getting a constant weight (Friedrich et al., 1999).

- Inoculation: the inoculation of the isolated bacteria took place in a basal medium (0.5 g NH4Cl, 0.5 g NaCl, 0.3 g K2HPO4, 0.4 g KH2PO4, 0.1 g MgCl2. 6H2O, and 0.1 g yeast extract (Lin, Lee, Casale, & Shih, 1992)) in addition to the treated feather (0.04 g) as a sole carbon and energy source , pH was adjusted to 10 using 1N NaOH. Both white and black feathers were used separately in this experiment.

The ability of each isolate to degrade keratin was tested against white and black feather. Feather degradation was observed qualitatively via weighing the remains of the dried feather after the incubation time and measuring the percentage of degradation as display in the following equation:

The % of feather degradation $= \frac{W1 - W2}{W1} * 100$ (eq.1)

Molecular identification of isolated Keratin degrading Alkaliphiles:

The isolates showed the ability to degrade keratin were identified by sequencing according to the protocol of MicroSeq® 500 16S rRNA Bacterial Identification Kits.

The Bacterial genomic DNA of each strain was isolated using the PrepMan™ Ultra Sample Preparation Reagent (PN 4322547). The genomic DNA was used as a template for PCR amplification of the 16S rRNA gene using forward primer 5'- AGTTTGATCATGGTCAG-3' and reverse primer 5' GGTTACCTTGTTACGACT 3’ (Tork, Aly, & Nawar, 2010). The PCR product of each of the tested isolates was purified and sequenced. The generated sequences were analyzed by Finch TV software and the phylogenetic tree was generated via Seaview software using the closest published type strains sequences.

2.3 Isolates Characterization

The selected isolates were characterized morphologically and biochemically, also motility test in addition to optimal temperature and pH were detected. The pH and temperatures were tested at various conditions at the same time using Central Composite Design (CCD) matrix to determine the optimum temperature and pH required for the best growth of the selected isolates. The tested levels of pH and temperatures are listed in Table 1.

Morphologically, they were examined by Gram staining. Biochemically many tests were

performed (including; carbohydrates fermentation, IMViC tests and caseinase and lipase production) (Healing, 1993).

Table 1. Different pH and temperature levels tested during optimization using Central Composite Design (CCD) experiment

Variables	Tested Levels				
	-2	-1	0	+1	+2
pH	7	8	9	10	11
Temperature (℃)	20	25	30	35	40

3. Results

3.1 Isolation of Keratin Degrading Alkaliphiles

Twelve bacterial strains were isolated under the alkaline condition (pH up to 10) and selected according to the morphological differences. All the obtained isolates were tested for keratin degradation in a medium containing keratin as a sole carbon source. Among these isolates only four isolates (MFNC2, MFNC5, MFNCA and MFNCY) showed the ability to degrade both white and black feather and the degradation percent is indicated in table 2. These four isolates were subjected to molecular identification and were used for further studies.

Table 2. Feather degradation % of all the tested isolate

Isolates names	Degradation % with White feather	Degradation % with Black feather
MFNC 2	15	8.2
MFNC 5	9.1	3.2
MFNCA	5.5	18
MFNCY	25.1	32

3.2 Molecular Identification of Isolated Keratin Degrading Alkaliphiles

The four selected isolates were identified via analyzing the sequence of the 16S rRNA gene, the 16S rRNA. The obtained sequence for each of them was compared to type strains obtained from The Ribosomal Database Project (RDP) using sequence match tool and using the BLAST program in the GenBank database in the National Center for Biotechnology Information (NCBI). The 16S rRNA sequence of isolates MFNC2 and MFNC5 showed high levels of sequence similarity (99.6 % and 99.8 % respectively) with members of the genus *Lysinibacillus* and they have been given the names *Lysinibacillus* MFNC2 and *Lysinibacillus* MFNC5. The isolate MFNCA showed high sequence similarity (98.6%) with members of genus *Nocardiopsis* and given the name *Nocardiopsis* MFNCA. The isolate MFNCY showed high level of sequence similarity (98.9 %) with members of genus *Micrococcus* and given the name *Micrococcus* MFNCY. The four isolates; MFNC2, MFNC5, MFNCA and MFNCY were submitted to gene bank with accession numbers; KT803878, KT803879, KT803881 and KT803880 respectively. The phylogenic trees of the identified isolates are shown in figures 1,2 and 3.

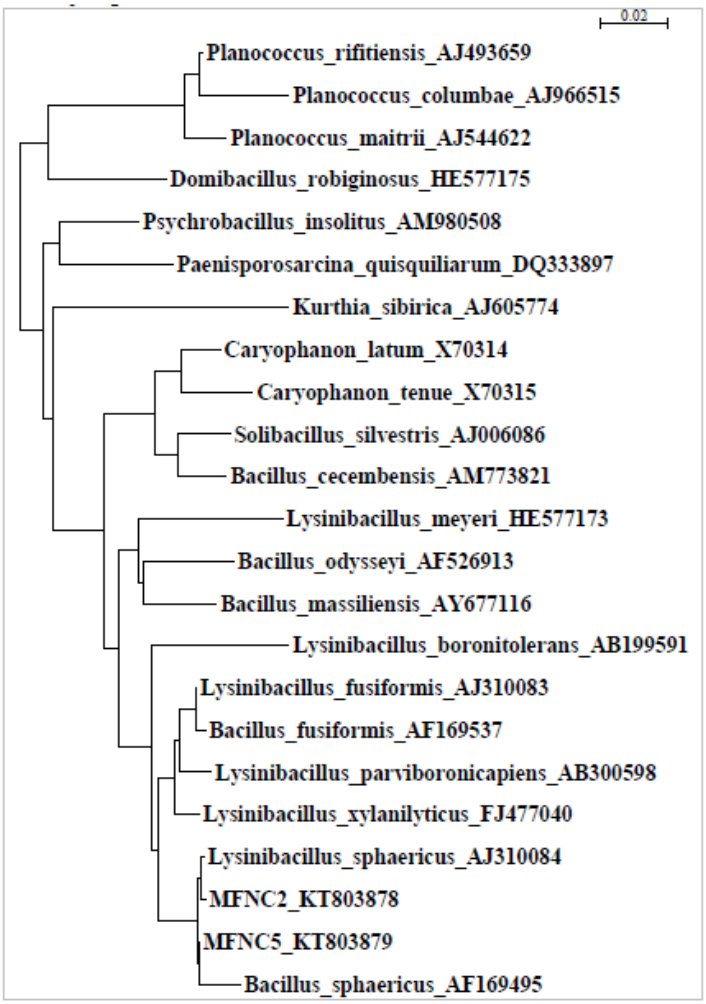

Figure1. The phylogenic tree of the strains MFNC2 & MFNC5 based on the full 16S rRNA gene sequences using the distance methods.

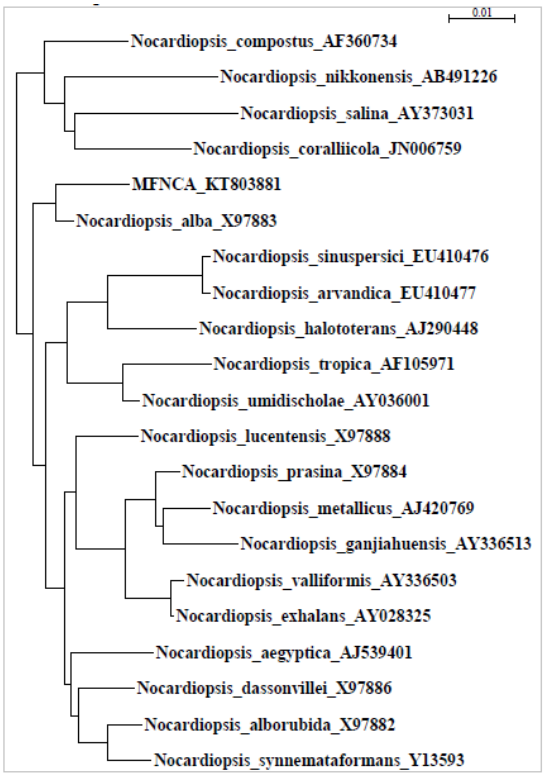

Figure 2. The phylogenic tree of the strain MFNCA based on the full 16S rRNA gene sequences using the distance methods.

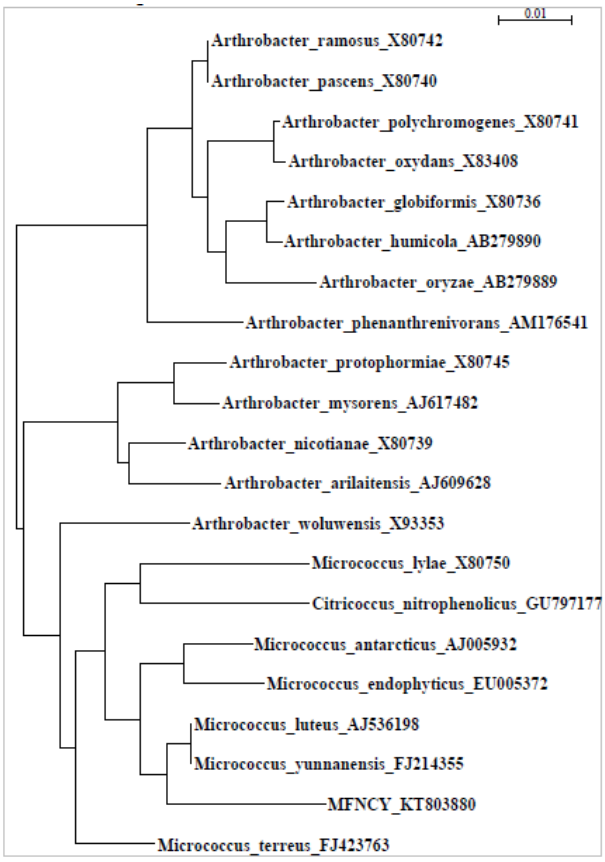

Figure 3. The phylogenic tree of the strain MFNCY based on the full 16S rRNA gene sequences using the distance methods.

3.3 Isolates Characterization

Morphological examination of the selected isolates showed different characters of the isolates as listed in table 3. Also biochemical tests which were performed were listed in table 4.

Table 3. Morphological characters of the isolates

Isolate name	Gram stain	Colony shape	Motility
Lysinibacillus MFNC2	Gram negative, Bacilli	Creamy colored colonies with irregular edges	Non-motile
Lysinibacillus MFNC5	Gram negative, Bacilli	Creamy colored colonies with irregular edges	Non-motile
Nocardiopsis MFNCA	Gram positive fungus-like branched networks of hyphea	white aerial mycelium and creamy substrate mycelium. And the colonies were of irregular edges	Non-motile
Micrococcus MFNCY	Gram positive, Tetracocci	Rounded yellowish single colonies	Non-motile

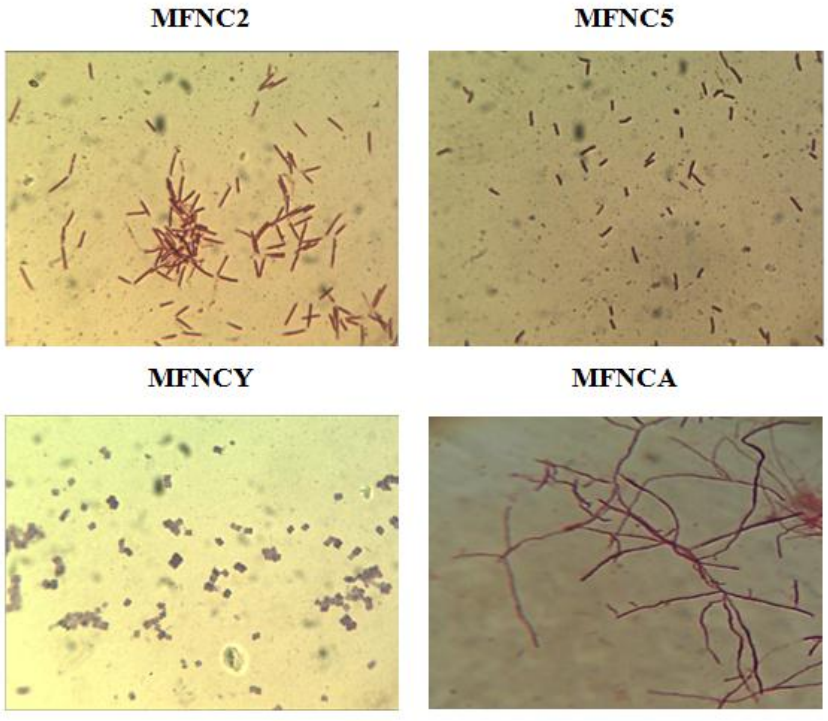

Figure 4. Gram staining of the four selected isolates.

Table 4. Biochemical characters of the isolates

Biochemical test	*Lysinibacillus* MFNC2	*Lysinibacillus* MFNC5	*Nocardiopsis* MFNCA	*Micrococcus* MFNCY
TSI	R/Y	Y/Y	R/Y	R/R
Glucose ferment.	+	+	+	-
Lactose ferment.	-	+	-	-
Sucrose ferment.	-	-	-	-
Ammonia production	+	-	+	+
Gas production	-	-	-	-
H2S	-	-	-	-
Citrate	-	-	-	-
Urease	-	+	-	-
Catalase	+	+	+	-
MR	-	+	-	-
VP	-	+	-	-
Indol	-	-	-	-
Caseinase	+	+	+	+
Lipase	-	-	-	-

The optimum pH and temperature for the isolates were determined at the same time using Central Composite Design (CCD) matrix, the results were taken as A_{600} for all isolates except for *Nocardiopsis* MFNCA which was evaluated by dry weight.

As showed in figure 5, *Lysinibacillus* MFNC2 and *Lysinibacillus* MFNC5 were able to grow optimally at 30 ℃ and pH 9 and grow well at temperature range of 20 ℃ to about 40 ℃ and pH range from 7.5 to 10.5, *Nocardiopsis* MFNCA showed optimum growth at 37 ℃ and pH 10 and grow well at temperature range from 25 ℃ to 45 ℃. Finally, the isolate *Micrococcus* MFNCY was grow optimally at temperature 37 ℃ and pH range 10 to 11 and grow well in temperature range 25 ℃ to 45 ℃ and pH range 8 to 11.

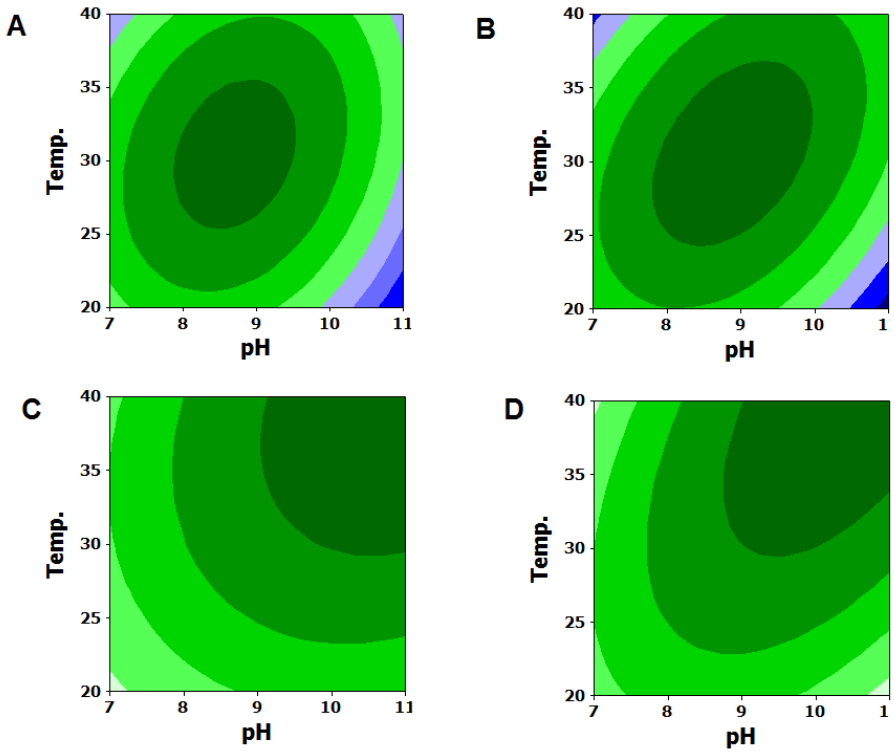

Figure 5. Contour plots of O.D and dry weight for the isolates vs temperature and pH, A: *lysinibacillus* MFNC2, B: *lysinibacillus* MFNC5, C: *Micrococcus* MFNCY, D: *Nocardiopsis* MFNCA.

4. Discussion

Biological treatment of feather was found to be the most suitable alternative not only because it allows the removal of feather which is intensively attained from poultry industry in a clean way but it also produces the high nutritional value feather meal which might be introduced again to animal feed (Saha et al., 2013) or used as organic fertilizer (Paul et al., 2013). This process can be performed by fungi (e.g. *Cunninghamella echinulata*), actinomycetes (e.g. *Streptomyces* spp.), or bacteria (e.g. *Bacillus subtilis* and *Bacillus licheniformis*) (Gopinath et al., 2015; More, 2013; Selvam, Vishnupriya, & Yamuna, 2013). Feather degradation mainly depends on keretinase enzymes which are responsible for releasing peptides from keratin (Nickerson & Durand, 1963) after reduction of native keratine to reduced keratin by disulfite reductase enzyme (Sangali & Brandelli, 2000).

This study represents four bacterial strains for their ability to degrade feather in a whole cell fermentation process. The organisms were isolated from alkaline environments and unsurprisingly, they all showed a tendency to grow optimally at alkaline conditions close to 11 in case of *Micrococcus* MFNCY. This strain showed the ability to grow in pH close to 13 (data not shown).

The four strains in the study succeeded to degrade the feather in different degrees, *Lysinibacillus* MFNC5 was the weakest organism in degradation as it degraded 9.1 % and 3.2 % of the white and black feather respectively, while *Micrococcus* MFNCY was the most

promising organism as it degraded 25.1 % and 32 % of the white and black feather respectively, a result that would be improved after optimization via further experiments. Additionally, biochemical characterization of these strains indicates the production of some other important enzymes (e.g. urease, caseinase and catalase) which can be considered as a target for further studies.

In addition to the keratinolytic activity of *lysinibacillus* which previously explained, it characterized by its ability to produce insecticidal toxins (Specifically *Lysinibacillus sphaericus*) so this organism widely used as insecticide instead of using chemicals that have adverse effects on humans, other animals, and aquatic life (Berry, 2012). Many reports about *Nocardiopsis* showed its ability to degrade feather (Saha et al., 2013; Suneetha, Kumar, & Nimesh, 2014). Many members of *Micrococcus* have been isolated from poultry feather waste, these organisms confirmed to produce proteases and showed its ability to degrade feather (Laba, Choinska, Rodziewicz, & Piegza, 2015).

These organisms showed various degrees of both white and black feather degradation indicating different abilities due to the difference in number, activity, and expression level of enzymes induced under these conditions. The use of these organisms in co-culture strategy along with optimization of different culturing conditions would lead us to better degradation of feather, a study that is already performed (data not shown). Furthermore, the molecular characterization of the enzymes produced in presence of feather is currently under investigation. The use of these organisms, in whole cell assays, for feather degradation as well as the characterization of their enzymes would open the door for further industrial applications that might be performed under alkaline conditions.

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