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JOURNAL OF APPLIED SCIENCES RESEARCH

JOURNAL home page: http://www.aensiweb.com/jasr.html 2014 January; 10(1): pages 46-52.

Published Online :15 January 2014 **Research Article**

Enhanced Keratinase Production and Feathers Degradation by a Mutant Strain of *Bacillus subtilis*

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Received: 12 November 2013; **Revised:** 14 December, 2013; **Accepted:** 20 December 2013.

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ABSTRACT

Keratin-rich wastes in the form of feathers, hair, nails, and horn are highly available as byproducts of agro-industrial processing. The increased needs for energy conserving and recycling, summed with the huge increase in poultry industry, have strongly stimulated the search for alternatives for the management of recalcitrant keratinous wastes. keratinases, which are produced by several bacteria that have been often isolated from soils and poultry wastes, show potential use in biotechnological processes involving keratin hydrolysis. Different bacterial strains were collected and isolated, then tested for their keratinase activity. The best keratinas producer was selected and identification morphologically, physiologically and by 16S rRNA sequencing was identified as *Bacillus subtilis* NRC-MGD-K34. The present study is focused on the improvement of *Bacillus subtilis* for keratinase production; chemical mutagensis using ethyl methane sulphonate (EMS 25, 50, 75; 100 µl/ml) was undertaken for 20 min. Ten mutants were isolated from *B.s*. They had show improved keratinase according to increasing the clear zone around their colonies grown on a solidified screening medium containing 5% casein, more feathers degradation and keratinase specific activities. The best mutant obtained was M3 mutant which had 27.44 units/ml enzyme activity which was more than three folds the wild type. SDS-PAEG protein banding patterns and analysis of the wild type and their mutants were done.

Keywords: Bacillus subtilis, mutation, EMS, Keratinase assay, protein analyses, 16S rDNA sequencing.

INTRODUCTION

Feathers are composed of over 90% protein and produced in large amounts as a waste by poultry processing worldwide. Accumulation of feathers will lead to environmental pollution and feather protein wastage [23,8]. Traditional ways to degrade feathers such as alkali hydrolysis and steam pressure cooking may not only destroy the amino acids but also consume large amounts of energy. Biodegradation of feathers by keratinase from microorganisms may provide a viable alternative. *Bacillus* [34,29,20,5], fungi [9,7] and *Actinomycetes* [14,8] have previously been shown to be able to produce feather-degrading keratinases.

Keratinase and related products have many applications [12]. For example, the feather hydrolysates of *Bacillus licheniformis* PWD-1 and *Vibrio* sp. strain kr2 [35,11] can be used as feed additives, while the keratinase from *Bacillus subtilis* S14 exhibits remarkable dehairing capabilities [19]. Moreover, keratinase from *B. licheniformis* PWD-1 can degrade the infectious form of prion, PrP^{sc}, in the presence of detergents and heat treatment [18], which is very important for the utilization of animal meal as feed. Usually, it is important to improve the enzyme yield for application purposes and so various methods including the optimization of cultural conditions and medium composition, or heterologous gene expression have been applied [28,2]. Given the effectiveness of traditional mutagenesis approach for isolating mutants that produce improved yields of various microbial enzymes such as lipase and α-galactosidase [31,32], it is conceivable that a similar strategy may be successfully applied to improve the ability of keratinase-producing strains for the production of this important enzyme.

Almost all keratinases are inducible and different keratin-containing materials such as feathers, hair and wool can be used as substrates for keratinase production [12].

Ethyl methyl sulphonate (EMS) exerts its effect by random mutation in genetic materials by nucleotide substitution particularly by guanine alkylation, for augmenting alkaline b-keratinase production by *Brevibacillus* sp. strain AS-S10-II in submerged fermentaion [25,33].

Successful application of MNNG as a mutagenesis tool to generate, from a wild-type keratinase-producing *B. subtilis* strain, mutants with elevated keratinolytic activity and their convenient screening on casein agar plates were indicated by Cai *et al*., [4].

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This study aimed to improve keratinase - producing microbial strain through mutagenesis.

Materials and Methods

Bacterial strain:

Bacillus subtilis NRC-MGD-K34 (identified in this study) was selected among different bacterial strains from the available stock culture at Microbial Genetics Dept., National Research Center, as high keratinase production strain.

Identification of Bacterial isolate:

Initial identification of the isolated microorganism was performed based on their morphological, physiological and biochemical characteristics Bergey's Manual of Systematic Determinative Bacteriology [16,30].

Molecular identification of bacterial isolate:

The 16S rDNA region was partially amplified (approx 1000 bp) by polymerase chain reaction (PCR). Bacterial isolates were cultivated on LB plates at 37°C for 18 hours. Crude lysate of cells [24] (fresh preparation) was obtained and subjected to PCR amplification using the forward primer (FP) 5'-AGAGTTTGATCATGGCTCAG-3' and the reverse primer (RP) 5'- GGTTACCTTGTTACGACTT-3'. Three µl of cell lysate were amplified in a 50 µl reaction mixture by using DreamTaq Green PCR Master Mix (Fermentas). The PCR was performed by using DNA thermal cycler (NYXTECHNIK).

The PCR program consisted of one cycle of DNA denaturation at 95°C (5 min.), 35 cycles of 95°C (2 min), 48°C (1 min) and 72°C (4 min), plus one additional cycle of a final chain elongation at 72°C (20 min).

A sample of PCR product was analyzed using agarose gel electrophoresis and compared with 1Kb Ladder DNA marker (300 bp-10000 bp) (AXYGEN).

The PCR product was purified using Ron's PCR-Pure Kit (BIORON) and sequenced (MACROGEN).

Culture media:

The basic medium used for isolation and fermentation of the feather-degrading microorganism contained the following constituents (g/L): NaCl (0.5), KH_2PO_4 (0.7), K_2HPO_4 (1.4), $MgSO_4$ (0.1) and feathers (10), pH 7.2. Cultivation was done using 500 ml Erlenmeyer flasks containing 100 ml medium. Feather agar medium containing the basic medium and 20 g/L of agar was used for screening the microorganism in plates. For the medium used for mutants screening, 10 g casein was used instead of feathers. Luria-Bertani (LB) medium (Trypton 1% (w/v), yeast extract 0.5% (w/v), NaCl 0.05% (w/v), pH 7.2) was used for inoculum preparation and bacteria maintenance Cai *et al.*, [4].

Mutagenesis and screening:

Bacillus subtilis NRC-MGD-K34 strain was cultivated in LB medium at 37 °C for overnight, followed by centrifuging 10 ml of the culture at 4000 rpm for 15 min. The cell pellet was diluted in 0.1 mol/L sterile phosphate buffer (pH 7.2) and adjusted to a concentration of 10^6 /ml; then 1 ml of the cell suspension was incubated with (25, 50, 75 and 100 µl) EMS solution at room temperature for 20 min. Finally the reactions were stopped, 100 µl serially diluted aliquots were plated on casein plates and cultivated at 37 °C for 48 h.

For determination of the keratinolytic activity, flask cultivation was carried out at 37 °C and 200 r/min for 30 h.

Keratinolytic activity determination:

The keratinolytic activity was assayed as follows: 1.0 ml of crude enzyme properly diluted in Tris-HCl buffer (0.05 mol/L, pH 8.0) was incubated with 1 ml keratin solution at 50 °C in a water bath for 10 min, and the reaction was stopped by adding 2.0 ml 0.4 mol/L trichloroacetic acid (TAC). After centrifugation at 4000 rpm for 30 min, the absorbance of the supernatant was determined at 280 nm against a control. The control was prepared by incubating the enzyme solution with 2.0 ml TCA without the addition of keratin solution. One unit (U/ml) of keratinolytic activity was defined as an increase of corrected absorbance of 280 nm [9] with the control for 0.01 per minute under the conditions described above and calculated by the following equation:

$$U=4\times n\times A280/(0.01\times 10)$$

Where n is the dilution rate; 4 is the final reaction volume (ml); 10 is the incubation time (min) according to Cai *et al*., [4].

Determination of residual hydrolysates:

The residual hydrolysates were composed of cells, amino acids, small peptides and undigested feathers. After cultivation, cultures containing the residual hydrolysates were centrifuged at 5000 rpm, and filtered through Witman filter paper, and then dried to a constant weight. The weight of the residual hydrolysates was determined by subtracting the weight of the filter paper according to Cai *et al.*, [4].

SDS-PAGE protein analysis:

Total protein NRC-MGD-K34 strain was extracted and analyzed as described by Horvath and Riezman [13]. Luria-Bertani (LB) medium (100 ml) was inoculated into 500-ml flasks with one loop of bacterium and shake for 48 hours at 220 rpm at 30°C. The suspension was centrifuged for 10 min at 10,000 rpm at 4°C. The pellet was washed twice with high salt TNT-1 buffer (50 mM Tris HCl pH 7.5, 1.0 M

NaCl and 0.05% Triton X-100) followed by two washes with TNT-2 buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl and 0.05% Triton X-100). One ml of aliquot was solubilized by heating in Laemmli buffer (10% (w/w) glycerol, 5% (w/w) β-mercaptoethanol, 1% (w/v) SDS, 0.188 M Tris-HCl pH 6.8 and 0.01% (v/v) bromphenol blue) at 100°C for 5 min. The aliquot was centrifuged at 13,000 rpm for 5 min and the supernatant containing solubilized proteins was fractionated by size using 15% SDS-PAGE according to Laemmli [17] to compare the products secreted by the parental strains and those secreted by the new fusant strains. After size fractionation, the proteins were visualized by staining with Coomassie Blue R-250 dye [27].

The electrophoresis condition and gel staining:

It was preformed according to Rabilloud *et al*., [27].

Results and Discussion

Different bacterial strains were collected or isolated from different locations in Egypt. They were tested for their proteases and keratinase activities. The highest keratinase activity was selected and used mutagenesis treatments to select over-producer keratinase mutants. This isolate was identified and named *Bacillus subtilis* NRC-MGD-K34 through different morphological, physiological and chemical identification following the classification of Bergey's Manual of Systematic Determinative Bacteriology [16,30].

Further identification was done by isolation of the isolate's 16SrRNA gene and its partial sequencing (Fig. 1). The obtained partial DNA sequences of 16S rRNA was analyzed through search a nucleotide database using NCBI/BLAST/BLASTN (http:// www.ncbi.nlm.nih.gov/blast/Blast.cgi). The database indicated 99% to *Bacillus subtilis* subsp. *spizizenii*.

GGCACGTGGCGGCGTGCTATACATGCAGTCGAGCGGACAGATGGGAGCTTGCTCCCTGATGTTAG
CGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGG
GGCTAATACCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTT
ACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCAACGATGCGTA
GCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGT
TTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGAC
GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCC
CGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATT
CCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGT
CTGTAACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAA
GCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGA
CAATCCTAGAGATAGGACGTCCCCTTCCGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCT
CTTGTCCTGG

Fig. 1: 16S rRNA DNA partial sequence of *Bacillus subtilis* NRC- MGD - K34.

Induction of mutation:

In the strategy adopted to improved keratinase activity of *Bacillus subtilis* NRC-MGD-K34 strain, classical mutagensis was applied. Ethylmethan sulphonate (EMS) as a chemical mutagen was used at 25, 50, 75 and 100 µl for 20 min. Table 1 shows number of *Bacillus subtilis* survival and its mutation. Results indicted that number of survival was decreased by increasing the EMS concentration. Some researchers have employed random mutagensis for keratinase studies by exposing the bacterial culture to chemical mutagenesis like EMS [22]. Prescott and Dunn's [22] and French *et al* (2006) reported that EMS was a powerful chemical mutagen and its effect on cells is related to its concentration in a medium. Our finding about mutagenesis induced by EMS was in good agreement with the above reports and confirmed the usefulness of this potent mutagenic agent for inducing mutagenesis in *Bacillus*. Low frequency of survival and mutation may be due to the high toxicity of EMS rather than its mutagenic effect; the toxicity of EMS explained as it disrupts many metabolic processes, it inhibits the amino acid accepting ability of tRNA and the synthesis of protein and interferes with the action of variety of enzymes [21].

Survived colonies were randomly selected and tested on casein plates after incubation at 30ºC for 24 h. Those which produce more clear zones than the WT were selected as keratinase modified mutants (Fig. 2).

Keratinase efficiencies were studied in *Bacillus subtilis* NRC-MGD-K34 and ten of its mutants where residual feather, casein hydrolysis, keratinase specific activity and amino acids production were determined (Table 2).

Table 1: Effect of different concentrations EMS on No. of survivals and mutation of *Bacillus subtilis* NRC-MGD-K34 strain.

	EMS concentration µl/ml				
	0.0	25.0	50.0	75.0	100.0
No. of survival	200 x 10^4	161 x10^4	150 x 10^4	150 x10^4	99 x 10^4
No. of mutation	0.0	3	7	7	10

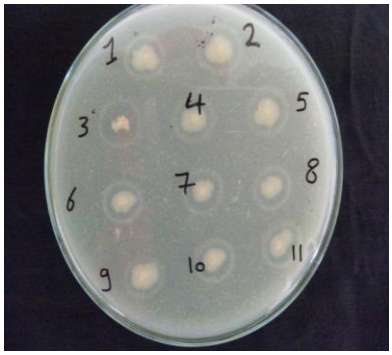

Fig. 2: Keratinase activity as indicated by the clear zone of casein hydrolyses of W.T (11) and EMS mutants of *Bacillus subtilis* NRC-MGD-K34 strain.

Table 2: Keratinase activities of W.T and mutants of *Bacillus subtilis* NRC-MGD-K34 strain.

B. subtilis mutants	Residual feathers (gram)	Clear zones of Casein* (cm)	Keratinase (units/ml)	Amino acid (O.D $_{280}$)
W.T	0.21	0.3	8.88	0.101
M1	0.19	0.6	17.08	0.160
M2	0.16	0.5	20.96	0.171
M3	0.15	1.1	27.44	0.177
M4	0.13	0.6	14.24	0.151
M5	0.19	0.7	10.24	0.105
M6	0.18	0.7	10.20	0.133
M7	0.15	0.9	15.24	0.165
M8	0.19	0.8	18.88	0.167
M9	0.18	0.4	14.16	0.168
M10	0.19	0.8	15.28	0.128

* Clear zones of casein hydrolysis after 48 hours

Table (2) showed that all tested mutants were more efficient in feathers degradation. The best feathers hydrolyses was the mutant M4 where only 0.13 g of feather was remained which was less than the WT by about 62% followed by M3 and M7 mutants. In casein hydrolyses, as indicator for proteases activity, all mutant produce higher clear zones than the WT. The best casein hydrolysis was mutant M3 followed by M7 and M8 mutants. For keratinase specific activity, the *Bacillus subtilis* NRC-MGD-K34 wild type only produced 8.8 Units /ml. All mutants had higher keratinase activities ranged from 1.2 to more than 3 folds the wild type. The best muatant was M3 in keratinase activity. For amino acids concentration, the OD_{280} reading was used as indicator. The residual amino acids after feathers hydrolyses represents the remaining amino acids after its consumption for bacterial growth. The best amino acids producer was M3 mutant where OD_{280} reading was 0.177 comparing with the WT which produce only OD_{280} reading 0.101.

SDS-PAGE protein fingerprinting:

Protein banding Patterns of ten mutants are illustrated in Fig. 3 and Table (3). There were observable differences in the protein banding pattern for all ten mutants and the wild type. The results of SDS-PAGE analysis revealed that, the monomorphic bands (0), polymorphic without unique (22 bands), unique bands (5), polymorphic with unique (27 bands), total number of bands and polymorphism equal 100%. Zhang *et al*. (2009) reported that some minor differences in banding patterns between the control and the mutants which resulting from the EMS concentration 100 µl/ml. The molecular weight ranged from 4.1 - 66 kDs. There were common banding found in all strains. Andersson (1980) reported EMS is a mutagenic agent that induces point mutations in a DNA molecule by A-T transition to G-C. In the presence of EMS, native sequences of genes are changed and their related products are structurally modified, causing in-activation of functional proteins. On the other hand, significant differences were determined as three types (mono & poly & unique) as shown in Table (3). The similarity was approximately 40% between the mutants M2, M3, M5 and Ml0 and Bs W.T and the similarity approximately 60% between the mutants M1, M4, M6, M7, M8, M9 and B.s W.T such results were obtained by Kawasaki *et al* [15].

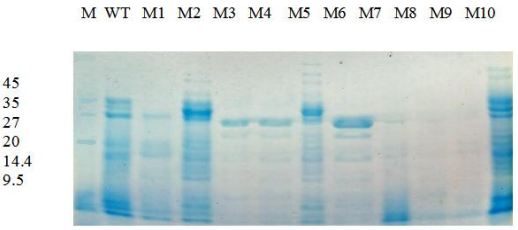

Fig. 3: SDS-PAGE protein banding patterns of *Bacillus subtilis* strain and its selected mutants after treatment with different concentration of EMS.

Table 3: Protein analysis of *Bacillus subtilis* strain (W.T) and some of its mutants.

No. of bands	MW (kDa)	W.T	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	Polymorphism
1	66	-	-	-	-	-	+	-	-	-	-	+	Polymorphic
2	61	-	-	-	-	-	+	-	-	-	-	+	Polymorphic
3	5.6	-	-	+	+	-	+	-	-	-	-	-	Polymorphic
4	45	+	-	+	+	-	+	-	-	-	-	+	Polymorphic
5	39.9	+	-	+	+	-	-	-	-	-	-	+	Polymorphic
6	37.7	-	-	+	+	-	+	-	-	-	-	-	Polymorphic
7	37.7	+	+	+	+	-	+	-	+	-	-	+	Polymorphic
8	35	-	-	-	-	+	-	+	+	+	-	-	Polymorphic
9	32.2	-	-	-	-	-	-	+	-	-	-	+	Polymorphic
10	28.2	-	-	-	-	+	+	+	-	-	-	+	Polymorphic
11	27	+	-	+	+	-	+	-	-	-	+	+	Polymorphic
12	24.5	-	+	+	+	-	+	-	+	-	-	-	Polymorphic
13	21.9	+	+	-	-	+	-	-	+	+	+	+	Polymorphic
14	21.2	-	-	+	+	+	+	+	-	-	-	+	Polymorphic
15	21	-	-	+	+	-	+	-	-	-	-	+	Polymorphic
16	20.8	+	+	+	+	-	+	-	-	-	-	+	Polymorphic
17	20.5	+	+	+	+	-	+	+	-	+	-	+	Polymorphic
18	20	-	-	+	+	+	+	-	-	+	-	+	Polymorphic
19	18	+	-	+	+	-	+	+	-	-	+	-	Polymorphic
20	14.4	-	-	-	-	-	-	+	-	-	-	-	Unique
21	9.5	+	+	+	+	-	+	-	-	-	-	+	Polymorphic
22	8	-	+	-	-	-	-	-	-	-	-	-	Unique
23	7	+	+	+	+	-	+	+	+	+	+	-	Polymorphic
24	6.5	-	-	+	+	+	+	+	+	-	+	-	Polymorphic
25	6	-	-	-	-	-	-	-	-	-	+	+	Unique
26	5	-	-	-	-	-	-	-	-	-	-	+	Unique
27	4.1	-	-	-	-	-	-	-	-	-	-	+	Unique
Total bands		10	8	16	16	6	18	9	6	5	6	18	

(+): The existence of protein band and (-) absence of protein band

The difference in protein banding pattern reflects mutation differences among obtained mutants. One mutation could produce or prevent more than one peptide if this mutation occurred in a regulatory sequence. The same could be happened if the mutation occurred in a branched pathway and hence effect different protein production.

Conclusion:

In this paper we report the successful application of EMS as mutagenesis tool to generate, from a wild-type keratinase-producing *B. sabtitis* NRC-MGD-K34 strain, mutants with elevated keratinolytic activity and their convenient screening on casein agar plates. They had show ability of feather degradation by making a clear zone around their colonies grown on a solidified screening medium containing 5% casein and mineral salts. There were observable differences in the protein banding pattern for all ten mutants and the wild type.

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