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Keratinolytic activity from new recombinant fusant AYA2000, an endophytic *Micromonospora spp.*

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ABSTRACT

Two different endophytic strains ESRAA1997 and ALAA2000 were isolated from the Egyptian herbal plant Anastatica hierochuntica. The two strains produced alkaline serine protease and they were identified based on their phenotypic and chemotypic characteristics as two different strains of Micromonospora spp. Both strains grew and produced keratinase using different keratinous waste substances as the sole source of carbon and nitrogen. In our study, the activity and properties of keratinase enzymes of the both wild strains ESRAA1997 and ALAA2000 have been altered by genetic recombination through protoplast fusion between them, leading to a potent keratinolytic fusant *Micromonospora spp.* AYA2000 with improved properties (activity, stability, specificity and tolerance to inhibitors). Whereas using a mixture of yeast extract, peptone and malt extract as a supplement to the bovine hair medium increased keratinase production by 48%, addition of 1% glucose suppressed enzyme production by *Micromonospora spp*. AYA2000. The enzyme was purified by ammonium sulphate precipitation, DEAE-cellulose chromatography followed by gel filtration. The molecular mass, determined using SDS–PAGE, was 39kDa. AYA2000. The enzyme exhibited remarkable activity towards all keratinous wastes used and could also adapt to a broad range of pH and temperature with optima at pH 11 and 60°C. The enzyme was not influenced by chelating reagents, metal ions or alcohols. These properties make AYA2000 keratinase an ideal candidate for biotechnological application.

KEYWORDS

Endophytic Actinomycetes;
Protoplast fusion;
Keratinase.

INTRODUCTION

Keratinous wastes such as hair, feather, wool, horn and hoof are increasingly generating and accumulating as a waste by products from poultry processing, reaching millions of tons per year worldwide and there is a demand for developing biotechnological alternatives for recycling such wastes[1]. Over the past decades, native keratinous material degradation by micro-organism has attracted a great deal of attention as these enzymes could be applied in food, textile, medicine, cosmetic, leather and poultry processing industry[2].

In terms of chemical ecology, endophytic microorganisms residing inside the healthy tissue of plants are

rich source of bioactive metabolites[3]. However, up till now there are no studies that have been conducted on keratinolytic enzymes biosynthesized by endophytes, although it could be readily hypothesized that they might be required to produce proteases for their survival and reproduction inside the host.

Although microorganisms are extremely good in presenting us with an amazing array of valuable products, they do not tend to overproduce their metabolites. The application of recombinant DNA technology by protoplast fusion for increasing the titers of secondary metabolites has been of great interest[4]. The industrial enzyme business adopted recombinant DNA technology methods to increase the production levels of enzymes and improve the stability, activity or specificity of industrial enzymes[5].

The present work aims to apply the protoplast fusion technique between two different genomes of keratinolytic endophytic Micromonospora species isolated from the Egyptian herbal plant Anastatica hierochuntica to construct new recombinants with new properties. Moreover, the purification and characterization of the fusant AYA2000 keratinase were reported. To our knowledge, this is the first report on the genetic improvement of keratinase by protoplast fusion between endophytic Actinomycetes that can degrade different keratinous wastes.

MATERIALS AND METHODS

Sources of keratin substrate

For keratinase production, several keratinous materials from different origins, such as human (stratum corneum from sole, nail and hair), bovine (nail, hair and skin), sheep (wool), birds (duck and chicken feathers) were purchased from commercial suppliers. These keratinous materials were washed with tap water and finally with distilled water. The washed substances were dried at 60°C overnight, soaked in chloroform: methanol (1 : 1, v / v), washed with water three times, dried overnight at 60°C and then cut into short fragments and ground to be used in media[6].

Isolation and screening of keratinase producing endophytic actinomycetes

Endophytic Actinomycetes were isolated from the inner healthy stem tissue of Anastatica hierochuntica by surface-sterilization technique[7]. Isolates were initially screened for proteolytic activity by streaking on casein-yeast extract-peptone (CYP) agar plates which, composed of (g/l): casein hydrolysate, 10; peptone, 5; yeast extract, 1; K_2HPO_4, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.1; Na_2CO_3, 10; and 15.0, agar. Na_2CO_3 was sterilized separately and added to the rest of the medium after cooling. After 72 h, incubation at 30°C, protease production was observed as a clear zone around the colony of positive protease isolates[8]. Isolates, which showed strong protease activity were checked for their keratinase activity in liquid medium prepared as g % (K_2HPO_4, 0.05; KH_2PO_4, 0.05; $MgSO_4 \cdot 7H_2O$, 0.1; $CaCl_2$, 0.01; $FeSO_4.7H_2O$, 0.0015; $ZnSO_4.7H_2O$, 0.0005; NaCl, 0.03 and pH adjusted to 8.0) but casein was substituted by 2.0% (w/v) of various keratinous wastes. Hydrolysis of each keratinous waste and the level of keratinase production were estimated after incubation for five days at 30°C and 180rpm. The potent keratinolytic isolates were subjected to identification and protoplast fusion technique to construct new recombinants with new enzymatic properties.

Identification of keratinolytic endophytic isolates ESRAA1997 and ALAA2000

The isolates were examined for their phenotypic and chemotypic properties, using procedures described in previous studies[9-14].

Protoplasts formation and fusion

To form protoplasts, two different Micromonospora sp. ESRAA1997 and ALAA2000 were inoculated into 50ml of GER medium[15] supplemented with 0.15% glycine and $CaCl_2$ (20mM). The culture was incubated for 48 to 60 h at 28°C in 250ml flasks containing three glass beads (4 mm). The cells were harvested by centrifugation, washed with 10.3% sucrose, and resuspended in 4ml of L buffer (pH 7.6)[16] containing 5 mg/ml of lysozyme. Effective protoplasting usually occurred after 1 to 2 h of incubation at 30°C. Protoplast formation, monitored by microscopy, was generally greater than 95% of cells.

Equal volumes of protoplast suspensions from each strain were mixed and washed by centrifugation (3000rpm, for 10 min). The pellets were resuspended

in the least volume (0.5 ml) of remaining liquid by stirring. PEG solution (1.5ml) was added. After treatment with PEG, 6ml of medium P[17] was added to stop the action of PEG. After centrifugation (3000rpm, for 10 min) at 4°C and resuspension, protoplasts were diluted and plated on regeneration agar medium[18] containing antibiotic markers.

Degradation of keratinous wastes using the parental isolates and their fusants

Keratinase production of the keratinolysis parental isolates (ESRAA1997 and ALAA2000) and their fusants were investigated using the modified mineral salt broth medium[8] supplemented with 2% of each keratinous waste powder added separately as sole nitrogen and carbon sources. After the fermentation period the actinobacterial growth was monitored by measuring the dry weight basis of cell mass[19]. The degradation of each keratinous substrate was evaluated by determining the dry weight at various time intervals[20]. The supernatants of these strains were used as crude enzyme preparations. To evaluate the efficiency of protoplast fusion techniques some characteristics of the crude keratinase preparations of the parental strains and their fusant AYA2000 were compared.

Optimization of culture conditions for keratinase production by the fusant Micromonospora sp. AYA2000

The influence of several factors include the concentration of bovine hair (0.5-5.0 % w/v), some nutritional supplements, which added to bovine hair (BH) - salt broth medium, typical time course of enzyme production (1-10 days), incubation temperature (25-50°C) and pH of medium (3.0-10.0) on Keratinase production by the fusant AYA2000 were evaluated.

Analytical methods

Protein measurement and fusant AYA2000 keratinase activity

The protein content of the enzyme preparation was determined by the Folin's phenol reagent method[21] using bovine serum albumin (BSA) as a standard. Keratinolytic activity was determined as described previously[22]. One unit of the keratinase activity was defined as the amount of enzyme that increased absorbance by 0.1 under the used assay conditions (1 U = 0.100 corrected absorbance).

Purification and characterization of AYA2000 keratinase

All purification operations were done below 4°C. The culture filtrate of the fusant Micromonospora sp. AYA2000 was collected after seventh days of incubation by filtration through Whatman filter paper. The filtrate was centrifuged at 5000rpm for 30 min and the cell-free supernatant fluid was collected. The enzyme was precipitated from the supernatant by the gradual addition of solid ammonium sulphate (0-90% saturation), with gentle stirring, allowed to stand for 2 h and centrifuged at 10000 rpm for 30 min. Each precipitate was dissolved and dialyzed against Tris-HCl buffer, pH 8.0 in a refrigerator for 24 h. After dialysis, the partial purified keratinase fractions obtained from the ammonium sulphate precipitation (80% saturation fractions) were applied to the DEAE-Cellulose column chromatography. The equilibration and elution of the protein was performed with a linear gradient of 0.025M Tris-HCl buffer containing 0.05M NaCl at pH 8.0, with a flow rate of 30 ml/h. Fractions (5ml) were collected, protein content and keratinase activity for each fraction were estimated. The fractions possessing highest specific activity were concentrated and applied to the Sephadex G-100 gel fractionation and eluted with 0.05M Tris-HCl buffer at pH 8.0 with a flow rate of 30ml/h, enzyme activity and protein content in each fraction were measured.

Enzyme purity was tested by sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE)[23]. Molecular weight of the purified keratinase was estimated by comparing its relative mobility with different proteins low-molecular weight markers (14-97 kDa, Sigma Co.) as standard. The proteins were stained with a 0.1% solution of Coomassie brilliant blue R-250.

Effect of pH and temperature on the purified enzyme activity and stability

The optimal pH for the keratinolytic activity was investigated by measuring the enzyme activity in various pH buffers (50mM) between 3.0 and 12.0 (citrate, pH 3.0-6.0; phosphate buffer, pH 7.0; Tris-HCl, pH

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TABLE 1 : Differential cultural characteristics of the parental strains in comparison to their fusant strain AYA2000

Medium	Parents and fusant								
	ESRAA1997			ALAA2000			AYA2000		
	Growth	Color of colony	Soluble pigment	Growth	Color of colony	Soluble pigment	Growth	Color of colony	Soluble pigment
ISP2	Abundant	Orange	Pale yellow	Abundant	Yellowish white	Pale yellow	Abundant	Brownish orange	Pale yellow
ISP3	Abundant	Orange	-	Moderate	Whitish orange	Pale yellow	Good	Brownish orange	Pale yellow
ISP4	Abundant	Dull orange	-	Poor	Whitish orange	-	Abundant	Deep Brownish orange	-
ISP5	Good	Dull orange	-	Poor	Yellowish white	-	Good	Brownish orange	-
ISP6	Good	Deep orange	-	Moderate	Yellowish white	Pale yellow	Good	Deep Brownish orange	-
ISP7	Good	Dull orange	-	Good	Yellowish white	-	Good	Brownish orange	-
Glucose-asparagine agar	Moderate	Orange	-	Poor	Whitish orange	-	Good	Brownish orange	-
Czapek's agar	Moderate	Deep orange	-	Moderate	Yellowish white	Pale yellow	Moderate	Deep Brownish orange	-
Nutrient agar	Good	Dull orange	Pale yellow	Poor	Yellowish white	Pale yellow	Abundant	Deep Brownish orange	Pale yellow

8.0-9.0 and carbonate bicarbonate buffer, pH 10.0-12.0). Stability to pH was assessed by assaying the residual activity (%) after incubating the enzyme at room temperature for 24 h at given pH values ranging from 3.0 to 12.0. The dependency on temperature of the purified enzyme activity was determined in standard conditions maintaining a constant pH at a varying temperature. For heat stability measurement, the enzyme was incubated for 60 min in a temperature from 20 to 75°C, followed by the determination of the residual keratinolytic activity.

Effects of various chemicals on the purified enzyme activity

Keratinase was preincubated at room temperature for 30 min in the presence of 5mM of the following chemicals separately, inhibitors: phenylmethylsulphonylfluoride (PMSF), 3,4-dichlorocoumarine, ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT) and 0.1% SDS as well as metal ions (Na^+, K^+, Ca^{2+}, Cu^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Pb^{2+}, Hg^{2+}). On the other hand, the enzyme was kept in different separately organic solvents (20%, v/v) such as acetonitrile, acetone, ethanol and methanol at 4°C for 24 hours. The residual enzymatic activity was measured after incubation with each inhibitor, metal ion or solvent and the keratinase activity without any chemicals was taken as 100%.

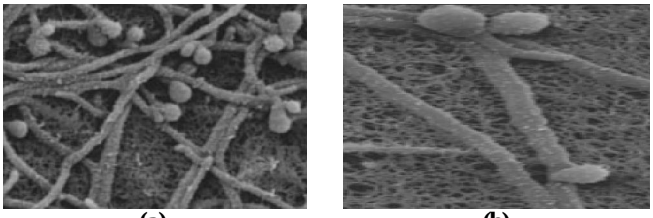
Substrate specificity of AYA2000 keratinase

The purified enzyme was incubated for up to 30 min with each substance (bovine hair, human hair, chicken feathers, sheep wool, stratum corneum, duck feathers, bovine skin, human nail and bovine nail separately). Then the enzymatic activity and substrate degradation were determined.

RESULTS AND DISCUSSION

Screening for endophytic actinobacterial species with keratinolytic activity

The initial screening of the preserved wild type of



(a) (b)

Figure 1 : Spore surface ornamentation of the parental strains (a) ESRAA 1997 and (b) ALAA 2000

endophytic actinobacterial isolates residing in the healthy inner stem tissues of Anastatica hierochuntica (a traditional Egyptian herbal plant) for proteolytic activity on casein agar medium and further checked for keratinase activity showed that superior producers of keratinase were detected in the culture medium of the endophytic strains with isolation numbers ESRAA1997 and ALAA2000. These strains were selected as parental strains for further studies.

Morphological, physiological and biochemical characteristics of selected keratinolytic isolates

The cultural, physiological and biochemical properties of strains ESRAA1997 and ALAA2000 (TABLE 1 and 2) are consistent with their classification in the genus Micromonospora[9-14,24,25]. The main characteristics of species of the genus Micromonospora, the absence of aerial mycelium and the formation of single spores have a rough and nodular surface on the substrate hyphae, were observed in the keratinolytic strains ESRAA1997 and ALAA2000 (Figure 1).

The isomer of diaminopimelic acid (A2pm) was meso, indicating that these strains have wall chemotype II. On the other hand, glucose, xylose, arabinose as whole-cell sugars were detected (whole-cell sugar pattern D)[24]. Characteristic phospholipids were diphosphatidylglycerol, phosphatidylinositol, and phosphatidylethanolamine, this pattern corresponds to phospholipid type II[25]. Major cellular fatty acids of the two strains were iso-15:0 and iso-16:0, this pattern refers to fatty acid type 3b[13]. Mycolic acids were absent and the predominant menaquinones were MK-10(H4) as well as the DNA G+C contents ranged from 69.1 to 70.2%.

The parental strains ESRAA1997 and ALAA2000 were differentiated from each other by the color of colony, soluble pigments, characteristic phospholipids and predominant menaquinones. On the basis of their differentiations in morphological, physiological and chemotaxonomic data, the parental strains ESRAA1997 and ALAA2000 should be classified as two different members of the genus Micromonospora (TABLE 1 and 2).

Protoplast fusion and evaluation of keratinous wastes biodegradability

The fusion was carried out between the hyperactive endophytic keratinolytic strains (ESRAA1997 × ALAA2000). The antibiotic resistance or sensitivity of the two Micromonospora strains were examined using five antibacterial agents (ampicillin, 100µg/ml; mitomycin, 5µg/ml; vancomycin, 75µg/ml; tetracycline, 30µg/ml and kanamycin, 50µg/ml) and the differing antibiotics responses of them were used as selective markers during the detection of fusants after protoplast fusion. The results displayed that ESRAA1997 strain showed resistance to kanamycin, mitomycin, tetracycline and ampicillin as well as sensitivity to vancomycin. On the other hand, ALAA2000 strain exhibited resistance to vancomycin and ampicillin as well as sensitivity to mitomycin, kanamycin and tetracycline. After the fusion procedure, 12 colonies were proved to have the ability to grow on the selective medium containing vancomycin, mitomycin, and tetracycline, confirming the true recombinants of the two endophytic strains. In a previous study[26] we found different responses of different marine Streptomyces isolates for one or more antibacterial agents as markers in protoplast fusion.

A comparison of the keratinase activity of the recombinant strains compared to their parents using different keratinous waste materials is presented in TABLE 3. It appeared that, out of the 12 fusants obtained, four fusants; AYA2001, AYA2002, AYA2003 and AYA2004 were similar to the first parent (P1, ESRAA1997), they failed to hydrolyse sheep wool, bovine hair or human hair. On the other hand, three fusants, AYA2005, AYA2006 and AYA2007 exhibited the same keratinase as the second parent (P2, ALAA2000) with bovine nail, hair and skin; sheep wool; stratum corneum; human nail and hair but failed to hydrolized feather. Three fusant strains, AYA2008, AYA2009 and AYA2010 showed keratinolytic activity different from their parents. Among all recombinants, fusant AYA2011 showed no keratinase activity. However, the recombinant strain AYA2000 presented the traits of the parents, ESRAA1997 and ALAA2000, with higher keratinase activity; it was se-

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TABLE 2 : Differential physiological and biochemical characteristics of the parental strains in comparison to their fusant strain AYA2000

Characteristics	Parents and fusants		
	ESRAA1997	ALAA2000	AYA2000
Cell wall amino acids	Glu, D-Ala, Gly, mDAP (1:1.50:1.16:0.60)	Glu, D-Ala, Gly, mDAP (1:1.44:1.33 : 0.70)	Glu, D-Ala, Gly, mDAP (1:1.20:1.50:0.74)
Whole cell sugars	Gala, Glu, Ara, Xyl, Rham.	Glu, Ara, mann. Xyl, Rib.	Gala, Glu, Ara, Xyl, Rib.
Major fatty acids (%)			
Iso- 15:0	14.82	14.44	18.44
Iso-16:0	34.98	15.86	36.22
Iso-17:1ω9c	12.66	11.12	3.12
17:1ω8c	1.20	11.28	11.34
Anteiso-17:0	6.86	10.30	8.34
10-methyl 17:0	10.80	9.76	10.24
10-methyl 18:0	18.60	2.64	2.42
18:1ω9c	1.48	12.40	10.18
Major respir. Quinones (%)			
MK11 (H4)	6.4	0.0	3.6
MK10 (H4)	70.0	62.2	60.0
MK10 (H6)	18.2	3.3	11.0
MK10 (H8)	3.0	2.2	3.1
MK9 (H2)	0.0	0.0	0.0
MK9 (H4)	2.4	19.8	13.0
MK9 (H6)	0.0	12.0	9.0
MK9 (H8)	0.0	0.80	0.3
Characteristi cphospholipids			
DPG	+	+	+
PI	+	+	+
PE	+	+	+
PIMS	+	-	+
Aerobic reduction of NaNO3	+	-	+
Tyrosinase activity	-	+	+
Hydrolysis of			
Starch	+	+	+
Cellulose	+	+	+
Casein	+	+	+
Gelatine	+	+	+
Utilization of (1%)			
Glucose	+	+	+
Glycerol	+	+	+
Erythritol	-	-	-
Adonitol	-	-	+
L-Arabinose	+	+	+
D-Galactose	+	+	+

Characteristics	Parents and fusants		
	ESRAA1997	ALAA2000	AYA2000
Cell wall amino acids	Glu, D-Ala, Gly, mDAP (1:1.50:1.16:0.60)	Glu, D-Ala, Gly, mDAP (1:1.44:1.33 : 0.70)	Glu, D-Ala, Gly, mDAP (1:1.20:1.50:0.74)
Whole cell sugars	Gala, Glu, Ara, Xyl, Rham.	Glu, Ara, mann. Xyl, Rib.	Gala, Glu, Ara, Xyl, Rib.
D-Mannose	+	+	+
D-Ribose	-	-	-
L-Rhamnose	-	-	-
L-Sorbose	-	-	-
Mannitol	+	+	+
N-acetyl glucosamine	+	+	+
Lactose	+	+	+
sucrose	+	+	+
Maltose	+	+	+
Raffinose	+	+	+
Malate	+	+	+
Citrate	+	+	+
Oxalate	+	-	+
Temp. range for growth	15 – 40	20 - 45	10 - 45
Optimum temp. for growth	25 - 32	28 - 37	20 -35
NaCl tolerance range for growth (%)	0 – 3.5	0 – 8.0	0 – 8.0
Optimum NaCl for growth(%)	1.5 - 2.5	3.0 - 5.0	2.0 – 5.0
Mol% G+C	69.1	70.2	72.5

TABLE 3 : Effect of different keratinous substrates on the keratinase productivity by the parental strains and their fusants

Parents and fusants	Substrate																	
	Duck feather		Chicken feather		Bovine nail		Bovine hair		Bovine skin		Sheep wool		Stratum corneum		Human nail		Human hair	
	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b	a	b
ESRAA1997(P1)	70	40	72	43	25	20	0	0	76	50	0	0	78	52	31	22	0	0
ALAA2000(P2)	0	0	0	0	50	29	65	39	15	19	65	38	35	27	59	32	69	42
AYA2000	81	54	83	55	58	30	87	64	76	42	80	62	79	57	67	40	85	60
AYA2001	65	42	70	45	88	46	0	0	60	50	0	0	70	48	90	63	0	0
AYA2002	88	53	80	53	50	25	0	0	90	56	0	0	94	60	22	19	0	0
AYA2003	82	50	70	42	44	25	0	0	62	60	0	0	90	50	35	20	0	0
AYA2004	64	41	68	50	30	22	0	0	54	30	0	0	78	41	20	12	0	0
AYA2005	0	0	0	0	55	28	85	58	22	20	90	61	20	18	62	40	90	70
AYA2006	0	0	0	0	62	33	92	46	20	18	95	60	24	18	66	42	80	65
AYA2007	0	0	0	0	38	29	60	42	90	64	85	65	85	61	40	33	80	68
AYA2008	75	58	48	34	66	20	0	0	80	43	75	32	90	50	20	8	0	0
AYA2009	0	0	0	0	10	14	0	0	12	8	0	0	12	15	15	10	0	0
AYA2010	88	43	90	52	0	0	0	0	15	8	0	0	20	10	0	0	0	0
AYA2011	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

a = keratinous substance hydrolysis (%), b = keratinase production (U/ml)

lected for further investigation. Moreover, bovine hair as a sole carbon and nitrogen source was selected as the best inducer for the production of AYA2000 keratinase. Therefore, the use of bovine hair, a cheap and readily

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TABLE 4 : Effect of protoplast fusion on crude keratinase activity and stability at different pH

pH	Enzyme activity (U/ml)			Enzyme stability (%)		
	ESRAA1997	ALAA2000	AYA2000	ESSRA1997	ALAA2000	AYA2000
3	0	0	0	0	20	22
4	0	2	0	10	25	31
5	0	3	0	40	36	45
6	20	5	20	70	48	79
7	45	9	52	70	61	87
8	50	9	70	100	78	100
9	50	18	75	90	90	100
10	25	62	75	85	100	100
11	7	10	60	80	100	100
12	5	0	36	78	76	85

TABLE 6 : Effect of bovine hair concentration on keratinase production by fusant Micromonospora sp. AYA2000

Bovine hair concentration (%)	Final pH value	Biomass (g/l)	Bovine hair hydrolysis (%)	Keratinase activity (U/ml)
0.5	7.55	4.2	41	10
1.0	8.28	4.5	45	39
2.0	8.76	4.9	88.0	75
3.0	9.00	5.4	80.8	60
4.0	9.28	6.2	76	43
5.0	9.42	6.4	60	27

TABLE 8 : Purification of keratinase enzyme from fusant Micromonospora sp. AYA2000

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)	Purification factor (fold)
Crude supernatant	320	5850	18.28	100	1
80% ammonium sulphate precipitation	74	2250	30.41	38.46	1.66
DEAE- cellulose column chromatography	21.28	1900	89.29	32.48	4.89
Sephadex G-100 column chromatography	12.60	1640	130.16	28.04	7.12

available substrate, could result in a substantial reduction in the cost of enzyme production. Previous reports, showed potent keratinolytic activity against keratin of hard structure by different species of Streptomycetes[1,28].

Efficiency of protoplast fusion technique on crude AYA2000 keratinase activity

Data illustrated in TABLE 3, 4 and 5 proven that protoplast fusion technique was a promising tool to over-

TABLE 5 : Effect of protoplast fusion on crude keratinase activity and stability at different temperatures

Temp. (°C)	Enzyme activity (U/ml)			Enzyme stability (%)		
	ESRAA1997	ALAA2000	AYA2000	ESSRA1997	ALAA2000	AYA2000
20	11	0	16	100	100	100
25	21	0	24	100	100	100
30	30	0	35	100	100	100
35	35	0	45	100	100	100
40	41	17	59	100	100	100
45	45	60	70	100	100	100
50	50	17.5	75	100	95	100
55	57	12.88	76	48	80	100
60	53	12.50	80	29	74	100
65	51	0	70	10	55	80
70	46	0	65	2	55	60
75	32	0	50	0	40	42
80	7	0	39	0	25	30

TABLE 7 : Effect of different supplements to the BH-medium on keratinase production by fusant Micromonospora sp. AYA2000

Supplements to BH medium	Final pH value	Biomass (g/l)	Bovine hair hydrolysis (%)	Keratinase productivity (U/ml)
Control (Bovine hair)	9.22	5.2	90	75
Yeast extract	9.00	7.4	77	65
Casein	9.28	6.2	64	43
Peptone	8.62	7.6	79	60
Malt extract	8.89	6.98	93	85
Beef extract	9.90	8.20	70	60
Yeast extract+ Peptone	9.10	7.40	92	70
Yeast extract+ Malt extract	9.12	7.80	100	90
Peptone+ Malt extract	9.00	8.00	93	82
Yeast extract+ peptone+ Malt extract	8.90	8.78	100	111
Glucose	7.50	8.00	35	25

produce keratinolytic enzymes with improved properties by the recombinant fusant (AYA2000). The increasing levels in the keratinase production by AYA2000 fusant were up to 9.62, 27.91, 35.0, 50.0, 81.81, 100, 100 and 100 % more than ESRAA1997 (P1) with stratum corneum, chicken feather, duck feather, bovine nail, human nail, bovine hair, sheep wool and human hair, respectively. On the other hand, it was increased by 3.45, 25.0, 42.85, 63.16, 64.10, 100, 100, 111.11 and 121.05% than ALAA2000 strain (P2) using bo-

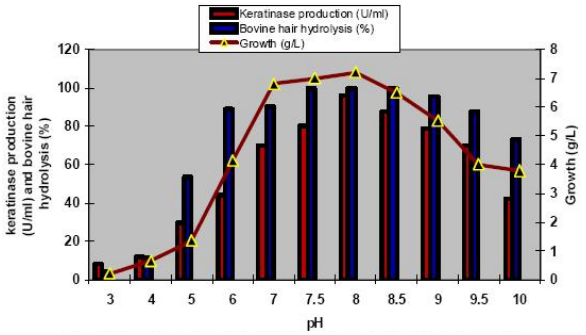

Figure 2(a) : Effect of pH of medium on growth, keratinase production and bovine hair hydrolysis by fusant *Micromonospora spp.* AYA2000

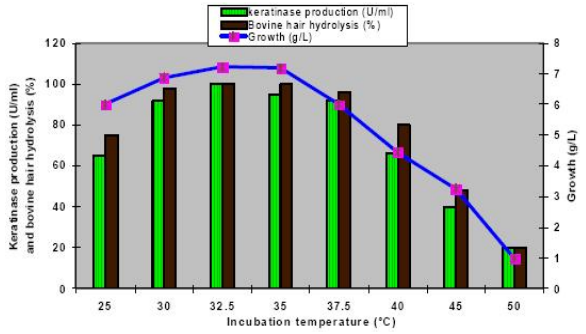

Figure 2(b) : Effect of incubation temperature on growth, keratinase production and bovine hair hydrolysis by fusant *Micromonospora spp.* AYA2000

vine nail, human nail, human hair, sheep wool, bovine hair, , chicken feather, duck feather, stratum corneum, and bovine skin, respectively (TABLE 3). Crude AYA2000 keratinase was stable enough in the range of 20 to 60 °C and at pH from 8 to 11 compared to 20 to 50°C with pH 8 and 20 to 45°C with pH range 10-11 for keratinase of ESRAA1997 (P1) and ALAA2000 (P2), respectively. Recombinant DNA technology was reported as a promising method to increase the production levels of enzymes and to improve the stability, activity or specificity of industrial enzymes and bioactive benzopyrone derivatives from new recombinant fusant of marine Streptomyces[4,5,26].

Optimization of keratinase production by fusant AYA2000

The highest keratinase activity was achieved at 32.5°C as the optimal temperature and at pH 8 in the seventh day of incubation (124.12 U/ml) after which it declined (Figure 2 a, b and c). Both bovine hair degradation and keratinase activity were increased proportional to the hair concentration up to 2% (75 U/ml) and

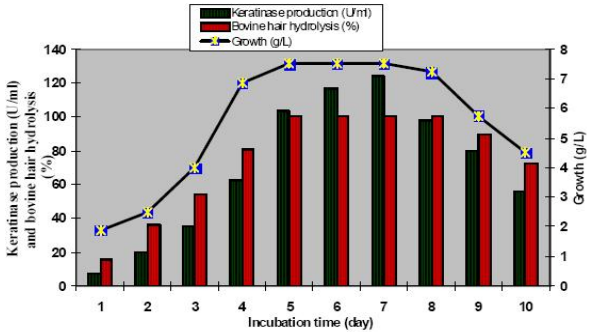

Figure 2(c) : Typical time course of growth, keratinase production and bovine hair hydrolysis by fusant *Micromonospora spp.* AYA2000

it was decline thereafter (TABLE 6). These results were similar to that of Wang and Yeh[27], they reported that maximum keratinase activity of Bacillus subtilis TKU007 was achieved with 1% hair as a substrate in 48 h and higher hair concentrations might cause substrate inhibition or repression. Using of a mixture of some supplements (malt extract, yeast extract and peptone) to BH-liquid medium exerted a favorable effect on AYA2000 keratinase production (from 75 to111 U/ml) (TABLE 7). However, none of the other supplements exerted this favorable effect. The growth of fusant Micromonospora sp. AYA2000 caused a significant increasing in the pH of the medium during bovine hair degradation, indicating strong keratinolytic activity. This tendency to increase the pH of the culture medium may be results from the production of ammonia due to the deamination of peptides and amino acids, originating from keratin degradation[28]. The level of AYA2000 keratinase activity was increased by 48% after optimization experiments. Previous reports indicated that temperature, pH and the nature of supplements presented in the medium have varied effects in different microbial species[27-29].

Purification of AYA2000 keratinase

In the present investigation, the purification of keratinase enzyme produced by fusant *Micromonospora sp*. AYA2000 was very efficient. Keratinase was purified from the culture filtrate by 80% saturation of ammonium sulfate (the fraction showed the highest keratinase activity) followed by DEAE- cellulose column chromatography and gel filtration (Sephadex G-100). The purification steps are summarized in TABLE 8 and figure 3. An overall specific activity 130.16 U/mg protein, recovery of 28.04% and 7.12 purification fold

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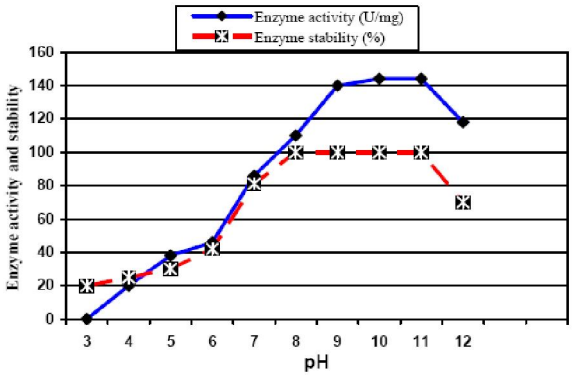

Figure 4(a) : Effect of pH on purified keratinase activity and stability from fusant AYA2000

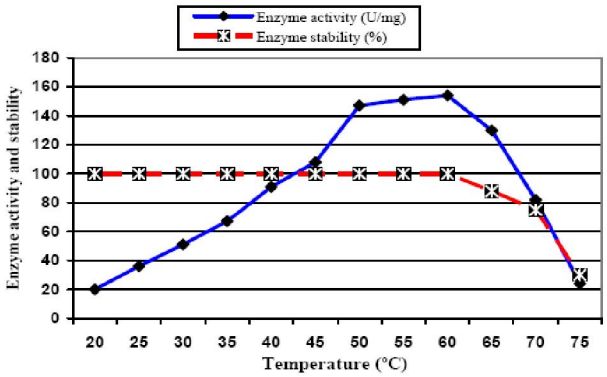

Figure 4(b) : Effect of temperature on purified keratinase activity and stability from fusant AYA2000

TABLE 9 : Effect of different metal ions, protease inhibitors and other chemicals on fusant AYA2000 keratinase activity

Reagent	Residual activity (%)*
*Control	100
Metal ions	
Na+	100
K+	100
Ca2+	118
Cu2+	82
Fe3+	102
Mg2+	110
Mn2+	85
Zn2+	100.5
Co2+	113
Pb2+	36.4
Hg2+	64.2
Protease inhibitors and other chemicals	
EDTA	100
PMSF	0
3,4-DCC	10
DTT	90
SDS	88.6
2,4-DNP	100
Acetonitrile	100
Acetone	120
Ethanol	115
Methanol	112

***The activity of fusant AYA2000 keratinase without any reagent was defined as 100%**

of keratinase were obtained. Homogeneity of the purified keratinase was revealed by SDS-PAGE, as seen by a single protein band (Figure 3). Keratinase enzyme has been purified from different Actinomycetes[29-31]. The molecular mass of the enzyme was 39kDa according to standard of protein molecular weight. However, the

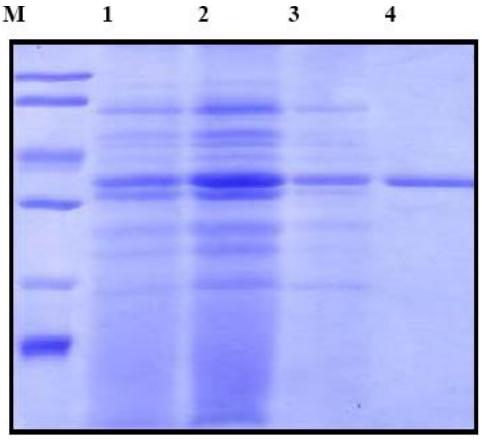

Figure 3 : Purification of keratinase from fusant Micromonospora sp. AYA2000. Lane M: molecular weight marker (97, 66, 45, 30, 20 and 14kDa), lane 1: crude extract, lane 2: fraction of 40-80% ammonium sulfate precipitation, lane 3: fraction of DEAE- cellulose column chromatography and lane 4: fraction of Sephadex G-100 column chromatography

TABLE 10 : Substrate specificity of fusant AYA2000 keratinase

Substrate	Relative activity (%)
Bovine hair	100
Human hair	96
Chicken feather	93
Sheep wool	95
Stratum corneum	87
Duck feather	92
Bovine skin keratin	83
Human nail	69
Bovine nail	58

molecular weight of AYA2000 keratinase was similar to the majority of microbial keratinases that have molecular weight vary from 20 to 50kDa included the keratinase of Streptomyces S7 and Streptomyces pactum DSM 40530[8,29].

Characterization of purified AYA2000 keratinase

As illustrated in figure 4 (a and b), the optimum pH for keratinolytic activity of fusant AYA2000 was in the range of 10-11(144 U/mg) and decreased out of the optimal pH range. Furthermore, keratinase seemed stable enough in the pH range from 8 through 11 and the optimum temperature for the keratinolytic activity was 60°C at pH 10-11(154 U/mg). Keratinase showed 100, 100, 100, 100, 100, 100, 100, 100, 100, 85, 60 and 50 residual activities after 60 min standing at 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75°C, respectively. Data obviously indicated that maximal AYA2000 keratinase activity similar to other alkaline keratinolytic enzymes reported for other Streptomycetes at usual temperature range[8,29,32] with a peak at pH 11 and 60°C. The stimulation of keratinase activity by alkaline pHs suggests a positive biotechnological potential to formulate detergents, in leather industry and in bioremediation processes[2,29,31,33].

The effects of various protease inhibitors, metal ions and other reagents at a concentration of 5mM on the keratinase activity were assessed (TABLE 9). The complete inhibition of enzyme activity by phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor, reveals that keratinase produced by fusant AYA2000 belongs to serine protease type. To confirm that, 3,4-dichlorocoumarin, another specific serine protease inhibitor, was used and the enzyme lost 90% of its activity after 30 min of incubation. It was partially inhibited by SDS and dithiothreitol (DTT), but not by ethylene diamine tetra acetic acid (EDTA), metalloprotease inhibitor, or 2,4-dinitrophenol (2,4-DNP). As a result (TABLE 9), Pb^{2+}, Hg^{+}, Cu^{2+} and Mn^{2+} inhibited keratinase activity to 36.4%, 64.2%, 82.0% and 85.0% respectively. In view of the properties of pH, temperature and inhibitors profile of AYA2000 keratinase, it can be classified as a thermostable alkaline serine protease, which is common for most of the keratinases described from Streptomyces pactum DSM 40530, Streptomyces albidoflavus and Streptomyces fradiae ATCC 14544[29-31]. On the other hand, Mg^{2+}, Co^{2+} and Ca^{2+} activated keratinase activity to 110, 113 and 118%, respectively. Other metal ions such as K+ and Na+ had no effect on the enzyme activity. Moreover, the residual keratinase activity after keeping for 24 hours in the presence of the tested organic solvents (25% v/v) at 4°C were determined (TABLE 7). The keratinase activity was stable in the tested organic solvents, since precipitation occurred in the culture supernatant kept in organic solvents at 4°C. It was presumed that stabilization of Pseudomonas aeruginosa keratinase in organic solvents occurred because the precipitation in organic solvent maintained the stereo configuration and thus the activity of the enzyme[34].

Moreover, data in TABLE 10, confirmed that AYA2000 keratinase is potent broadly specific toward a wide variety of keratinous wastes but with different degree. Our studies showed that among all the substrates tested, hair demonstrated the highest degree of hydrolysis and susceptibility whereas nail was least susceptible for hydrolysis. Relative specific activity of the enzyme decreased in the following order: bovine hair 100%, human hair 98%, chicken feather 97%, sheep wool 96%, stratum corneum 96%, duck feather 94%, bovine skin keratin 90%, human nail 69% and bovine nail 58%.

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