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Thermophilic cellulolytic microorganisms from western Algerian sources: promising isolates for cellulosic biomass recycling

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Abstract

Cellulosic biomass is considered as one of the most promising sources for the production of alternative renewable bioenergy and other valuable products. The abundance of cellulosic waste such as agricultural, industrial and forest waste, and the need for their biodegradation and their bioconversion into fermentable sugars, has increased the demands for more effective cellulase producing microorganisms. For this purpose, the present study was conducted to isolate thermophilic cellulolytic microorganisms. 111 thermophilic microorganisms (91 bacteria and 20 yeasts) were isolated from 10 western Algerian sources (thermal and non-thermal) and tested for the production of cellulase. The results revealed the presence of 19 thermophilic cellulolytic isolates. Macroscopic and microscopic examination has indicated the presence of 16 thermophilic bacteria and 3 thermophilic yeasts. These isolates were tested for the degradation of cellulosic biomass (printable paper, filter paper and cotton) for 14 days of incubation at 60°C. The obtained results showed a great potential of these thermophilic cellulolytic microorganisms to produce thermostable cellulolytic enzymes, and can be used in the recycling of cellulosic biomass for bioenergy production after optimization studies in the future.

Keywords: cellulosic biomass; recycling; bioenergy; thermophilic microorganisms; thermostable cellulases; Algerian sources.

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1. Introduction

In this rapidly changing world, demand for energy is increasing every year. According to Enerdata[1] world energy consumption in 2012 has increased by 1%, this has led to an augmentation in CO2 emissions by 1.4% [1]. Fossil fuels which are the main sources of energy in the world are an exhaustible resource and threaten our planet with a continued increase in pollution that causes global warming and which may adversely affect our future. This is why finding more renewable energy sources is now no longer something of luxurious.

Biomass which is the oldest source of energy in the world, can be today an effective alternative for the production of bioenergy. Technologies of bioenergy production from biomass require the intervention of microorganisms, these microorganisms have the ability to transform lignocellulosic materials using lignocellulolytic enzymes to fermentable sugars, for finally use them to produce ethanol, methane (gasification), Hydrogen, etc.

A large variety of enzymes are involved in the degradation of lignocellulosic compounds; Laccase, Manganese peroxidase, Lignin peroxidase for lignin, Pectin methyl esterase, pectate lyase, polygalacturonase, rhamnogalacturonan lyase for pectin, Endo-xylanase, acetyl xylan esterase, β-xylosidase, endomannanase, β-mannosidase, α-L-arabinofuranosidase, α- glucuronidase, ferulic acid esterase, α-galactosidase, p-coumaric acid esterase for Hemicellulose, and finally for cellulose bioconversion, three types of enzymes are implicated: exo-1,4-β-glucanases (cellobiohydrolase), endo-1,4-β-glucanases and β-glucosidases (cellobiases) [2].

Enzymes from mesophilic microorganisms are generally characterized by a remarkable instability, and require the use of techniques to reduce the temperature, which increases the cost. In addition to that, the enzymatic treatment carried out at 50°C causes a slow hydrolysis and gives a low yield of sugar (incomplete hydrolysis) [3].

Thermostable enzymes offer a real alternative, these enzymes as their name implies, have a very high stability; a higher specific activity, allowing for extended hydrolysis times and decreasing the amount of enzyme needed for saccharification [4].

The goal of this work is to isolate thermophilic cellulolytic microorganisms from local sources, and test their ability to degrade cellulosic biomass for a possible application in paper recycling field.

2. Materials and methods

All chemicals used in this study are of technical grade and obtained from commercial sources.

2.1. Preparation of colloidal cellulose

Cellulose used in this study to test the cellulolytic activity is a cellulose MN, this type of cellulose don't dissolve in water, that's why it was treated with pure HCl for one hour. After a series of washing with distilled water to remove HCl; the cellulose that is formed in this reaction is called “colloidal cellulose” which dissolves perfectly in distilled water.

2.2. Isolation of total thermophilic microflora

16 Samples were taken from 7 thermal and non thermal western Algerian sources (Table 1).

To induce the growth of total microflora, a method of sources enrichment was followed by adding 1g of soil to 10 ml of nutrient broth solution containing 0.1% yeast extract and 0.1% peptone. For liquid sources 0.1% yeast extract and 0.1% peptone were added directly to the sources, the tubes containing the samples were incubated at room temperature for 24h.

For the thermophilic microflora isolation, 200 ml of each enrichment solution was added to tubes containing 20 ml of an enrichment broth comprising 0.5% peptone, 0.3% beef extract, and 0.02% cellulose MN.

[1] http://www.enerdata.net

To induce the production of thermostable cellulases and the proliferation of thermophilic microorganisms, the tubes were heated for 15 min at 70°C and then incubated at 60°C for 24h [5].

Table 1. Sources of sampling.

Sources	number of samples	Type of Samples
1- Hammam Bouhnifia/Mascara[1] (Hot spring)	3	- Water source - Water and soil river - Sparkling water source
2- Hammam Bouhdjar/Aïn Timouchent[2] (Hot spring)	1	- Water source
3- Mediterranean Sea /Mers Elhadjadj, Oran	1	- Water sea
4- Daya Morsli/Oran (wetland)	2	- Water - Soil
5-Laghouat[3]	1	- Soil
6-Oran	4	- Aches (waste incinerator USTO[4]) - Compost - Semi-degraded hay (Hai Bouamama[5]) - Semi-degraded hay (Aïn Beïda[6])
7- Miscellaneous	4	- Pickled vegetables - Brined black olives - Brined green olives - Rotted citrus waste
Total	16	

2.3. Isolation of thermophilic microflora producing thermostable cellulases

Isolation of thermophilic microorganisms which produce thermostable cellulases has been carried out in liquid media containing: 0.2% cellulose MN, 0.5% peptone, 0.3% beef extract and 0.1% olive oil. The results were recorded after 24, 48 and 120 hours of incubation at 60°C.

The purification of the isolates was done on nutrient agar plate, the isolated colonies were then transferred to nutrient broth media (NB) and stored at -20°C in glycerol 50%.

2.4. Selection of thermotolerants isolates from thermophilic isolates

In addition to their ability to grow at 60°C, the isolates were tested at 37°C to see if they are thermophilic or thermotolerant.

2.5. Selection of hyperthermophilic isolates

To test if the isolates are hyperthermophilic, they were cultured in a nutrient broth and incubated at 80°C for 72h.

[1] Mascara is an Algerian wilaya (department) in the north west of the country.
[2] The wilaya of Ain Témouchent, located in the west of Algeria between the provinces of Oran, Tlemcen and Sidi Bel Abbes.
[3] The wilaya of Laghouat is an Algerian wilaya, located at 400 km south of the capital Algiers.
[4] University of Sciences and Technology of Oran Mohamed Boudiaf.
[5] Hai Bouamama or El Hassi is a neighborhood in the south-west of the municipality of Oran, crossed by the highway 2 (RN2).
[6] Aïn Baïda or Ain El Beida, is a neighborhood in the municipality of Es Senia, Oran.

2.6. Detection of extracellular cellulases production

The isolated microorganisms were inoculated on two minimum media (0.1% peptone, 0.1% beef extract, 1.5% agar), the first one contains 0.2% cellulose MN, and the second one contains 0.2% colloidal cellulose. The incubation was done at 60°C for 48 hours.

To reveal the presence of clear zone, Congo red solution (1%) was poured on culture dishes, half an hour later, a solution of NaCl (1N) was added to intensify the color.

2.7. Macroscopic and microscopic study

The macroscopic appearance of colonies was examined using a Stereo microscope. The microscopic appearance of the isolates was studied using a light microscope after staining the microorganisms with a Gram stain.

2.8. Cellulose product degradation by total thermophilic cellulolytic microflora on submerged fermentation

To test the cellulosic degrading capacity of the isolated microorganisms, a mixed culture was applied in a 250 ml Erlenmeyer flask containing 150 ml of liquid culture medium composing of filter paper (0.5%), cotton (0.5%), peptone (0.1%) and beef extract (0.1%).

2.9. Application of thermophilic cellulolytic isolates for printable paper degradation

Cellulolytic isolates were inoculated into tubes containing 20 ml of minimal media + ribbon of printable paper (0.5 cm wide). The test of paper degradation was viewed by using a Stereo microscope and also by observing the physical condition of the paper ribbon, in particular the consistency of the paper, the color and transparency.

Isolates that gave the best results were selected for microscopic observation, for this, sections were made in fragile areas using a Stereo microscope. The paper sections were colored according to the following protocol[1]:

- Congo Red for 1 min;
- Rinsing with water;
- Gentian violet for 1 min;
- Rinsing with water;
- Drying in the open area;
- Microscopic observation.

3. Results and discussion

3.1. Isolation of thermophilic microflora producing thermostable cellulases

Contrary to our expectations, most of the sources tested in this research have given unexpected results. Table 2 presents the results observed in 24, 72 and 120 hours of growth.

After the microbial isolates purification, the final number of isolates was 111 strains, with 19 cellulolytic thermophilic microorganisms. Potential sources of thermophilic cellulolytic microorganisms are showed in Fig.1.

With 16% of the isolates, the hot spring Hammam Bouhnifia at Mascara is a very important source of thermophilic microorganisms waiting for exploration. This source has a very important microbial diversity with thermophilic bacteria and yeasts. By cons, Hammam Bouhdjar; the other studied hot spring, did not give results for thermophilic cellulolytic microorganisms.

32% of isolates were obtained from semi-degraded hay (Hai bouamama and Ain Baïda), this source seems to be very important for the isolation of thermophilic microorganisms.

[1] This protocol was established by us

Also different brines can be a potential source of thermophilic microorganism's isolation (30% of the isolates).

Table 2. Isolation of thermophilic microflora producing thermostable cellulases.

Sources	Type of Samples	Cellulolytic activity		
		24h	48h	72h
1- Hammam Bouhnifia/Mascara (Hot spring)	- Water source	-	+	+
	- Water and soil river	+	+	+
	- Sparkling water source	-	+	++
2- Hammam Bouhdjar/Aïn Timouchent (Hot spring)	- Water source	-	-	-
3- Mediterranean Sea /Mers Elhadjadj, Oran	- Water sea	-	-	-
4- Daya Morsli/Oran (wetland)	- Water	-	++	++
	- Soil	-	-	++
5-Laghouat	- Soil	-	-	-
6-Oran	- Aches (waste incinerator USTO)	-	+	++
	- Compost	-	-	+
	- Semi-degraded hay (Hai Bouamama)	++	++	++
	- Semi-degraded hay (Ain Baïda)	-	+	++
7- Varied	- Pickled vegetables	++	++	++
	- Brined black olives	+	+	++
	- Brined green olives	++	++	++
	- Rotted citrus waste	-	-	-

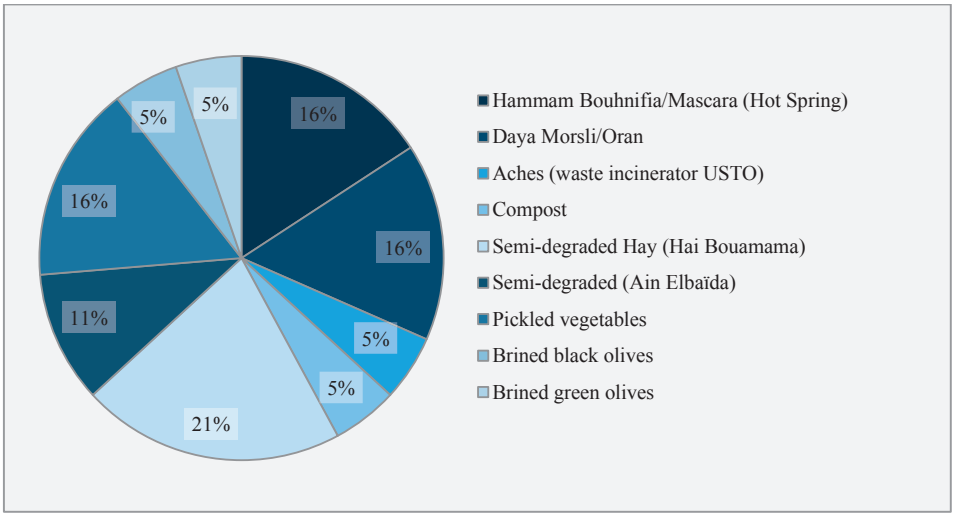

Fig. 1. Potential sources of thermophilic cellulolytic microorganisms.

Most of the thermophilic microorganisms are isolated from the hot springs, but there are so-called unconventional sources for isolation of microorganisms that produce thermostable enzymes (Table 3).

3.2. Selection of thermotolerants isolates from thermophilic isolates

There was no growth after 24 hours of incubation at 37°C, so the isolated microorganisms are thermophilic and not thermotolerant.

Table 3. Examples of unconventional sources of microorganisms producing thermostable enzymes

Sources	Microorganism	Enzyme	References
Soils	*Bacillus sp.*	Amylase	[5]
Decomposed plant	*Clostridium absonum CFR-702*	Cellulase	[6]
Semi-degraded hay	*Clostridium sp*	Cellulase	This study
Compost	*Bacillus MH-1*	Endochitinase	[7]
Compost	*Bacillus stearothermophilus CH-4*	β-N-acetylhexosaminidase	[8]
Compost	*Yeast*	Cellulase	This study
Fermented anchovy	*Bacillus sp. KYJ963*	β-Amylase	[9]
Pickled vegetables	*Bacillus*	Cellulase	This study
Landfill	*Bacillus circulans*	Xylanase	[10]
Compost treated with artichoke juice	*Bacillus sp.*	Inulinase	[11]

3.3. Selection of hyperthermophilic isolates

After 72 hours of growth at 80°C, two hyperthermophilic isolates were detected, the isolate C3 (Hammam Bouhnifia - Sparkling water source) and the isolate C9 (Semi-degraded hay - Hai Bouamama).

3.4. Detection of extracellular cellulases production

The detection of the extracellular cellulase production was made by observation of the clear zone on the growth media. Fig. 2 shows the degradation of the colloidal cellulose; however there were no significant results for the untreated cellulose (Cellulose MN).

The enzymes produced by microorganisms may be useful if they are excreted from the cell. This is why the experiences that make this study are based on the extracellular enzymes. These enzymes can be easily recovered, and yield interesting results concerning the enzymatic degradation and bioconversion of cellulosic biomass. Dashtban et al. (2009) have pointed out the importance of the extracellular cellulase excreted by microorganisms (fungi and bacteria) in the bioconversion of lignocellulosic residues [12].

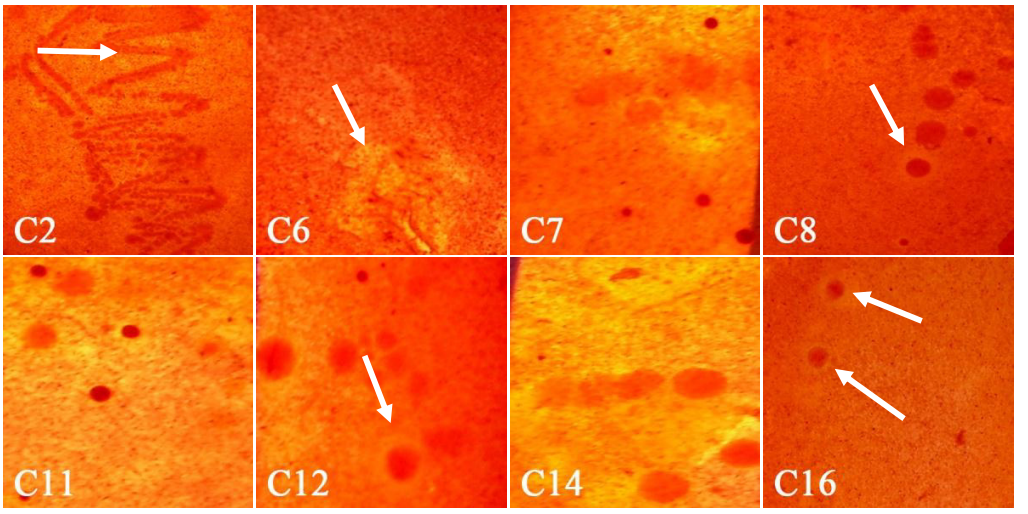

Fig. 2. Degradation of colloidal cellulose. C2 Hammam Bouhnifia/Mascara (Sparkling water source); C6 Daya Morsli (water); C7 Semi-degraded hay (Aïn Beïda); C8, C11, C12 Semi-degraded hay (Aïn Beïda-Oran); C14 Pickled vegetables ; C16 Brined black olives (X10).

3.5. Macroscopic and microscopic study

The macroscopic study allowed us to detect different forms of cellulose lysis, which vary depending on the microorganism's type.

In aerobic microorganisms we have observed a yellowish clear zone around the colony, however, anaerobes isolates precipitate the cellulose which results in a zone of lysis in the form of nebula (Fig. 3).

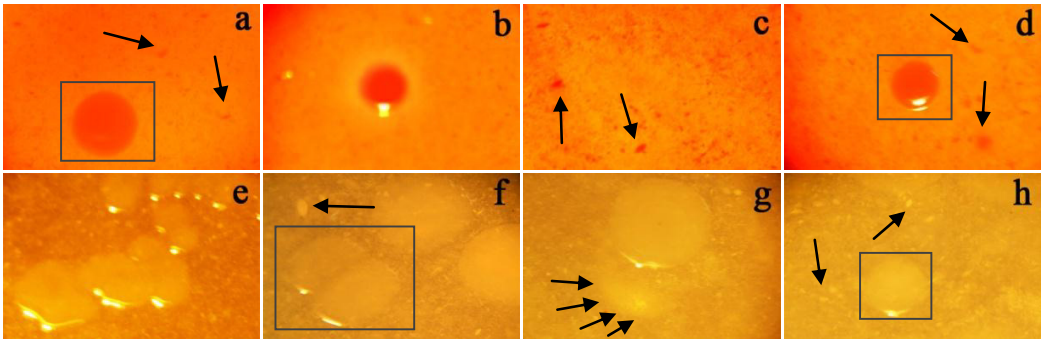

Fig.3. Morphological diversity of some thermophilic cellulolytic colonies. a the isolate C12 is characterized by a flattened colony with a clear zone, the arrows indicate the presence of microcolonies; b isolate C13: clear zone of degraded cellulose; c anaerobic lenticular microcolony (Clostridium sp.); d synergy between anaerobic (arrow) and aerobic microorganisms (rectangle); e C8 colony is characterized by a creamy consistency; f, g ; synergy between anaerobic (arrow) and aerobic microorganisms (X10).

3.5.1. The isolated bacteria

16 cellulolytic bacterial strains were visualized with the light microscope, the Gram-positive bacteria are the most dominant (10 bacteria of the family Bacillaceae were isolated), but other types also exist, Fig. 4 shows some cellulolytic microbial diversity. Several thermophilic Bacillus species have been studied for their production of thermostable cellulase (*B. halodurans* [13], *B. licheniformis* [14], *B. subtilis* [15], *Bacillus amyoliquefaciens* [16]).

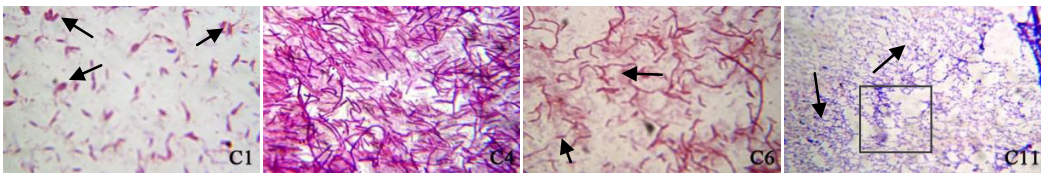

Fig. 4. Microscopic observation of isolated cellulolytic bacteria. C1 Bacillus (Gram-variable) with arrangement like a Chinese letter. C4 Gram-positive bacteria with a long size. C6 Bacillus Gram-variable bacteria. C11 Encapsulated Gram-positive Cocci (X1800).

There is few studies on the production of thermostable enzymes from gram-negative bacteria, these bacteria are rarely studied for this purpose and not used for the production of thermostable enzymes. In this study a thermophilic gram-negative bacterium was isolated (isolate C3). Ruby et *al.*(2000) attribute the difficulty of isolating some microorganisms to their interaction with other organisms [17]. Similarly, Rhee et *al*. (2000) have succeeded to isolate a new gram-negative thermophile which has an obligate commensalism with another thermophilic *Bacillus* strain [18].

Anaerobic bacteria are also important as a source of thermostable enzymes, it's the genus *Clostridium*, which is the most used (C. *thermobutyricum et C. thermopalmarium C. thermocellum, C. thermolacticum, C. thermoalcaliphilum,*) [19]. Several thermophilic strains of *Clostridium* were isolated in this study; these strains provide cellulose degradation under anaerobic conditions, which is very important in the field of biofuels productions.

3.5.2. The isolated yeast

3 cellulolytic yeasts were isolated; these isolates are characterized by degradation of cellulose under anaerobic conditions, and have various forms of storing bodies (Fig. 5).

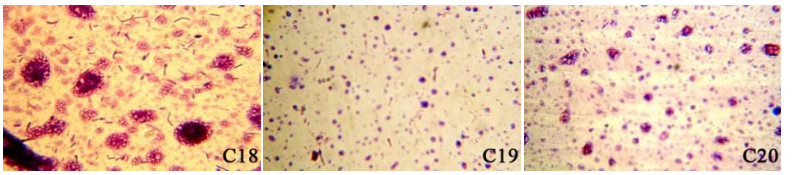

Fig. 5. Morphological diversity of the thermophilic cellulolytic isolated yeast.

These isolated yeasts are very important in industrial point of view, as they ensure the production of valuable products as ethanol [10] at high temperature. Several studies have shown the existence of thermophilic yeast as *Hansenula polymorpha* with a maximum temperature for growth at 48°C [21]. The genus *Candida* also includes thermophilic yeast; *Candida thermophila* is a thermophilic yeast that has a maximum temperature for growth at 50°C [22]. Fossi et al. showed the existence of thermophilic yeast in the soil [5], 7 yeasts were isolated with a maximum temperature for growth at 40°C. The cited studies have indicated that the upper limit of the temperature in the known thermophilic yeast doesn't exceed 50°C. In this work we isolated 3 yeasts that can grow at 60°C, which offers very important biotechnological perspectives.

3.6. cellulose product degradation by total thermophilic cellulolytic microflora on submerged fermentation SmF

After two weeks of incubation at 60°C; dry weight of cellulosic biomass has increased from 1g to 0.7g, so a degradation of 30% occurred.

To test the ability of microorganisms isolated for the bioconversion of cellulose, we must use insoluble cellulose because there are no clear relationships between cellulase activities on soluble substrates and those on insoluble substrates, soluble substrates should not be used to screen or select improved cellulases for cellulose bioconversion [23].

In 1981, Ng et al. have observed an acceleration of cellulose hydrolysis on mixed cultures combining *Clostridium thermocellum* (thermophilic cellulolytic strain) and *Clostridium thermohydrosulfuricum* (thermophilic strain, non-cellulolytic, capable of degrading many sugars including cellobiose) [24].

3.7. Application of thermophilic cellulolytic isolates on printable paper degradation

Paper degradation was viewed with a Stereo microscope (Fig. 6). The degradation of the cellulose fibers was visualized using a light microscope (Fig. 7).

After 14 days incubation at 60°C we obtained the following results:

- The control (Fig. 6: 1) has not changed color or consistency, it remained intact.
- The isolates C8 (Semi-degraded hay - Aïn Beïda), C14 (Pickled vegetables) and C2 (Hammam Bouhnifia - Sparkling water source) (Fig. 6: 2, 5 and 7): we noticed a swelling of the paper and a detachment of fibers. There are accessory proteins called swolenins and expansins, these proteins affect the saccharification of cellulose and they act by swelling (up) the cellulose network which disrupts the structure of chemical bonds and facilitates the action of cellulase, the presence of these proteins during the cellulolytic hydrolysis increases the conversion of cellulose [25].
- The isolate C4 (Daya Morsli - Soil) (Fig. 6: 3): yellowing of paper probably due to the degradation of cellulose which exposes the lignin to the light and gives a browning. In fact Paper made of mechanical pulp is very sensitive to photochemical reactions of lignin. These papers tend to turn yellow when exposed to light for any length of time [26].
- The isolates C1 (Hammam Bouhnifia - Sparkling water source) and C9 (Semi-degraded hay - Hai Bouamama) (Fig. 6: 4 and 6): The paper became transparent and fragile, the isolate C9 has been selected for microscopic

observation, sections were made in fragile areas and have been colored by a double staining; Congo red for the cellulose and Gentian violet for the Bacteria. The Fig. 6 (picture 8) shows the result; it was noted that the cellulose fiber took a red color, but in the center the color changes to pink, sign of cellulose degradation. The arrows indicate the presence of immobilized bacteria throughout the fiber; all around these bacteria we have noticed a clear area. These results let us assume that the bacteria C9 has an enzyme complex called cellulosome. Cellulosome is an extracellular multifunctional protein complex containing cellulase and xylanase with cellulose-binding domain (CBD). These enzymes have the ability to bind to the insoluble cellulose allowing them an efficient degradation of insoluble cellulosic materials [27].

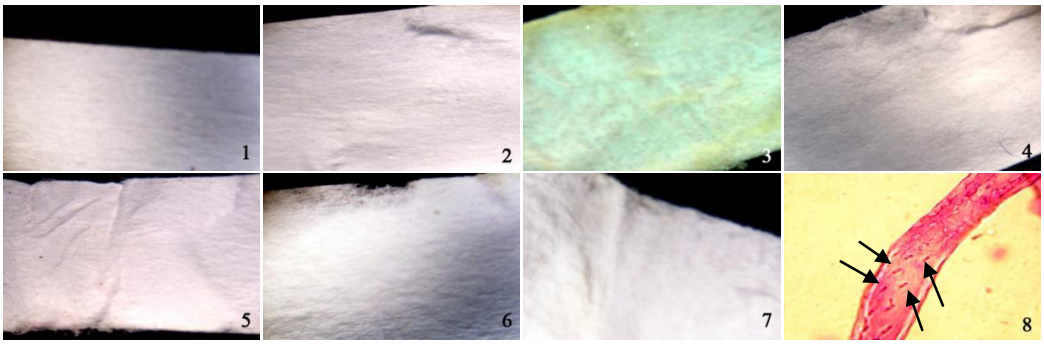

Fig. 6. Printable paper degradation. 1 Control, 2 isolate C8, 3 C4 isolate, 4 isolate C1, 5 isolate C14, 6 isolate C9, 7 isolate C2 (X10), 8 microscopic observation of a cellulose fiber degraded by the isolate C9 (X1800).

4. Conclusion

In this primary study, we succeeded to isolate several cellulolytic thermophilic and hyper thermophilic microorganisms (bacteria and yeast). These microorganisms offer a promising opportunity to recycle paper and produce biofuels and are waiting to be studied by molecular identification and optimization test.

This study also allowed us to value the Algerian sources, which is a gain, especially for the study of the Algerian hot springs microbial diversity that are not yet well studied.

We also recommend the unconventional sources (brine and agro-waste such as hay) for the isolation of thermophilic and even hyperthermophilic microorganisms.

The double staining protocol proposed in this study allowed us to see in situ the action of cellulolytic bacteria with less expensive way and can be used in other studies.

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