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Original Research Article

Newly isolated *Bacilli* from *Rosa damascena* cv. Taifi and their evaluation for cellulose degrading efficiency

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Keywords

Cellulose degrading enzymes, *Bacilli*, CMC, *Bacillus* broth, *Rosa damascena* cv. Taifi

A B S T R A C T

Cellulose degrading enzymes were evaluated in newly isolated different BSFA Taifi Bacteria, mainly *Bacilli* from the medicinal plant *Rosa damascena* cv. Taifi leaves, flowers, halo-, rhizo- and endophytes. The enzyme assay was typically performed on plates containing Crystalline Microcrystalline Cellulose (CMC) in the agar at a final concentration of 0.1–0.5 % (w/v). After incubation at 30ºC, the plate was flooded with 1% (W/V) Congo red which gave a more rapid and highly discernable result. Twelve BSFA Taifi *Bacillus* isolates grown-up in Nutrient broth showed cellulase activity. Among twenty one BSFA bacterial isolates grown-up in *Bacillus* modified broth growth media, eighteen showed cellulase activity and the other three strains may be pseudocellulolytic. Four Bacterial BSFA Taifi isolates grown-up in tryptic soya broth showed cellulase activity. Collectively thirty four Bacterial BSFA Taifi strains were isolated. A higher clearing zone of bacterial *Bacillus* cellulase enzyme (10±2 mm) was obtained for each of the five *Bacillus* BSFA Taifi strains (104, 111, 112, 114 and 131) in *Bacillus* modified broth growth media and *Bacillus* BSFA Taifi strains (197, 216, 220, 230 and 269) in nutrient broth.

Introduction

Cellulose is an extensively existing natural reserve with an enormous potential for bioproducts. It is found in leaves, stems, and stalks from sources such as corn fiber, corn stover, rice straw, rice hulls, sugarcane bagasse, woody crops, and forest residues. It is also present in coconut biomass, sawdust, citrus peel waste, paper pulp, municipal cellulosic solid waste, industrial waste, and paper mill sludge. From four different invertebrates (termite, snail, caterpillar, and bookworm) eight isolates of Cellulose Degrading Bacteria (CDB) were isolated and their extracellular cellulase activities

were reported by Pratima et al. (2012). *Bacillus pumilus EWBCM1*, a strain isolated from earthworm gut (*Eudrilus eugeniae*) at 37°C and pH 6 in submerged fermentation, showed cellulase activity (Shankar and Isaiarasu., 2011). The cellulolytic enzymes of *Bacillus* species were utilized for the agricultural wastes to convert them into valuable products (Ozaki *et al.,* 1990). Krishna (1999) reported that *Bacillus subtilis CBTK 106* can produce a substantial amount of cellulase activity.

Cellulase secreted by cellulolytic bacteria degrades cellulose. Cellulases are at present the third major industrial enzyme universally. Microbial origin cellulase enzyme is of industrially applicable main enzyme. The current $5.8 billion world enzyme industry, the world's enzyme demand, will rise 6.8 percent annually to $8.0 billion in 2015. Cellulases add to 8% of the international industrial enzyme demands (Bon and Maria, 2007). The cellulase market has been predicted in the U.S.A to be as high as US$ 400 million per year (Zhang *et al.,* 2006). Cellulases are applied in textile manufacturing for biopolishing of fabrics and producing stonewashed looks of denims. In domestic laundry detergents they are used for improving fabric softness and brightness (Hill *et al.,* 2006). Cellulases have vital roles in food manufacturing and in bioconversion of cellulosic biomaterials into commodity chemicals (Lynd *et al.,* 2005).

Cellulases those are either free or cell-associated to hydrolyze and metabolize insoluble cellulose. According to their mode of catalytic action, cellulase systems were classified. Endoglucanases (Endo-1, 4-β-D-Glucan Glucanohydrolases) are active against acid-swollen amorphous cellulose, soluble derivatives of cellulose such as CMC, cellooligosaccharides (Wood, 1989). Exoglucanase (1, 4-β-D-Glucan Cellobiohydrolases) are active against crystalline substrate such as Avicel, amorphous celluloses and cellooligosaccharides and inactive against cellobiose or substituted soluble celluloses such as CMC. The Exoglucanases (1, 4-β-D-Oligoglucan Cellobiohydrolases) are also inactive against amorphous cellulose or CMC.

β-Glucosidases or β-D-Glucoside Glucohydrolases hydrolyze soluble cellodextrins and cellobiose to glucose. It is inactive against crystalline or amorphous cellulose**.** Cellobiose phosphorylase catalyzes the reversible phosphorolytic cleavage of cellobiose (Ayers, 1959).

Some cellulolytic bacterial species are *Trichonympha, Clostridium, Actinomycetes, Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus albus, and Methanobrevibacter ruminantium* (Milala, 2005; Schwarz, 2001). Bacteria belonging to *Cellulomonas, Bacillus, Thermomonospora, Erwinia, Acetovibrio, Microbispora,* and *Streptomyces* can produce cellulases (Bisaria, 1991). The bacterium, *Cellulomonas,* grows more rapidly on a Petri dish and the production of extra-cellular cellulases by it is simple to measure. *Bacillus Subtilis, Bacillus spharicus, Bacillus circulans, Sinorhizobium fredii, Pseudomonas, Paenibacillus azotofixans, Gluconacetobacter, Azospirillum, Cytophaga* and *Vibrio* are cellulolytic bacterial species (Emtiazi, 2007).

The present work is aimed at screening and isolating bacteria from the samples collected from different regions (flower, leaves, stems and roots) of the medicinal plant *Rosa damascena* cv. Taifi which produce cellulase enzyme using selective media with enrichment.

In this paper we report the cellulase enzymatic activity of rose (locally as *ward Taifi*) extract from *Rosa damascena* cv. Taifi (Collected from *Al Hada* region in Taif and was grown in the green house of Taif University). This project study on *Rosa damascena* cv. Taifi was conducted at the Molecular Biotechnology Research Unit (MBRU) Department of Biotechnology, Taif University from Dec 2012 to May 2013.

Materials and Methods

Materials, chemicals and rose extract

The Chemicals used were Liquid nitrogen ; Nutrient agar ; Nutrient broth; NaOH; *Bacillus* Agar, Nutrient Agar, Potato dextrose agar, Peptone broth, Carboxymethylcellulose (CMC), glycerol, NaCl and materials used for cellulase studies were tooth picks, Congo red, petridishes, transfeppettes, yellow tips, water bath, falcon tubes 1.5ml, burette, centrifuge, pH meter, shaker, etc.

An overview of the entire experiment is shown in Fig.1.

Preparation of rose extract

Initially, water was sterilized by autoclaving at 121°C at 15psig for 20min for the extraction. The flower leaves, and roots of Taif roses, *Rosa damascena* cv. Taifi were washed with sterilized distilled water and ethyl alcohol. All the parts which were taken in mortar and pestle were homogenized to fine powder separately with liquid nitrogen and the powdered material was extracted using sterilized distilled water. The rose extract collected was kept in a shaking incubator at 30°C for 2 hrs and then stored at -80°C until further use. This rose extract was used later for the study of cellulase activity.

Media preparations and screening

Initially, the mud of *Rosa damascena* cv. Taifi was collected from the cleaned roots and it was washed with 3.5 liters of sterilized distilled water. The supernatant was collected after filtration using Whatman filter paper No.1. Different nutrients media such as *Bacillus agar*, nutrient agar, tryptic soya agar & potato dextrose agar were made by autoclaving at 121°C at 15psig for 20min.

The sand water filtrate was poured into the plates made from the different media and kept in incubation temperatures 30°C, 40°C and 50°C. The growth in *Bacillus* agar at 50°C was predominant. An aliquot of the extracts from flower leaves and root were smeared in the plates of each media and kept at 30°C for 24hours.

All the plates containing total 321 colonies were marked. Colonies were picked out from the corresponding media plates and transferred to NA and peptone broths media prepared by autoclaving at 121°C at 15psig for 20min. All the 321 tubes containing the colonies were organized and kept at -80°C.

From the 321 tubes, colonies were inoculated in 77–171 BA plates, in 172–268 NA plates and in 269–321 TS plates.

Colonies were found from plates kept at various temperature environments (30°C/ 40°C/ 50°C. Colonies 77–98 found from BA 30°C; Colonies 99–120 found from BA 40°C; Colonies 121–132 found from BA 50°C; Colonies 133–148 found from Roots at 30°C; Colonies 149–171 found from Flower at 30°C; Colonies found in 172–268 in NA plates at 30°C/ 40°C/ 50°C; Colonies found in 269–321 in TS plates at 30°C/ 40°C/ 50°C.

The *Bacillus* agar medium was prepared by using the premix from Fluka's Hichrome *Bacillus* agar (49.2g of premix was added to 1L of distilled deionized water, the pH was adjusted to 7.1 and the solution was autoclaved at 121ºC for 15 minutes) and freshly prepared extracts were used during this project.

The *Bacillus* medium broth was prepared using the premix from Fluka's Hichrome *Bacillus* agar, 49.2g of premix was added to 1L of distilled deionized water, the pH was adjusted to 7.1 and the solution was autoclaved at 121ºC for 15 minutes (The agar was removed by filtration using Whatman filter paper to get clear broth). The *Bacillus* broth was poured into sterilized 20ml bottles.

The Hichrome *Bacillus* agar media contained Peptic digest animal tissue 10.00 g/L; Meat Extract 1g/L D-Mannitol 10g/L; Sodium chloride 10g/L; Phenol Red 0.025g/L and Agar 15g/L as composition. Final pH (at 25ºC) 7.4±0.2

The solutes were shaken until they were dissolved and the pH was adjusted to 7.4 using 1N NaOH (8ml). The volume was then adjusted to 1L by adding deionized water. The sterilization was done by autoclaving at 121ºC for 20 minutes at 15psi (1.05kg/cm) on liquid cycle.

The composition of Hichrome *Bacillus* media broth was the same like Hichrome *Bacillus* agar except for the agar.

The tryptic soya agar media with the composition of 40g/1lit tryptic soya agar in distilled water was adjusted to pH7.4 and the solution was autoclaved for 20minutes at 15 psi (1.05kg/cm) at 121ºC on liquid cycle and was used. The composition of tryptic soya media broth was the same like Potato dextrose agar except for the agar.

The carboxy methyl cellulose (CMC) was obtained from Loba Chemie, (Mumbai, India) with viscosity 1% aqueous solution at 20ºC.

The Congo red indicator solution was obtained from Loba Chemie, (Mumbai, India). pH 3.0–5.2 measured based on the range blue violet to red orange.

The agar plates were made by weighing twenty eight grams of Nutrient agar (obtained from Himedia, India) and dissolving it in a 1L of sterilized distilled water. The solution was autoclaved after adjusting the pH to 7.4 for 20minutes at 15 psi (1.05kg/cm) at 121ºC on liquid cycle. The autoclaved medium was swirled gently to distribute the melted agar evenly throughout the solution and allowed to cool to 50ºC–60ºC. Then under sterile conditions, 20ml of this medium was poured on to 90mm Petridishes and was set to cool. When the medium sets completely, Petridishes were inverted and stored at 4ºC and took out from storage 1–2 hours earlier to use (Sambrook and Russell, 2001). Suspensions of the bacterial cultures were covered completely on the agar plates and were allowed to dry. The inverted agar plates were then incubated for 24 hours at 37ºC. The colonies were marked when growth appeared.

Screening of cellulase producing bacterial strains

Screening for cellulase activity in isolated bacterial strains from *Rosa damascena* cv. Taifi were typically performed on plates containing crystalline microcrystalline cellulose (Avicel) in the agar at a final concentration of 0.1–0.5% (w/v). After incubation at 30ºC, a zone of clearing surrounding the colonies indicated that cellulose was produced (Kluepfel, 1988).

The colonies which did not show any clearing zone were also found (Schlegel and Schmidt, 1986).

For a quick screening of cellulase producing bacteria, after the incubation of the agar medium contains 0.5% (W/V) carboxymethyl cellulose (CMC) as sole carbon source and flooded with 1% of (W/V) Congo red (Teather and Wood, 1982). After 20 minutes, the dye was decanted and the plates were again flooded with 5M NaCl which was decanted after 20–30 minutes. Positive colonies were identified to be surrounded by a pale orange to clear zone against red background. The cellulolytic bacteria can be screened directly on such plate, but replica plating from master plate is preferred for isolation of active colonies as flooded reagent impairing isolation. Kasana *et al.* (2008) found that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red, gave a more rapid and highly discernable result (Kasana *et al.,* 2008).

Bacterial cellulase producing strains and their distinctiveness

Bacteria novel cellulase are significant for their relatively higher growth compared to fungi, exhibiting increased function and synergy, real resistant to ecological changeable conditions. Some bacteria (true cellulolytic), synthesize the complete enzyme system (produce some endoglucanases and ß-glucosidases) resulting in hydrolysis of the crystalline material available in nature. Bacteria not capable to synthesis the enzyme complete system was termed "pseudocellulolytic" (Coughlan and Mayer, 2013).

Endoglucanase were produced by *Bacillus* sp (Fukumori *et al.,* 1985) such as *Bacillus subtilis* (Kim and Pack, 1988), *Bacillus licheniformis* (Dhillon *et al.,* 1985), *Bacillus megaterium, Bacillus amyoliguefaciens* (Lee *et al.,* 2008), *Bacillus* sp (cloned in *E. coli*) AC-1 (Zhang *et al.,* 2006) and *Bacillus coagulans Co4* (Adeleke et al., 2012). Endoglucanase were also produced by *Thermomonospora* sp (George et al., 2001), *Cellulomonas* sp.YJ5 (Yin *et al.,* 2010), *Pseudomanas fluorescens* (Bakare et al, 2005) and *Clostridium josui* (Fujino et al, 1989). The exocellobiohydrolase were produced by *Cellulomonas uda* (Nakamura and Kitamura, 1988) and *Microbispora bispora* (Yablonsky *et al.,* 1988). An extracellular alkaline carboxy methyl cellulase (CMCase) from *Bacillus subtilis* strain AS3 has been purified and characterized (Deka *et al.,* 2013).

Result and Discussion

Cellulase activity of bacterial strains

A total of 245 bacterial strains (Fig. 2) were isolated from *Rosa damascena* cv. Taifi, from them, Thirty four BSFA Taifi bacterial *Bacillus* strains (Fig. 3 & 5) were found to be positive for cellulase production by their zone forming ability in NA-CMC.

Higher Bacterial cellulase activity was observed in five BSFA Taifi bacterial *Bacillus* strains (104, 111, 112, 114, 131) grown in *Bacillus* agar media and five BSFA Taifi (197, 216, 220, 230, 269) strains grown in nutrient agar media (Table 3). All four Bacterial *Bacillus* BSFA Taifi strains (279, 289, 307, 314) grown in tryptic soya (TS) media showed considerable cellulase activity (Table 1) Among two hundred and forty five bacterial isolates from three different media, thirty four BSFA Taifi bacterial *Bacillus* strains exhibited the cellulase enzyme activity (13.5%).

Table.1 Ratio of bacteria isolated from dental caries

Bacterial BSFA Taifi strains	Observations of cellulase activity	Media
BSFA Taifi 279	8±2	Tryptic soya (TS)
BSFA Taifi 289	8 ±2	
BSFA Taifi 307	6±2	
BSFA Taifi 314	8±2	

Table.2 Cellulase activity observed in *Bacillus* agar media for Bacterial BSFA Taifi strains isolatedfrom *Rosa damascena* cv. Taifi

Bacterial BSFA Taifi strains	Observations of Cellulase activity	Media
BSFA Taifi 80	2±2	*Bacillus* agar
BSFA Taifi 87	Neg.	
BSFA Taifi 93	2±2	
BSFA Taifi 96	Neg.	
BSFA Taifi 97	Neg.	
BSFA Taifi 98	6±2	
BSFA Taifi 99	6±2	
BSFA Taifi 104	10±2	
BSFA Taifi 108	8±2	
BSFA Taifi 109	6±2	
BSFA Taifi 110	8±2	
BSFA Taifi 111	10±2	
BSFA Taifi 112	10±2	
BSFA Taifi 113	6±2	
BSFA Taifi 114	10±2	
BSFA Taifi 131	8±2	
BSFA Taifi 150	2±2	
BSFA Taifi 151	6±2	
BSFA Taifi 153	6±2	
BSFA Taifi 162	2±2	
BSFA Taifi 169	2±2	

Table.3 Cellulase activity observed in Nutrient agar media for Bacterial BSFA Taifi strains isolated from *Rosa damascena* cv. Taifi

Bacterial BSFA Taifi strains	Observations of Cellulase activity	Media
BSFA Taifi 172	8±2	Nutrient Agar
BSFA Taifi 180	2±2	
BSFA Taifi 183	2±2	
BSFA Taifi 190	6±2	
BSFA Taifi 197	10±2	
BSFA Taifi 206	8±2	
BSFA Taifi 216	10±2	
BSFA Taifi 220	10±2	
BSFA Taifi 222	8±2	
BSFA Taifi 230	10±2	
BSFA Taifi 269	10±2	

Fig.1 Flowchart of the methodology

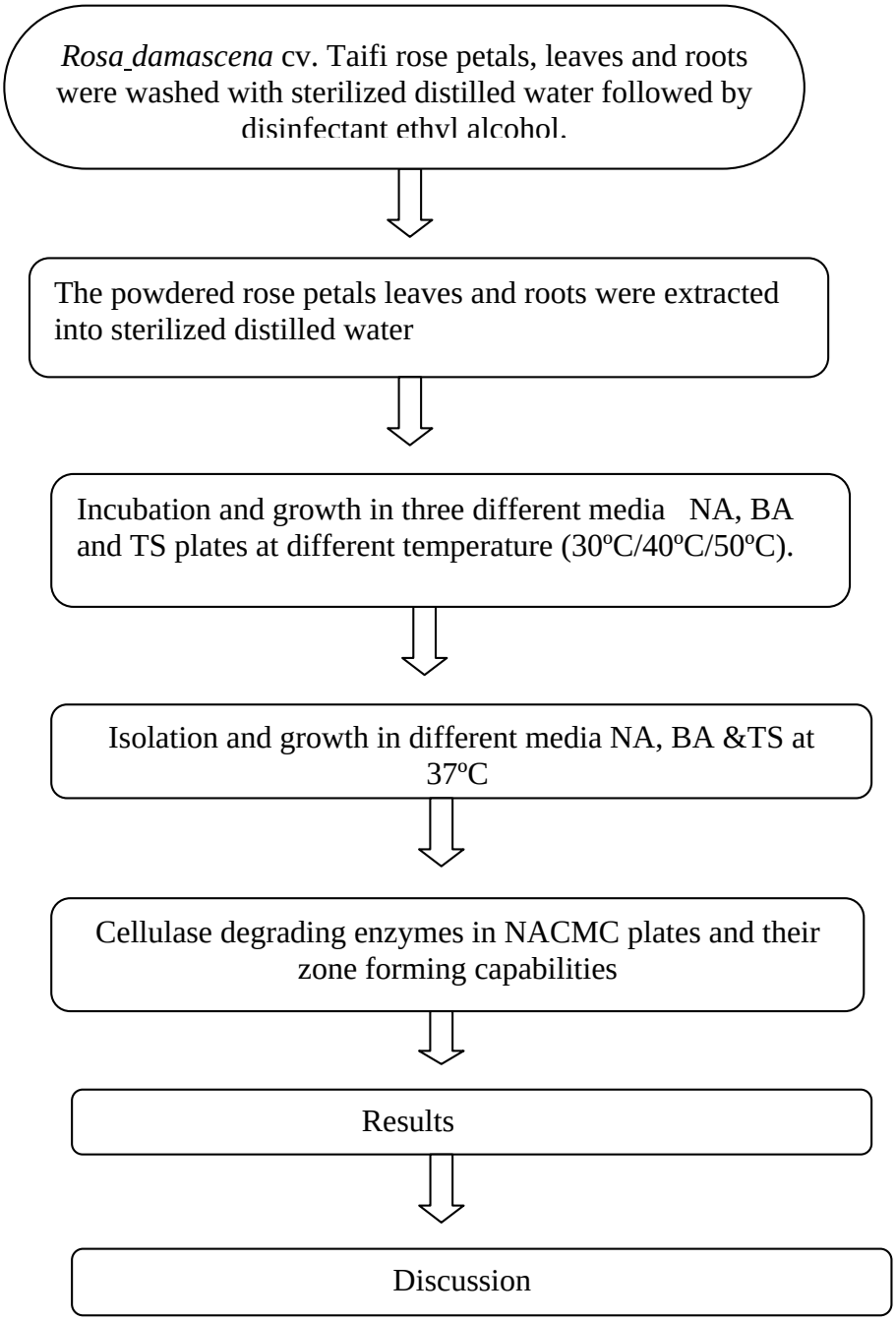


Fig.2 Total Number of BSFA Taifi (245) isolates grown in different media (BA, NA, TS)

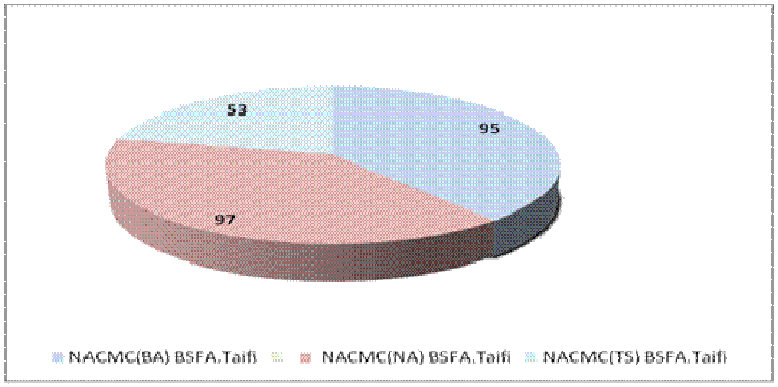


Fig.3 Number of Active BSFA (34) isolates grown in different media (BA, NA, TS) having cellulase activity confirmed by cellulose Congo red method (Teather and Wood, 1982)

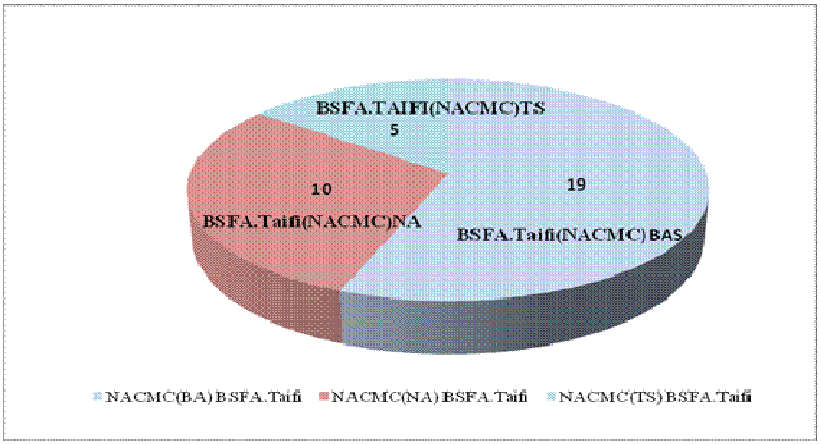


Fig.4 Cellulose degrading bacterial isolates from *Rosa damascena* cv. Taifi showed growth as the medium turned cloudy. Thirty four BSFA Taifi bacterial isolates showed positive on screening media (cellulose Congo-Red agar) producing a clear zone. The moderate cellulase activity of bacterial BAFA Taifi strains in BA, NA and TS broth were noticed

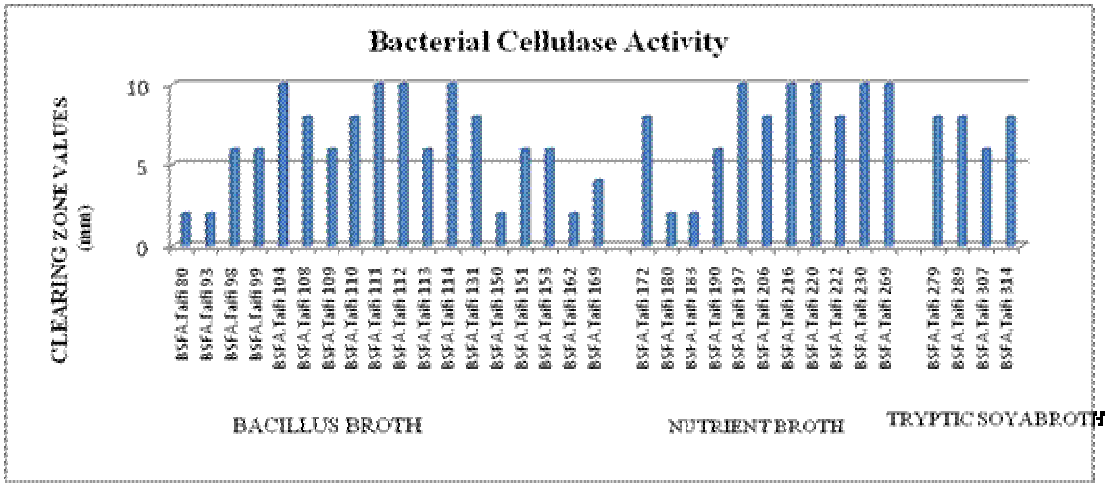
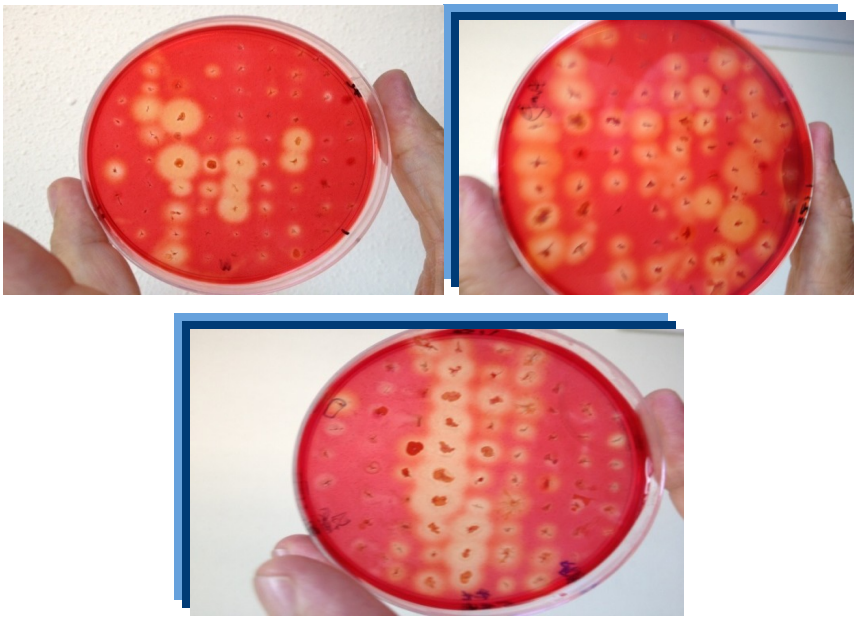


Fig.5 Cellulose degrading enzymes assay on CMC Congo-red agar stained and destained by 100 mM NaCl. The clearing zones indicate the enzymatic hydrolysis of cellulose



Eleven BSFA Taifi bacterial isolates grown in nutrient broth showed cellulase activity (Table 3). Among the twenty one Bacterial BSFA Taifi *Bacillus* isolates from *Bacillus* broth, eighteen showed cellulase activity (Table 2). The other three strains may be pseudocellulolytic. Four BSFA Taifi bacterial isolates from tryptic soya broth showed cellulase activity.

The diameters of the zones of inhibition of the bacterial isolates were ranged from 11.77±0.74mm to 18.40±1.10mm. The results obtained for BAFA Taifi were in line with the past findings denoted that bacteria.

Cellulase has been detected in various fungi and bacteria (Ayers, 1959; Shankar and Isaiarasu, 2011; Bisaria, 1991). However, in this study, newly isolated *Bacilli* from *Rosa damascena* cv. Taifi using three different media (*Bacillus* broth, Nutrient broth media and Tryptic soya broth) showed an excellent result for cellulase activity. These results are encouraging and promising. Most BAFA Taifi strains taken for the study behaved differently.

The cellulase production can be studied in the future for *Bacilli* isolated from *Rosa damascena* cv. Taifi and other available Taif roses varieties in the Kingdom. Further study is to be done for cloning and to perform the purification of cellulase enzymes and their characterization.

The most significant enzymes producer strains will be optimized for other industrial enzymes as previously done (Hussin et al., 2007, 2009, 2010 and 2012) and their application for bioconversion of plant biomass into various products (Idriss *et al.*, 2002; Farouk *et al.*, 2013).
Besides diverse chemical industries, cellulases are extensively used in animal feed, food, textile and paper industry and fuel bioconversions. The need for the

novel bacterial cellulases with specific higher function for industrial applications must be isolated or currently existing cellulases should be enhanced by means of available mutation techniques. Extra attempts are required for BAFA Taifi strains and cellulases to have significant industrial impact after optimization of their physico-chemical factors. Further study is required to characterize the cellulose efficiency and secretion in various growth media.

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