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Isolation of Pigment Producing Actinomycetes from Rhizosphere Soil and Application It in Textiles Dyeing.

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ABSTRACT

The toxicity problems caused by synthetic dyes to the environment have created amounting interest towards natural pigments. Pigments from microbial sources as natural dyes are potentially good alternative ones to synthetic pigments. The main objective of this study is to isolate rhizospheric actinomycetes capable to produce natural dyeing pigments which have antimicrobial activity against pathogens. To achieve this goal, 12 different actinomycetes isolates were obtained from rhizosphere soil of garden of plant department of women's Faculty for Arts, Science and Education, Ain Shams University and garden of National Research Centre and tested for pigment production. Six isolates were found to produce pigments on the tested solid media represented in international *Streptomyces* project (ISP). These isolates were inoculated onto yeast malt (ISP2), glycerol asparagine (ISP5), tyrosine (ISP7) and tryptone (ISP1) broth media to produce pigment in liquid state. Only one isolate (No. 10) was the best one in pigment production and produce reddish and greenish black pigments after 7 days of incubation. This isolate was identified as morphological, physiological and biochemical characteristics and the identification was confirmed by 16s rDNA as *Streptomyces torulosus*. Three pigmented media were used to produce natural pigments by *Streptomyces torulosus*. Three different pigments were obtained after one day and developed until 7 days of incubation. These pigments were reddish black, brown and deep green with ISP2, ISP7 and ISP5, respectively. The produced pigments were assessed for dyeing application in textile industry. The results leaded to accept these pigments as natural dye for clearing deep brown and reddish brown colour to different grade of textiles. The colour was detected to be stable after wash processes.

Keywords: Actinomycetes, Natural pigment, *Streptomyces torulosus*, Textile dyeing.

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INTRODUCTION

Actinomycetes are gram-positive filamentous bacteria and widely distributed in soil, water and other natural ecosystem [1]. The population and type of actinomycetes in an environment are determined by numerous physical, chemical and biological factors [1]. And it is known as a good producer for microbial bioactive compounds [2]. A lot of these compounds have been isolated and characterized, many of which have been developed into drugs and other bioactive materials [3]. Many kinds of antibiotics are produced by actinomycetes and moreover of these antibiotics contain pigments [4]. Various pigments are produced by actinomycetes using natural and artificial media. These pigments are usually described in different colours such as blue, violet, red, rose, yellow, green, brown and black. The pigments may be dissolved into the medium or retained in the mycelium [4]. Production of pigments by actinomycetes has been utilized as an important cultural characteristic in describing the organisms [5]. Pigments production is affected by initial medium pH, aeration and incubation temperature, as well as carbon and nitrogen sources. Synthetic dyes have some limitations, (i) hazardous chemicals used in production process and creating worker safety concerns (ii) they may produce hazardous wastes and (iii) the dyes are not environmentally safe [6]. Due to the toxicity of these dyes there has been a great effort to study and gadget the various natural dyes in the dyestuff industry. The present work deal with isolation of pigment producing actinomycetes from soil and characterize the efficient actinomycetes isolate in pigment production. As well as try to use the produced pigment in textiles dyeing.

MATERIALS AND METHODS

Isolation and screening of pigment(s) producing actinomycetes:

The actinomycetes used in this study were isolated from4 soil samples collected from different location sat garden of plant department of women's Faculty for Arts, Science and Education, Ain Shams University and garden of National Research Centre, Egypt. Soil samples were taken at 5 cm depth and then kept in clean plastic bags. Over the surface of solidified starch nitrate agar plates, 0.1ml of the soil dilutions was spread out by sterilized glass rod. The plates were incubated at 30^{o}C for 7 days. The different actinomycetes morphological characteristics were isolated and purified on starch nitrate agar plates and then stored on starch nitrate slants.

Twelve actinomycetes were isolated in this study and subjected to screen the efficient isolates in pigment(s) production by inoculating0.1ml of heavy spore suspension (5 days old) onto starch nitrate agar plates. The inoculated plates were incubated at 30^{o}C for 20 days. The morphological characteristics of actinomycetes colony such as colour of aerial mycelium and substrate mycelium, elevation and surface characteristics were detected.

Six isolates were chosen from primary screening and subjected through advanced screening on 4 standard agar media [tryptone (ISP1), yeast malt (ISP2), glycerol asparagine (ISP5) and tyrosine (ISP7)]. Hundred microliter of heavy spore suspension (5 days old) was inoculated onto agar plate's media and incubated for 20 days at 30^{o}C.

Identification of the most efficient isolate in pigment(s) production:

The most efficient actinomycetes in pigment(s) production was identified according to cultural, morphological and physiological characteristics using Bergey's Manual of Systematic Bacteriology [7]and the description of *Streptomyces* sp. was detected by the International *Streptomyces* Project (I.S.P) introduced by Shirling and Gottlieb [8]. *Streptomyces* isolate 10 was the most efficient isolate in pigment(s) production and was grown on starch nitrate agar medium for 5 days of incubation at 30°C.The spore chain morphology and spore surface ornamentation were examined by light microscope on oil immersion using Olympus CX 4 microscope with camera (Olympus SC 100) by cover slip technique [9].

Molecular identification of *Streptomyces* isolate 10:

The identification of *Streptomyces* isolate 10 was done using 16s r DNA analysis .The total genomic DNA of *Streptomyces* isolate 10 was extracted from 3 days old cultures growing on starch nitrate broth

medium by enzymatic lysis using lysozyme (20mg/ml) and sucrose buffer. The potassium acetate buffer was added to precipitate bacterial proteins. Total DNA was purified using isopropanol extraction as described by Darwesh et al. [10]. The 16s r DNA genes of isolated DNA was amplified by polymerase chain reaction (PCR) technique using forward primer 16RW01 (5-AACTGGAGGAAGGTGGGAT-3) and reverse primer 16DG74(5-AGGAGGTGATCCAACCGCA-3) [11]. The final 50 µl reaction mixture contained1x PCR buffer (NEB, England), 1nmol of dNTPs, 1 pmol of 2 mM $MgSO_4$, 0.25 pmol of forward and reverse primers, 1 unit of Taq DNA polymerase (NEB, England) and 10 mol template DNA was prepared in sterilized PCR tubes. The PCR amplification included initial denaturation of DNA at 95^oC for 5 min, followed by 35cycles of 95^oC for 30 sec, 55^oC for 30 sec and 72^oC for 45 sec. The mixture was kept for 10 min at 72^oC for complete extension. The amplified PCR product was tested using gel- electrophoresis (agarose gel) and purified by QIA quick Gel Extraction kit (QIAGEN,USA) and run on agarose gel to get the purified 16s rDNA fragments for sequencing. Identification was achieved by comparing the contiguous 16s rDNA sequences obtained with the 16s rDNA sequence data from the reference and type strains available in public data bases Gen Bank using the BLAST program (National Centre for Biotechnology Information) The phylogenetic reconstruction was done using the neighbor-joining (NJ) algorithm, with bootstrap values [12].

Production of pigment(s) by *Streptomyces torulosus* using different media:

Yeast malt (ISP2), tyrosine (ISP7) and glycerol asparagine (ISP5) media were used in this study for pigment(s) production. A disc (1 cm diameter) from mycelia and spores of 5-day-old cultures of *Streptomyces torulosus* grown on starch nitrate agar medium was inoculated into 250ml conical flasks containing 100ml of broth media. The flasks were incubated into incubator shaker (120rpm) at 30$^oC$for 15 days. The produced pigment was extracted by filtration through What man filter paper No. 1 and the supernatant was detected and scanned using spectrophotometer (Jen way 6405 UV/VIS) to identify the suitable wavelength of each pigment.

Antimicrobial activity of the produced pigments:

Pathogenic microorganisms, used in this study, were obtained from the American type culture collection (ATCC; Rockville, MD, USA).Two tested Gram positive bacteria; *Staphylococcus aureus* ATCC-47077 and *Bacillus cereus*ATCC-12228, as well as two Gram negative bacteria; *Escherichia coli* ATCC-25922 and *Salmonella typhi*ATCC-15566were used. Two yeast strains; *Candida albicans* ATCC-10231 and *Saccharomyces cerevisiae* ATCC- 9763, as well as one fungal strains; *Aspergillus niger ATCC-16888*were subjected for this study. The antimicrobial activity of the produced pigments extracts was assayed by agar wells diffusion technique [13]. Agar plates were prepared using nutrient agar medium for bacteria and potato dextrose agar medium for yeasts and fungi. The plates were inoculated with 0.1 ml containing 10^6cfu/ml of fresh cultures and spore suspensions of pathogenic microbes. Wells of 6 mm in diameter were dug on the inoculated agar plates using a sterile cork borer. The tested pigments (70 µl) were added to the wells. The sterilized water was tested as control [14]. The plates were left for two hours at 4°C to allow the diffusion and then incubated for 24 h at 37°C, except *Aspergillus niger* which was incubated at 28°C for 72 h. Antimicrobial activities of pigments were determined by measuring the three replicates of the inhibition zones around the well in mm [15].

Dyeing experiment

Lab scale dyeing experiments were carried out on wool and polyamide 6 fabrics using the produced dyes without mordanting [16].The dye bath was prepared with the produced dye at a liquor ratio 40: 1 and with 1 g/l amphoteric leveling agent (Albegal B).The pH of dyeing solution was adjusted to 3.0 by glacial acetic acid. The dyeing was started at 50°C for 10 min, then the dye bath temperature was raised to boil over 30 min and the dyeing continued for 45 min. After dyeing, the temperature was lowered to 60°C, and then the dyed samples were rinsed and washed off in an aqueous solution 2 g/l non-ionic detergent (Hospital CV) at 60°Cusing a liquor ratio 50: 1 for 30 min, rinsed and dried [4].

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RESULTS AND DISCUSSION

Isolation and screening of actinomycetes isolates:

The main objective of this study is to obtain some rhizospheric actinomycetes isolates capable of producing pigment(s). To achieve this goal, four soil samples were collected from different rhizospheric locations at garden of plant department of women's Faculty for Arts, Science and Education, Ain Shams University and garden of National Research Centre, Egypt. Twelve actinomycetes were isolated from collected soil samples and screened for pigment(s) production. About 6 pigmented positive actinomycetes isolates were obtained from a total of 12 isolates. Pigmented actinomycetes were around 50% and non pigmented isolates were about 50%. The morphology of these isolates was observed and recorded (Table 1). The six pigmented isolates were advanced screened on yeast malt, glycerol asparagine and tyrosine agar media to evaluate the diffusible pigment production and the results were illustrated in Table (2). Actinomycetes isolate 10 gave white aerial mycelium on ISP1, grey on ISP2, green on ISP5 and deep beige on ISP7. Also this isolate was gave white substrate mycelium on ISP1, black with diffusion on ISP2, green with diffusion on ISP5 and brown on ISP7 (Fig. 1).

From these results, actinomycetes isolate 10 was the best one in pigments production and these pigments diffused into media. These results in agreement with the data recorded by Amsaveni et al. [1], who isolated 14 soil actinomycetes isolates producing pink, yellow, deep pink, greenish brown and brown diffused pigments in the Kuster's broth medium.

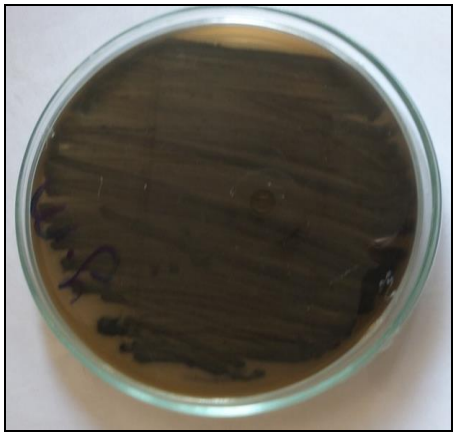

Figure 1: Pigmented substrate mycelium of actinomycetes isolate 10 on yeast malt agar medium.

Table 1: Morphological characterization of actinomycetes isolates.

Isolate number	Colour of aerial mycelium	Colour of substrate mycelium	Diffusible pigment	Elevation	Surface
1	White	Beige	-	Flat	Smooth
2	White	Pal beige	-	Raised	Wrinkled
3	Grey	White	-	Raised	Rough
4	White	Pal grey	-	Raised	Smooth
5	Creamy	Pal beige	-	Flat	Slimy
6	Beige	Pal grey	-	Raised	Rough
7	Grey	Beige	+	Raised	Wrinkled
8	Grey	Deep beige	+	Raised	Wrinkled
9	Grey	Coffee	+	Raised	Rough
10	Pal grey	Pal grey	+	Flat	Wrinkled
11	White	Creamy	+	Raised	Smooth
12	White	White	+	Flat	Smooth

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Table 2: Characterization of six actinomycetes growth at different agar media

Media	Parameters	Actinomycetes isolates					
		7	8	9	10	11	12
Tryptone (ISP1)	Growth	+	+	+	+	+++	+
	Aerial mycelium	White	White	Grey	White	Creamy	White
	Substrate mycelium	Beige	Beige	Pale beige	White	Light grey	Beige
Yeast molt (ISP2)	Growth	+++	+	+++	++++	+++	+
	Aerial mycelium	White	White	Grey	Grey	White	White
	Substrate mycelium	Beige	Beige	Yellow	Black with diffusion	Brown	Grey
Glycerol asparagine (ISP5)	Growth	+++	+++	+	+++	++	+++
	Aerial mycelium	White	Grey	Grey	Green	White	White
	Substrate mycelium	Beige	Bale brown	Beige	Deep green with diffusion	Brown	White
Tyrosine (ISP7)	Growth	++++	+++	++++	+++	+++	+++
	Aerial mycelium	White	Grey	White	Deep beige	White	White
	Substrate mycelium	Beige	Pink	Deep beige	Brown	Brown	Beige

Where; (-) no growth,(+)weak growth,(++) moderate growth, (+++)good growth and (++++) very good growth.

Identification of the most efficient isolate in pigment production:

Actinomycetes isolate 10 was the most efficient isolate in pigments production and the best one in production of diffusible pigments. This isolate was inoculated on 4 solid agar media (ISP1, ISP2, ISP5 and ISP7) with pH 7.2 and exhibited a weak growth on ISP1 while it showed a good growth on all other media. The aerial mycelium colour was ranged from deep beige to green on all media. The colour of the substrate mycelium was showed black on ISP2, green on ISP5 and brown colour on ISP7. The soluble pigments were observed on ISP2 with black colour, ISP5 with green colour, and ISP7 with brown colour (Table 2).The morphological characteristics such as shape of spore chains of this isolate was observed under light microscope and the results showed long chains of spiral segmented with hook shaped mycelium and more than >10 spore. The spores were spherical as it was illustrated in Figure (2).

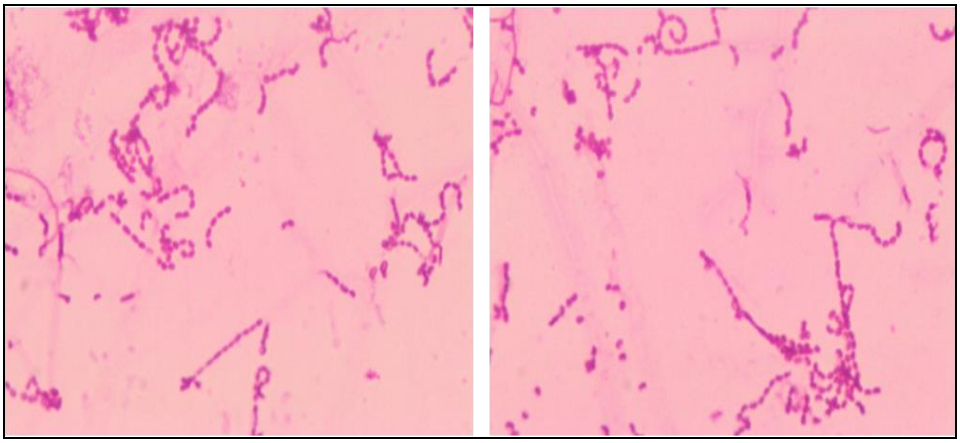


Figure2: Light microscope photograph (400x) of *Streptomyces* isolate 10 grown on starch nitrate agar for 5 days, 30ºC.

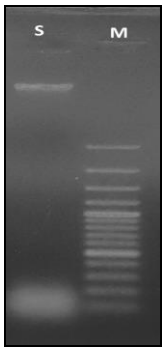
The physiological characteristics of this isolate were represented in Table (3). This isolate was positive in melanin pigment production, nitrate reduction test, starch hydrolysis and not able to hydrolyze of gelatin. Also this isolate was able to produce H_2S by fermentation and degrade the xanthine, L-tyrosine and casein. As well as, it was hydrolyzed urea and sensitive to Streptomycine antibiotic.

Table 3: Physiological Characteristics of the actinomycetes isolate 10

Test	Reaction
Melanin pigment production.	+ ve
Nitrate reduction.	+ ve
H_2S production.	+ ve
Gelatin hydrolysis.	- ve
Starch hydrolysis.	+ ve
Xanthine degradation.	+ ve
L-Tyrosin degradation.	+ ve
Casein degradation.	+ ve
Growth at 45°C	- ve
NaCl-tolerance (2-5 %w/v)	2.5 %
Resistance to Streptomycin (25µg ml^{-1})	- ve
Urea hydrolysis	+ ve

Molecular identification of Streptomyces sp.isolate10:

The morphological, biochemical and physiological identifications of *Streptomyces* isolate 10 were confirmed using molecular biology techniques. The total DNA of *Streptomyces* sp. isolate10 was isolated and detected on gel electrophoresis (Figure 3). The DNA fragment was amplified by PCR technique using reverse and forward 16s rDNA genes. The size of PCR product was 370 bp which was detected on 1% agarose gel electrophoreses compared with protein marker (Figure 4). The new sequence of *Streptomyces* sp. isolate 10 was compared with available 16S rDNA gene sequences from organisms in the GenBank databases. The partial sequence of 16S rDNA of *Streptomyces* sp. was aligned by Blast program. The nucleotide alignment and distance matrix showed high similarity value (95 %) with species: *Streptomyces torulosus*. The phylogenetic tree of *Streptomyces torulosus* compared with closed species in the Gene Bank was illustrated in Figure (5).



Where: M=DNA marker, S=DNA fragment of sample

Figure 3: DNA fragment isolated from *Streptomyces* isolate 10

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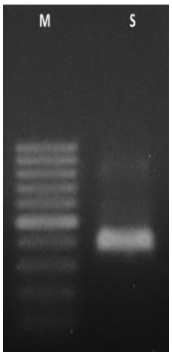
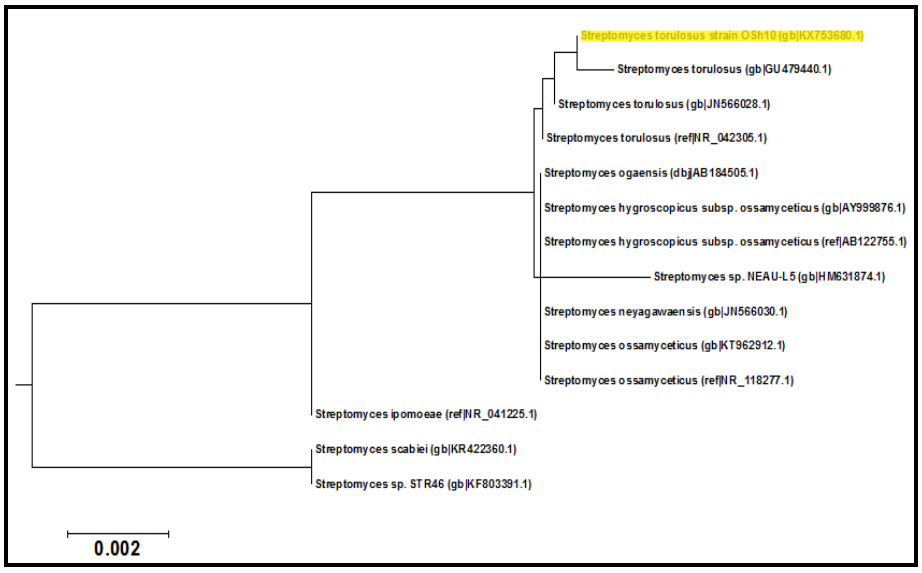


Where: M=DNA marker, S=PCR product of sample

Figure 4: Agarose gel electrophoresis for PCR product of 16s rDNA analysis of *Streptomyces*. isolate 10.



Where query sequence is isolated *Streptomyces torulosus.*

Figure 5: Phylogenetic tree constructed from the 16s rDNA sequence of *Streptomyces torulosus* and their related strains in Gene Bank.

Production of pigment(s) by *Streptomyces torulosus* using different media three deferent media were used to produce natural pigment(s) by *Streptomyces torulosus*. These media were yeast malt (ISP2), tyrosine (ISP7) and glycerol asparagine (ISP5). Hundred mL of each medium was inoculated by *Streptomyces torulosus* spores and mycelia. The pigment(s) were produced after 7 days of incubation under shaking conditions as illustrated in Figure (6) and some properties of the produced pigments were summarized in Table (4). Different colours such as black, deep green and brown were obtained in this study. Subhash and Kulkarni [9] produced red colour melanin by *Streptomyces bikiniensis* using tyrosine medium and used it as antimicrobial agent. The second aim of this study is to produce antimicrobial pigment and dyeing agent in the same time. To achieve this goal, we evaluated the antimicrobial activity and capability of produced pigment for dyeing different textiles.

Table 4: Summarize for some properties of the produced pigments by *Streptomyces torulosus*.

Properties	Pigment production media		
	Yeast malt (ISP2)	Glycerol Asparagine (ISP5)	Tyrosine (ISP7)
Colour	Black	Deep green	Brown
λ_{max} (nm)	525	390	390

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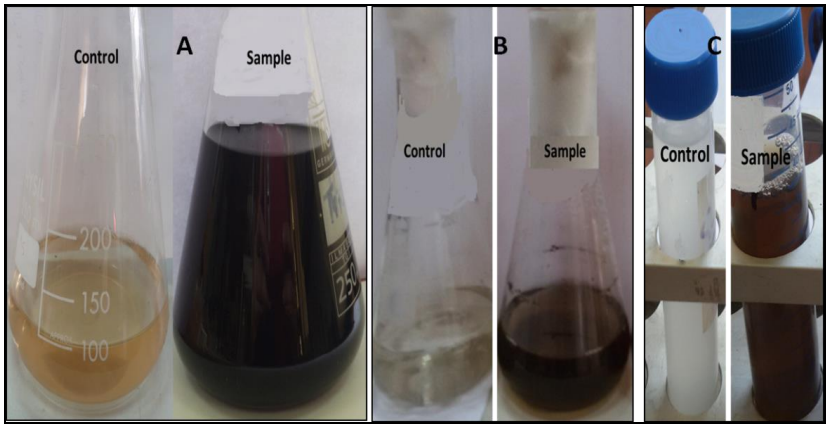


Where, A; yeast malt medium, B; glycerol asparagine medium, C; tyrosine medium, control; non-inoculated medium and sample; produced pigment

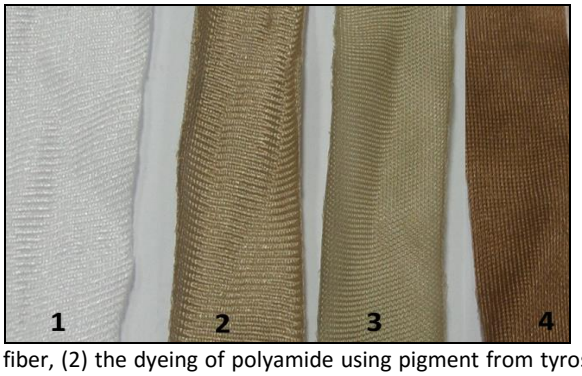
Figure 6: Pigments produced by *Streptomyces torulosus* using different media.

Antimicrobial activity:

The antibacterial activity of the produced pigments against both Gram positive (*Bacillus cereus* and *Staph. aurous*) and Gram-negative (*E. coli* and *salmonella typhi*) strains was evaluated in this study. The results indicated that the pigment did not have any activity against the tested microbes. Unfortunately also, in case of antifungal activity of the produced pigment(s), these pigments didn't have any activity against tested fungi. The obtained result in disagreement to that recorded by Amal et al. [4], who found that dark brown pigment produced by *Streptomyces virginiae* showed activity against Gram negative and positive bacteria (*Pseudomonas aeurogenosa* and *Staph. aurous*) as well as fungi (*F. oxysporium, Botrytis allii*, *Diplodiaoryzae*, *A. flavus* and *A. niger*). These results lead to find technology for enhancing the antimicrobial activity of these pigment(s).

Dyeing process:

The main objective of this study is to obtain natural dyes. This goal is considered the one of the most benefit option for replacing the toxic synthetic dyes by ecofriendly natural one. Fortunately, this aim was investigated in this study. Three different dyes with various colours were produced by *Streptomyces torulosus* using three pigmented media. These dyes were used for dying of wool and polyamide fabrics. The results of dyeing processes were illustrated in Figure (7, 8). The pigments from ISP2, ISP5 and ISP7 media gave different clear colours on polyamide and wool fabrics. These results lead to solve many problems causing by synthetic dyes.



Where (1) control of polyamide fiber, (2) the dyeing of polyamide using pigment from tyrosine medium, (3) pigment from asparagine medium and (4) pigment from yeast malt medium.

Figure 7: Dyeing of polyamide fiber using different natural dyes produced by *Streptomyces torulosus* in different media

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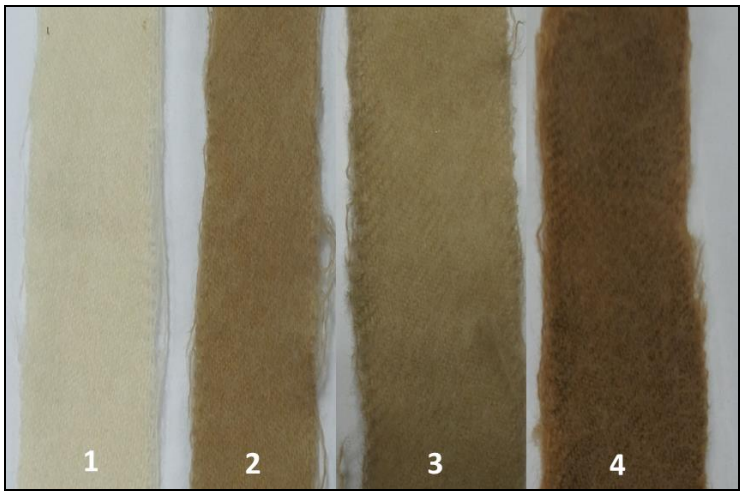

Where (1) control of wool fiber, (2) the dyeing of wool using pigment from tyrosine medium, (3) pigment from asparagine medium and (4) pigment from yeast malt medium.

Figure 8: Dyeing of wool fiber using different natural dyes produced by *Streptomyces torulosus* in different media

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