CLONING, SEQUENCING AND EXPRESSION OF *KERATINASE* GENE FROM INDIGENOUS *Bacillus licheniformis* N5 STRAIN

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Feather contains more than 90% protein. Large amounts of feathers are produced by poultry slaughterhouse worldwide. In the United States alone, over one million tons of feathers are produced yearly (Chandler, 2007). Additionally, approximately, 950 metric tons of feathers are generated in Germany per year (EDFA, 2007).

Accumulation of feathers leads to environmental pollution and feather- protein wastage. Alkali hydrolysis and cooking under steam pressure are the traditional ways to degrade feathers. They have many disadvantages such as destroying the amino acids; consume large amounts of energy and decrease protein quality and digestibility (Riffel and Brandelli, 2006). The enzymatic hydrolysis of feather may be a viable alternative to steam pressure cooking (Grazziotin *et al*., 2006).

Keratin is a fibrous and insoluble structural protein extensively cross linked with hydrogen, disulphide and hydrophobic bonds. It is the major component of the epidermis and its appendages viz. (Anbu *et al*., 2007). Feather keratin exhibits an elevated content of several amino acids such as glycine, alanine, serine, cysteine and valine. The commonly known proteolytic enzymes could not degrade proteins due to their intensive cross-linkage (Gupta and Ramnani, 2006). Keratinase of *Bacillus licheniformis* and *Bacillus subtilis* are effectiveness in feather degradation (Manczinger *et al*., 2003).

Keratinases belong to the group of serine proteases capable of degrading keratin. It is an enzyme produced in a medium containing keratinous substrates such as feathers. Keratinases have many applications in traditional industrial sectors including feed for livestock (Grazziotin *et al.*, 2006), detergent, medicine, cosmetics and leather manufacturers (Farag and Hassan, 2004), in more recent field Keratinase is used for treatment of the mad cow disease (Langeveld *et al*., 2003). Biodegradable plastic manufacture, feather meal production, enhance drug delivery in some tissues arise as novel potentially high impact of enzyme applications (Brandelli, 2007). Although many applications of keratinases are still in the stage of infancy, a few have found their way to commercialization, particularly the use of bioresource for feather meal production. The crude enzyme can also serve as a nutraceutical product, leading to significant improvement in broiler performance (Odetallah *et al*., 2003).

A number of keratinolytic microorganisms have been reported, including some species of *Bacillus* (Tamilmani *et*

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al., 2008), Actinomycetes (Bockle *et al*., 1995) and some fungi (Gradisar *et al*., 2005). Total amino acids digestibility of raw feathers and commercial feathers meal has been increased by using *B. licheniformis* crude keratinase enzyme (Lee *et al.*, 1991). It was cited by Odetallah *et al.* (2003) that the digestibility of commercial feather meal had been increased after keratinase treatment and could replace up to 7% of the dietary protein for growing chicks. By using feathers as substrates, essential amino acids as well as ammonia were produced.

The aim of the present study is to improve keratinase producing activity of some bacterial strains through gene manipulation of the indigenous *Bacillus licheniformis* N5 strain.

MATERIALS ADND METHODS

Bacterial strains, media and plasmid

Strains

Different bacterial strains were collected and gathered from different sources and tested for their protease and feather degrading activities. Table (1) represents the collected strains used in the present studies. *E. coli* DH5α was used in transformation experiments.

Media

Lauria-Bertani medium (LB) (Davis *et al*., 1980) was used for bacterial growth. Minimal medium (Cai *et al*. 2008) consists of (g/l): NaCl (0.5) - K_2HPO_4 (1.4) - KH_2PO_4 (0.7) - $MgSO_4$ (0.1) supplemented with 10 g/l of feather or casein.

Plasmid

The plasmid vector pJET1.2 (Fermentas life sciences), which allows positive selection after cloning (the vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site), was used for cloning trials.

Bacterial screening for feather degradation

Keratinase producing bacteria were screened using the method described by Cai *et al.* (2008). Tested strains were grown in minimal medium supplemented with 10 g/l of feather or casein. Plates were incubated for 48h and the relative clear zones were determined. Further experiment was done to confirm positive selection of keratinolytic bacterial strains by cultivation of the tested strains in minimal broth medium containing feather as the sole source of carbon.

Cloning of keratinase gene

Bacillus licheniformis N5 is a local isolate from New Valley, Egypt was used for *keratinase* gene isolation. It was cultivated on LB plates at 37°C for 18 hours. Crude lysate of cells (Ostuki *et al.*, 1997) (fresh preparation) was subjected to PCR amplification using *ker* forward and reverse primers. Three µl of cell lysate were amplified in a 50 µl reaction mixture by using DreamTaq Green PCR Master Mix (Fermentas life sciences) which contains 1.25 units *Taq* DNA polymerase; 4 mM $MgCl_2$ and 0.4 mM each of dNTPs, and 10 pmol of forward and reverse primers.

PCR was performed by using DNA thermal cycler (Amplitronyx, NYXTECHNIK, USA). 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). The PCR product was purified using Ron's PCR-Pure Kit (BIORON), sequenced by MACROGEN (Korea**),**and cloned in pJET1.2 vector using CloneJET PCR Cloning Kit (Fermentas life sciences).

Assay of keratinolytic activity

Crud enzyme was prepared as described by Riffel and Brandelli (2006). The bacteria were cultivated in minimal medium supplemented with feathers (10 g/l) up to 8 days at 30°C. Culture supernatants obtained after centrifugation at 10,000 rpm for 5 min, were used as crude enzyme extracts. The keratinolytc activity of the enzyme preparation was determined using keratin (keratin powder) as substrate. One mL of 50 mM Tris-HCl, pH 7.5 containing 20 mg of keratin suspension was incubated with 250 µL of the enzyme solution for 30 min at 37°C with constant trichloroacetic acid (TCA), centrifuged at 10,000 rpm for 10 min and the supernatant was used to read its absorbance at 280 nm against a control. The control was treated in the same way, except that TCA was added before the addition of enzyme solution.

One unit of keratinolytic activity was defined as an increase of corrected A_{280} under the assay conditions. Keratinase specific activity was determined as described by Hossain *et al*. (2007).

RESULTS AND DISCUSSION

Bacterial screening for feather degradation

A number of bacterial strains were screened for keratinolytic ability using the method described by Cai *et al*. (2008). Tested strains were grown on minimal medium supplemented with 10 g of feather or casein. Plates were incubated for 24 h, clear zones and bacterial growth for all collected bacterial strains were determined. The results present in Tables (2 and 3) showed that, among all tested strains, *Bacillus licheniformis* N5 was selected, as the best keratinase producer therefore its genomic DNA was used as a donor of keratinase gene for cloning experiments.

Cloning of keratinase gene

The selection of forward and reverse *ker* gene primers depends on amplifying the complete coding region (i.e., CDS) of *B. licheniformis- keratinasae* gene (i.e., 1140 bp). The *keratinasae* gene (*ker*) sequence of *B. licheniformis* was obtained from the available data in the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) including several bases upstream and downstream the *ker* CDS. Primers derived from this sequence were obtained using Primer 3 software.

The DNA sequence was analyzed for its restriction endonuclease cutting using the Webcutter 2.0 software (http://rna.lundberg.gu.se/cutter2/). Results indicated that both of *Bam* HI and *Sac* I restriction enzymes cannot cut within it. These enzymes recognition sequences were added to the selected forward (*Bam* HI) and reverse (*Sac* I) primers to facilitate manipulation of the PCR product. The *ker* forward primer was: **ggatcc**ccaagctgaagcggtctatt and the *ker* reverse primer was: gagctcttggacaaccttcatcagaatg. By using the selected primers the whole *Ker* CDS plus 208 bases upstream and 80 bases downstream was amplified and the expected PCR-product size was found to be 1440 bp.

The keratinase gene of *B. licheniformis* N5 was amplified as described above. A sample of PCR product was analyzed using agarose gel electrophoresis and compared with 1kb Ladder DNA marker, results are present in Fig. (1).

The purified PCR product was cloned in pJET1.2 vector and transferred into *E. coli* DH5 as described by Sambrock *et al.* (1989).

Several transformants were successfully obtained. They were analyzed for occurrence of *ker* gene using PCR as mentioned above (Fig. 2). Among all *E. coli* transformants, E. *coli* (pKer-NRC-1) was chosen for further studies. Ker-NRC-1 plasmid was isolated, purified and digested with single and double restriction DNA endonuclease enzymes. After agarose gel electrophoreses and DNA staining, the obtained DNA fragments were compared with DNA ladders, results are illustrated in (Fig. 3). The digestion information was used to map the recombinant Ker-NRC-1plasmid (Fig. 4).

The pJET1.2 vector allows the DNA sequencing of the cloned insert and its neighbor nucleotides by using the forward and reverse primers specific for this vector and surrounding its cloning site. DNA sequencing of *B. licheniformis* N5 *keratinase* gene region of Ker-NRC-1 plasmid was determined (MACRGEN, Korea) and analyzed. Results indicated the successful cloning of the whole CDS of keratinase (Fig. 5A), which was 1140 bp representing 379 amino acids. It was deposited in the Gene Bank (GenBank accession number: KJ642621.1).

Keratinase similarity

The nucleotide sequencing of *B. licheniformis* N5 keratinase gene and its deduced amino acids (Fig. 5) were used to search the database for similarity relatedness using NCBI/BLAST/ BLASTN (http://www.ncbi.nlm.nih.gov/blast/ Blast.cgi). Results indicated that 99% similarities to several *B. licheniformis* strains for both the nucleotides and amino acids sequence.

Expression of Ker gene in E. coli transformants

The recombinant plasmid was constructed by cloning the keratinase gene in

pJET1.2 vector and transferred into *E. coli* DH5 under control of T7 promoter. *E. coli* recombinant strains grown on feather agar plate displaying a clearing zone (Fig. 6) indicated that a successful expression of *B. licheniformis Ker* gene in its new host (*E. coli* DH5α), whereas no detectable keratinase activity was observed in *E. coli* parental strain (Table 4).

Expression analysis of *E. coli* DH5α (recipient stain), *B. licheniformis* (donor strain) and some *E. coli* transformants as shown in (Table 5) suggested that the recombinant *E. coli* strains expressed and secreted keratinase and the expression level was differ, some recombinant stains (T5, T6 and T12) had lower activity compared with that of *B. licheniformis* on the other hand other recombinant strains (T2 and T9) expressed keratinase at high level resulting in 19.3% and 55.1% increase, respectively.

Feathers and several organic materials from animal origin are cheap source of N and C when they are degraded by keratinolytic bacteria, they could be used as organic fertilizers. This application will not only reduce the costs of commercial fertilizers but also will be more environmental friendly (Paul *et al*., 2013; Brandelli, 2008).

Produced amino acids from feather degradation has several applications including pharmaceutical drugs, feedstuffs and feed additive, biodegradable films, glues and foils (Onifade *et al*.,1998; Jeong *et al*., 2010; Brandelli *et al*., 2010; Brandelli, 2008).

Bacillus licheniformis keratinase had a feed additive and improved the performance of broiler chicken (Odetallah *et al*., 2005).

The current study is conducted to select the best promising keratinolytic bacterial strain to this goal a number of collected strains were screened for keratinase producing ability on Casein agar plate. The organisms producing zone of hydrolysis were considered as keratinolytic organisms. By comparing the diameter of clear zone and the diameter of colony, high keratinase activity indicated large clear zone relatively to the colony. The hydrolysis activity were further confirmed by cultivation of the tested strains in minimal broth medium supplemented with feather as a sole source of carbon, and measure the ability of keratinolytic bacteria to grow and degrade feather. Among all tested strains *Bacillus licheniformis* N5 was selected as the best keratinase producer strain. The result of these experiments was confirmed with the previous study with *Bacillus licheniformis* ALW1 (Azza *et al*., 2018). The *Ker* gene from chromosomal DNA was amplified by PCR, sequenced and cloned into pJET1.2 vector. The *ker* gene was transferred and expressed efficiently into *E. coli* DH5α under control of T7 promoter with different levels of expression. The difference in *keratinase* expression among the *E. coli* transformants may be probably

due to the difference in plasmid copy number within these transformants.

Our results suggested that the recombinant strain was able to express keratinase at high level reach to 55% increase, were in a harmony with the Radha and Gunasekaran (2009) observation that cloned *keratinase* gene from *B. licheniformis* MKU3 was successfully expressed in *Bacillus megaterium* as well as in *Pichia pastoris*. Compared with parents strains, up to 3-fold increased of keratinase level had been found in recombinant *B. megaterium* and 2.9-fold increased level had been found in the recombinant *P. pastoris*.

In conclusion our study describes the method of cloning *keratinase* gene from indigenous *Bacillus licheniformis* N5 strain using PCR technique. The clone contains the whole CDS sequence which successfully expressed in *E. coli*. This clone could be subcloned and transform other bacterial hosts including *Bacillus s*trains. In order to obtain superior keratinase producer strains for biotechnological application further studies should be carried out to purify and optimize the fermentation condition of the recombinant enzyme.

SUMMARY

DNA fragment coding for keratinase (*Ker*) gene from local isolate, *Bacillus licheniformis* N5 was generated using designed primers and polymerase chain reaction (PCR) technology. It was cloned into pJET1.2 vector and expressed into *E. coli* DH5α. Transformants acquiring desired gene was confirmed either by PCR or by screening the gene expression on selective medium. *E. coli* transformant harboring recombinant plasmid named Ker-NRC-1 was selected and the nucleotide sequence of ker gene was deposited in GenBank (GenBank accession number: KJ642621.1).The complete CDS of keratinase was consists of 1140 bp representing 379 amino acids equivalent to 38.8 kDa. Up to 55% increase of keratinase activity was observed among *E. coli* recombinant strains comparing with *Bacillus licheniformis* N5 donor strain.

Conclusions: *Bacillus licheniformis* keratinase was cloned and successfully expressed using T7 promoter in *E coli*. The recombinant plasmid could be used to insert the desired gene into other bacterial hosts including *Bacillus* strains to obtain a superior keratinase producer strain which could be potentially used in biotechnological application.

REFERENCES

Anbu, P., S. C. B. Gopinath, A. Hilda, T. Lakshmipriya and G. Annadurai (2007). Optimization of extracellular keratinase production by poultry farm isolate *Scopulariopsis brevicaulis*. Bioresour. Technol., 98: 1298-1303.

Azza, M. A. F., S. E. L. Mamdouh, A. I. Siham and A. E. Mohamed (2018). Biodegradation of feather waste by keratinase produced from newely isolated *Bacillus licheniformis*

ALW. J. Genetic Engineering and Biotechnology, 16: 311-318.

Brandelli, A. (2007). Bacterial keratinases: Useful enzymes for bioprocessing agroindustrial wastes and beyond. Food Bioprocess Technol., 1: 105-116.

Brandelli, A. (2008). Bacterial keratinases: Useful enzymes for bioprocessing agroindustrial wastes and beyond. Food Bioprocess Technol., 1: 105-116.

Brandelli, A., D. J. Daroit and A. Riffel (2010). Biochemical features of microbial keratinases and their production and applications. Appl. Microbiol. Biotechnol., 85: 1735-1750.

Bockle, B., B. Galunsky and R. Muller (1995). Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. Appl. Environ. Microbiol., 61: 3705-3710.

Cai, C. G., B. G. Lou and X. D. Zheng (2008). Keratinase production and keratin degradation by a mutant strain of *Bacillus subtilis*. J. Zhejiang Univ. Sci. B., 9: 60-67.

Chandler, N. J. (2007). Feather Meal: Its nutritional value and use in dairy and beef rations. Cited after, Belewu, M. A., A. R. Asafa and F. O. Ogunleke (2008). Processing of feather meal by solid state fermentation. Biotechnology, 7: 589-591.

Davis, R. W., D. Botstein and J. R. Roth (1980). A Manual for Genetic Engineering, Advanced Bacterial Genetics. Cold Spring Harber La., Cold Spring Harber, New York.

EDFA (2007). Recycling of feather and down. Environment/recycling. Cited after, Belewu, M. A., A. R. Asafa and F. O. Ogunleke (2008). Processing of feather meal by solid state fermentation. Biotechnology, 7: 589-591.

Farag, A. M. and M. A. Hassan (2004). Purification, characterization and immobilization of a keratinase from *Aspergillus oryzae*. Enzyme Microb. Technol., 34: 85-93.

Gradisar, H., J. Friedrich, I. Krisaj and R. Jerala (2005). Similarities and Specificities of Fungal Keratinolytic Proteases: Comparison of Keratinases of *Paecilomyces marquandii* Doratomyces microsporus to Some Known Proteases. Appl. Environ. Microbiol., 71: 3420-3426.

Grazziotin, A., F. A. Pimentel, E. V. de Jong and A. Brandelli (2006). Nutritional improvement of feather protein by treatment with microbial keratinase. Anim. Feed Sci. Technol., 126: 135-144.

Gupta, R. and P. Ramnani (2006). Microbial keratinase and their prospective applications: An overview. Appl. Microbiol. Biotechnol., 70: 21-33.

Hossain, M. S., A. K. Azad, S. M. Abu Sayem, G. Mostafa and Md. M. Hoq (2007). Production and partial characterization of feather-degrading keratinolytic serine protease from *Bacillus licheniformis* MZK-3. J. Biological Sciences, 7: 599-606.

Jeong, J. H., O. M. Lee, Y. D. Jeon and J. D. Kim (2010). Production of keratinolytic enzyme by a newly isolated feather-degrading Stenotrophomonas maltophilia that produces plant growth-promoting activity. Process Biochem., 45: 1738-1745.

Langeveld, J. P. M., J. J. Wang, D. F. M. Van de Wiel, G. C. Shih, G. J. Garssen, A. Bossers and J. C. H. Shih (2003). Enzymatic degradation of prion protein in brain stem from infected cattle and sheep. J. Infect. Dis., 188: 1782-1789.

Lee, C. G., P. R. Ferket and J. C. H. Shih (1991). Improvement of feather digestibility by bacterial keratinase as a feed additive. FASEB J., 59: 1312.

Manczinger, L., M. Rozs, C. Vagvolgyi and F. Kevei (2003). Isolation and characterization of a new keratinolytic *Bacillus licheniformis* strain. World J. Microbiol. Biotechnol., 19: 35-39.

Odetallah, N. H., J. J. Wang, J. D. Garlich and C. H. Shih (2003). Keratinase in starter diets improves growth of broiler chicks. Poult. Sci., 82: 664-670.

Odetallah, N. H., J. J. Wang, J. D. Garlich, J. C. H. Shih (2005). Versazyme supplementation of broiler diets improves market growth performance. Poult. Sci., 84: 858-864.

Onifade, A. A., N. A. Al-Sane, A. A. Al-Musallam and S. Al-Zarban (1998). Potentials for biotechnological applications of keratin-degrading microorganisms and their enzymes for nutritional improvement of feathers and other keratins as livestock feed resources. Bioresour. Technol., 66: 1-11.

Ostuki, K., T. V. Guayeurus and A. C. X. P. Vicent (1997). *Bacillus sphericus* entomocidal potential determined by polymerase chain reaction. Mem. Inst. Oswaldo Cruz., 92: 107-108.

Paul, T., S. K. Halder, A. Das and S. Bera (2013). Exploitation of chicken feather waste as a plant growth promoting agent using keratinase producing novel isolate *Paenibacillus woosongensis* TKB2.

Biocatal. Agric. Biotechnol., 2: 50-57.

Radha, S. and P. Gunasekaran (2009). Purification and characterization of keratinase from recombinant *Pichia* and *Bacillus* strains. Protein expression and purification; 64: 24-31.

Riffel, A. and A. Brandelli (2006). Keratinolytic bacteria isolated from feather waste, Brazilian Journal of Microbiology, 37: 395-399.

Sambrock, J., E. F. Fritsch and T. Maniatis (1989). Molecular Cloning: a Laboratory Manual. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Soliman, E. A. M., S. E. M. Abo-Aba, E. M. Maysa and A. M. Gomaa (2003). The role of plasmids on *Bacillus* alkaline protease production in local isolated strains. Ann. Agric. Sic., 48: 573-584.

Tamilmani, P., A. Umamaheswari, A. Vinayagam and B. Prakash (2008). Production of an Extra Cellular Feather Degrading Enzyme by *Bacillus licheniformis* Isolated from Poultry Farm Soil in Namakkal District (Tamilnadu). International J. Poultry Science, 7: 184-188.

Table (1): Collected bacterial strains, characters and their references or sources.

Bacterial strains	Code	Characters	Reference/ source
Bacillus cereus	6A15	W.T.	Bacillus Genetic Stock Center
B. cereus	Bc29	W.T.	Microbial genetic Dep.National Research center
B. licheniformis	5A3	Sm^r	Bacillus Genetic Stock Center
B. licheniformis	N5	Local isolate from New Vally	Soliman *et al* (2003)
B. subtilis	B.S Ain Shams	W.T.	Faculty of Science, Ain Shams
B. subtilis	B.S Alazhar	Local isolate	Faculty of Science, Al-Azhar Univ.
B. subtilis	DB100	W.T.	
B. megaterium	7A37	Sm^r	Bacillus Genetic Stock Center
B. pumilus	I1	Local isolate from qanater	Soliman *et al* (2003)
B. alvea	N3	Local isolates from new vally	Soliman *et al* (2003)
B. thuringiensis israelnsisi A	BTI. A	W.T.	Dr. Priest, F.G.,Heriot Univ., England
B. thuringiensis israelnsisi B	BTI. B	W.T.	Dr. Priest, F.G.,Heriot Univ., England

Table (2): Protease activities of collected bacterial strains after their growth on Minimal medium supplemented with casine for 24 hours.

Bacterial strains	Code	Clear zone Diameter (C) (mm)	Growth Diameter (G) (mm)	Relative area C/G
Bacillus cereus	6A15	21	6	11.250
B. cereus	Bc29	18	9	3.000
B. licheniformis	5A3	8	3	6.110
B. licheniformis	N5	16	4	15.000
B. subtilis	B. S Ain Shams	16	5	9.240
B. subtilis	B. S Alazhar	14	4	11.250
B. subtilis	DB100	10	3	10.110
B. megaterium	7A37	17	4	17.000
B. pumilus	II	17	8	3.515
Bacillus alvea	N3	21	13	1.600
B. thuringiensis israelnsisi A	BTI. A	13	5	5.760
B. thuringiensis israelnsisi B	BTI. B	11	8	0.890

Table (3): Bacterial growth in minimal medium supplemented with 10g/l feather.

Bacterial strains	Code	Growth After	
		3 days	5 days
Bacillus cereus	6A15	+	++
B. cereus	Bc29	+++	+++
B. licheniformis	5A3	+	++
B. licheniformis	N5	+++	+++
B. subtilis	B.S Ain Shams	+++	+++
B. subtilis	B.S Alazhar	+	++
B. subtilis	DB100	-	-
B. megaterium	7A37	++	++
B. pumilus	11	+++	+++
B. alvea	N3	++	+++
B. thuringiensis israelnsisi A	BTI. A	+	+
B. thuringiensis israelnsisi B	BTI. B	++	++

-: No growth; +: Weak growth; ++: Moderate growth; +++: strong growth

Table (4): Keratinase activity of *E. coli* DH5α and its *Ker*-gene transformants.

Transformant no	Growth mm	Clear zone mm	Relative area
E. coli DH5α	0.0	0.0	0.00
Tra. 1	4.0	6.0	1.25
Tra. 19	10.3	20.0	2.77
Tra. 23	6.6	13.0	2.87

Table (5): Keratinase production by recombinant *E.coli* and *B. licheniformis* N5

Strains	Keratinolytic activity (U / ml)
Control	0.00
Bacillus licheniformis	4.90
E .coli DH5α	0.00
Kar-T2	5.85
Kar-T5	0.40
Kar-T6	3.00
Kar-T9	7.60
Kar-T12	3.20

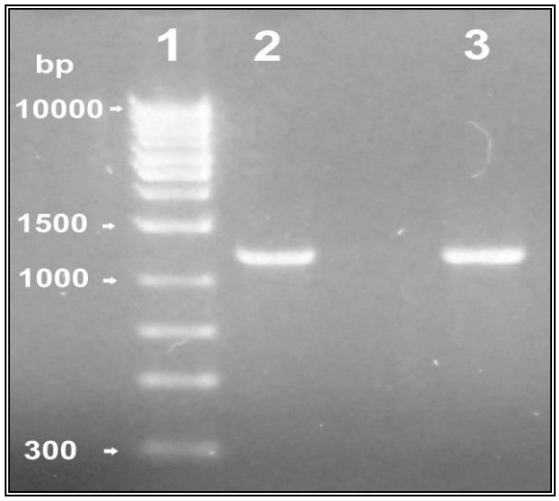

Fig. (1): Agarose gel electrophoreses of *keratinase* gene amplification by PCR.
Lane 1: 1 Kb Ladder DNA marker (AXYGEN, Bioscience, 300 bp to 10000 bp),
lane 2: *Keratinase* gene amplicon of *B. licheniformis* N5,
lane 3: *Ker* gene after PCR-clean up.

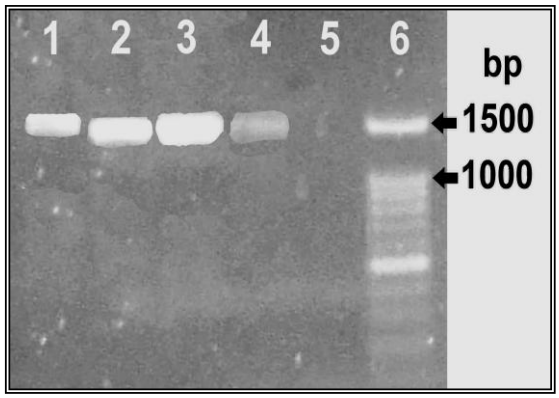

Fig. (2): Agarose gel electrophoreses of PCR products obtained from keratinase gene amplification of *E. coli* transformants.
Lane 1: *E. coli* (pKar-T13);
Lane 2: *E. coli* (pKar-T12);
Lane 3: *E. coli* (pKer-NRC-1);
Lane 4: *B. licheniformis* N5;
Lane 5: *E. coli* DH5α (as a negative control);
Lane 6: 100bp DNA marker (GeneDirX 100 to 1500 bp).

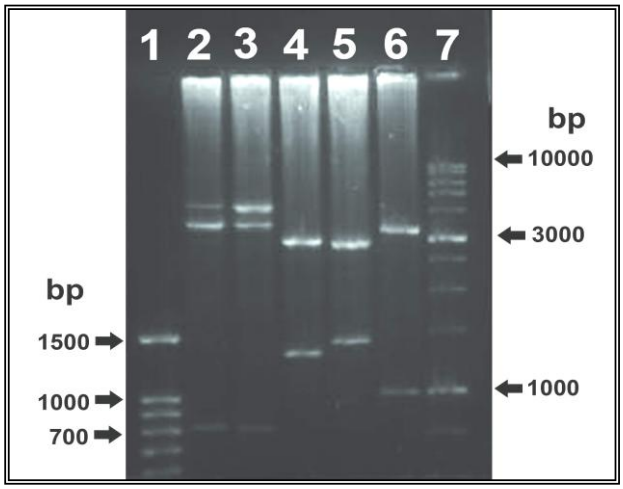

Fig. (3): Agarose gel electrophoreses of pKer-NRC-1 recombinant plasmid digested with different enzymes.
Lane 1: 100 bp DNA Ladder RTU (GeneDireX, 100 to 1500 bp); Lanes 2 to 6 are pKer-NRC-1 plasmid digested with *Bam* HI and *Eco*RI (lane2); *Bam*HI and *Eco*RI (lane 3); *Pst*I (lane 4); *Bam*HI and *Sac*I (lane 5); *Hind*III (lane 6); Lane 7: 1 kb DNA Ladder RTU (Nipon Genetics, Japan, 250 to 10000 bp)

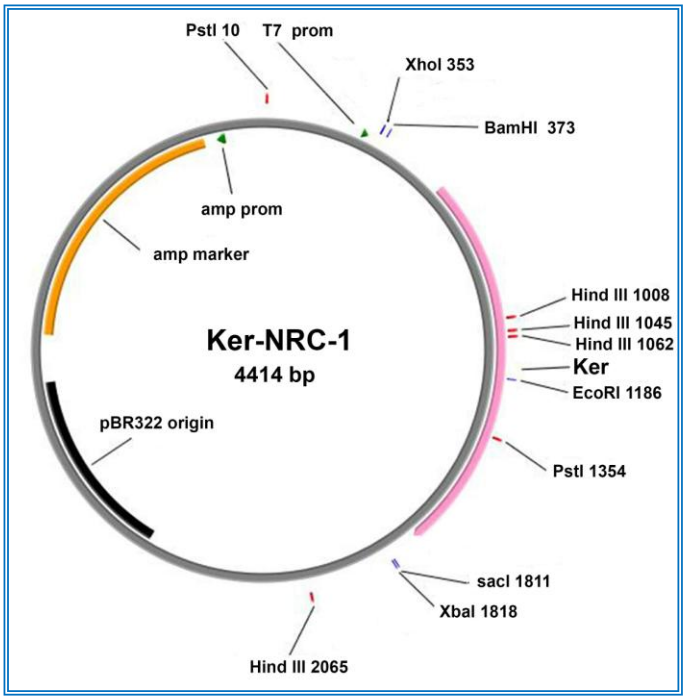

Fig. (4): The recombinant Ker-NRC-1plasmid.

(A)

ggatccccaagctgaagcggtctattcatactttcgaactgaacatttttctaaaacagtt
attaataaccaaaaaatttttaaattggtcctccaaaaaaataggcctaccatataattcat
ttttttttctataataaattaacagaataattggaatagattatattatccttctatttaaa
ttattctgaataaagaggaggagagtgagta**atg**atgaggaaaaagagtttttggcttggg
atgctgacggccttcatgctcgtgttcacgatggcattcagcgattccgcttctgctgctc
aaccggcgaaaaatgttgaaaaggattatattgtcggatttaagtcaggagtgaaaaccgc
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gctgcagcagggaacagcggatcttcaggaaacacgaatacaattggctatcctgcgaaat
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cacggcgacttatttgggaagctccttctactatgggaaaggtctgatcaatgtcgaagct
gccgctcaat**aa**catattctaacaaatagcatatagaaaaagctagtgtttttagcactag
ctttttcttcattctgatgaaggttgtccaagagctc

(B)

M M R K K S F W L G M L T A FM L V F T M A F S D S A S A A Q
P A K N V E K D Y I V G F K S G V K T A S V K K D I I K E S G
G K V D K Q F R I I N A A K A K L D K E A L K E V K N D P D V
A Y V E E D H V A H A L A Q T V P Y G I P L I K A D K V Q A Q
G F K G A N V K V A V L D T G I Q A S H P D L N V V G G A S F
V A G E A Y N T D G N G H G T H V A G T V A A L D N T T G V L
G V A P S V S L Y A V K V L N S S G S G S Y S G I V S G I E W
A T T N G M D V I N M S L G G A S G S T A M K Q A V D N A Y A
K G V V V V A A A G N S G S S G N T N T I G Y P A K Y D S V I
A V G A V D S N G N R A S F S S V G A E L E V M A P G A G V Y
S T Y P T N T Y A T L N G T S M A S P H V A G A A A L I L S
K H P N L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L
I N V E A A A Q

Fig. (5): The amplicon sequencing containing CDS (bold letters) of *Bacillus licheniformis* N5 keratinase gene, the start code are underlined (A) and its deduced amino acid sequences (B).

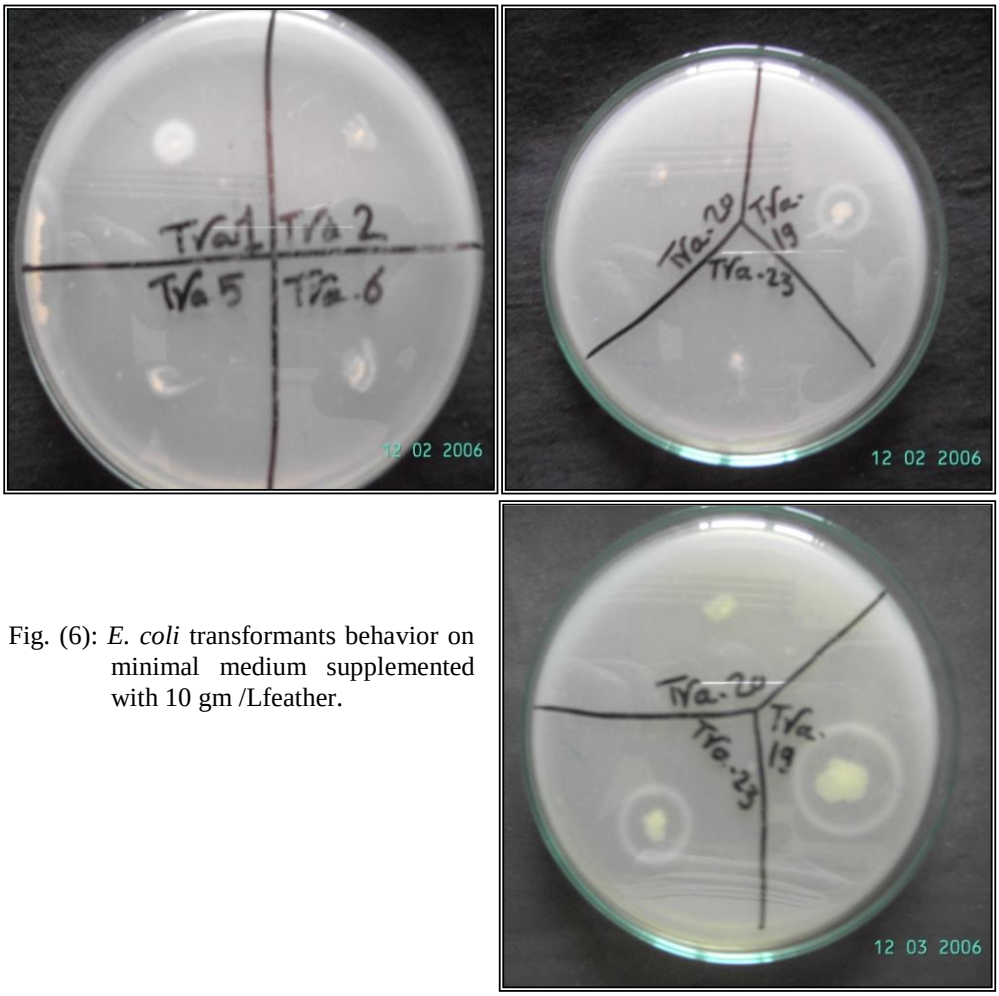

Fig. (6): *E. coli* transformants behavior on minimal medium supplemented with 10 gm /Lfeather.