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Optimization of CM Case Production by Actinobacteria strains isolated from Syrian Freshwater Habitats

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Abstract: Cellulases are a group of hydrolytic enzymes capable of degrading lignocellulosic materials and have wide range of applications. Recently discovered that one major obstacle facing the degradation of cellulosic biomass is the cellulose hydrolysis stage, so the objective of this work was focused on detecting new bacterial strains isolated from freshwater habitats with high ability in CMCase production, and determined several parameters of optimal production: time incubation, pH, carbon and nitrogen source.
Eight CMCase producing actinobacteriastrains were isolated and characterized by morphological and biochemical analysis. Three strains were grown on Congo red agar and showed high degradation efficiency above 90%, these strains were used to produce CMCase, S5 and M7 strains revealed high ability in production with optimal parameters pH=8, temperature=40C, incubation period= 72-96 h, Carbon source= starch, and nitrogen source= peptone or ammonium phosphate.
Key words : bioconversion, *Streptomyces*, *Micromonospora,* cellulosic biomass, endoglucanase.

Introduction and Experimental.

Introduction:

Cellulose is a linear polysaccharide of glucose residues with *β*-1,4-glycosidic linkages, and the abundant availability of cellulose makes it an attractive raw material for producing many industrially important commodity products, recently discovered that one major obstacle facing the degradation of cellulosic biomass is the cellulose hydrolysis stage(1). In addition, much of the cellulosic waste is often disposed of by biomass burning, which is not restricted to developing countries alone, but is considered as a global phenomenon (1,2,3).

With the help of cellulosic bioconversion system, which considered as sustainable approach to develop novel bioprocesses and products (2),cellulose can be converted to glucose which is a multi-utility product, in a much cheaper and biologically favorable process (2, 3), this process is controlled by the enzymes of cellulase system which represents the key step for biomass conversion, moreover enzymatic hydrolysis requires synergistic action of cellobiohydrolase or exoglucanase (E.C.3.2.1.91), endoglucanase or carboxymethyl cellulase (CMCase) (E.C. 3.2.1.4) and cellobiase or β-glucosidase (E.C.3.2.1.21) (3, 4). Endoglucanase or CMCase is responsible for random cleavage of *β*-1, 4-glycosidic bonds along a cellulose chain, whereaseExoglucanase is necessary for cleavage of the non-reducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and *β*-1, 4-glucosidase hydrolyses cellobiose and water-soluble cello-dextrin to glucose (2, 3).

Although, successful utilization of cellulosic materials as renewable carbon sources, the enzymatic demands of many industrial applications and the demands for more stable highly active and specific enzymes is dependent on the development of economically feasible process technologies for cellulase production and the isolation of new strains with high ability in cellulosic biomass degradation (5).

Actinobacteria, which are gram positive bacteria with high contents of G $^{+}$C, may serve as a novel source of CMCase due to their higher growth rate as compared to fungi(6), high ability to survive in extremely conditions (7, 8),and their ability to produce a diverse array of bioactive compounds like cellulase enzymes (9, 10), in addition,many research have been reported with actinobacteriacellulosic activities, and cellulase production(10, 1, 11).

In spite of the great role of actinobacteria in freshwater environments in biodegradation of cellulosic biomass and other organic compounds, researches focused on aquatic actinobacteria were low compared to the efforts focused on soil ones(12,13), so the present work was carried out to improve CMCase production by new actinobacteria strains isolated from freshwater habitats, and determine the optimized condition of CMCase: pH, temperature, incubation time, and carbon and nitrogen sources (14,15, 1).

Experimental

1.Isolation and screening of CMCase producing actinobacteria:

Water samples were collected from many fresh water habitats: Barada river, 16 Tishreenlake, and north Al-Kabeer river in Syria, and diluted upto10^{-4}, mounts of 1 ml were spread in triplicate over the surface of ISP4 (glucose, malt extract, yeast extract, distilled water, and agar-agar) (16). The initial pH of medium was controlled at 7.2 - 7.4. All plates were incubated at 30°C for 3- 4 weeks (17, 18).

Strains were characterized and identify to the genus level based on the standard morphological methods (16, 19), whereas physiological characteristics were determined on API gallery 32C, and 20E (20). Pure strains were isolated on CMC medium, and incubated at 30 °C for2-3 days, the most bioactive strains in cellulose degradation were obtained by usage of Congo Red as an indicator (21), and only these were taken for further study. Cellulose-degrading potential of the positive strains was also qualitatively estimated by calculating hydrolysis capacity (HC), that is, the ratio of diameter of clearing zone and colony (2, 22).

HC= halo diameter – colony diameter/ dish diameter*100

2. CMCase production:

100 ml of the production medium (CMC 10, yeast extract 3, KH2PO4 1, K2HPO4 1.5, MgSO4.7H2O 0.4, CaCL2.2H2O 0.05, FeSO4 0.00125 g/l) was inoculated with 10^{7}cfu of freshly strainthat have degradation efficiency above 90%,thenthe flask was loaded on a rotary shaker incubator at a speed of 200 rpm at 30°C for 72 hours, after incubation, fermented broth was centrifuged at 5000 rpm for 15 min, and thesupernatant was collected and used for the CMCase assay as crude enzyme (23).

3.CMCaseassay:

CMCase activity was assayed by measuring the amount of reducing sugars liberated during the action of the enzyme on a CMC substrate (24). Briefly, a reaction mixture composed of 1mL of crude enzyme solution added to 1ml of 0.1M of citrate buffer pH=5 plus 1mL of 0.5% carboxymethyl cellulose (CMC) was incubated at 50°C for 30 min, after incubation 3ml of DNS reagent was added then boiled for 15 m in boiling water bath, and 1ml of 40% sodium potassium tartarate was added after cooling to room temperature, OD of samples was measured at 540nm against a blank containing all the reagents minus the crude enzyme(5, 25, 26).

One unit of endoglucanase activity was expressed as the amount of enzyme required to release 1 μmol reducing sugars per ml under the above assay condition by using glucose as a standard curve (23, 27)

CMCase activity = absorbance * standard factor (IU/ml/min).

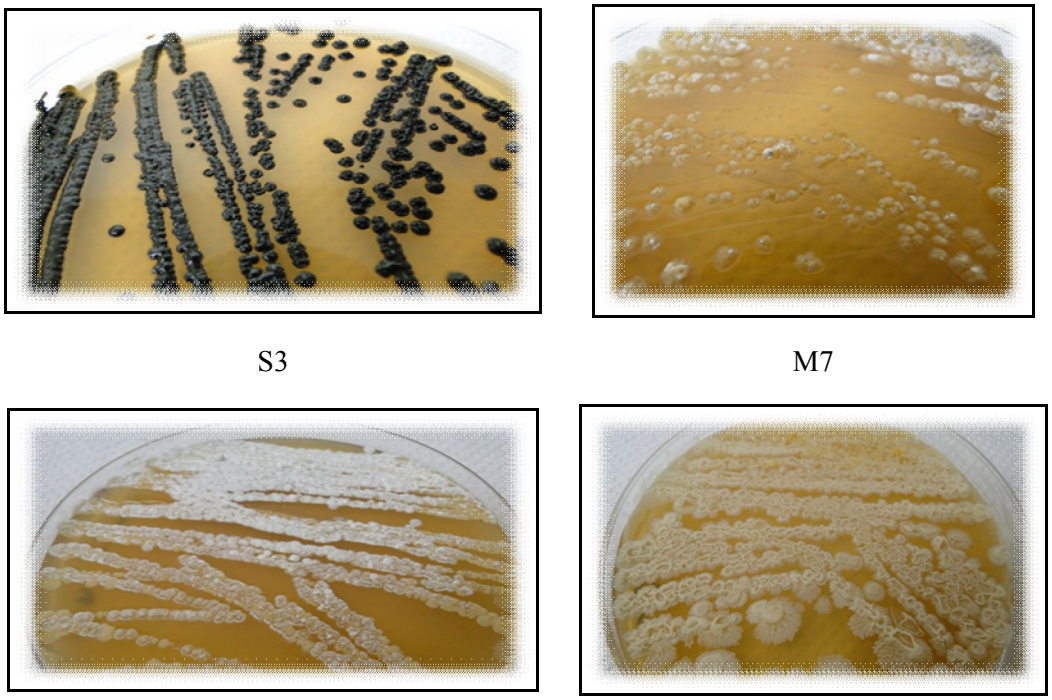
Where ase Standard factor = Conc. of standard(mg/ml)/Absorbance.

4. Optimization parameters for maximum CMCase production.

Many parameters were detection to reach the maximum production of CMCase, pH (5.0, 6.0, 7.0, 8.0, and 9.0),incubation period (24, 48, 72, 96, 12 h)optimal temperature(35, 40, 45, 50, 55, and 60°C) for 48 h, carbon sources (starch, glucose, galactose, and fructose) and nitrogen sources (casein, peptone, urea, and ammonium phosphate)(1).

Results and discussion

Most of isolated actinobacteria strains were characterized as *Streptomyces* (S1, S2, S3, S4, S5), 1 as *Nocardia* (N6), and 2 as *Micromonospora* (M7, M8). Strains were appeared with black, yellow and white color, they required about 3 weeks to reach the sporulation stage whereas they needed just 3 days to growth during screening stages.In addition, strains were tiny and appeared with granular, mucoide or curly texture, and the substrate mycelium was presence in most of the isolated strains, with melanoides pigments in some strains(figure 1, table 1, table 2).



S3 M7

S2 N6

Figure 1. Strains growth on solid medium.

Table 1. Morphological characteristics of Actinobacteria isolates.

Strain	Morphological properties			
	Color	Shape	mycilum	
			Aerial	Substrate
S1	Black	granular	Present	present
S2	crème turned to gray	curly	present	absent
S3	crème turned to white	granular	present	present
S4	orange turned to brown	mucoid	absent	present
S5	Yellow	granular	present	present
N6	Crème	mucoid	present	absent
M7	yellow turned to black	granular	present	present
M8	crème turned to black	curly	present	present

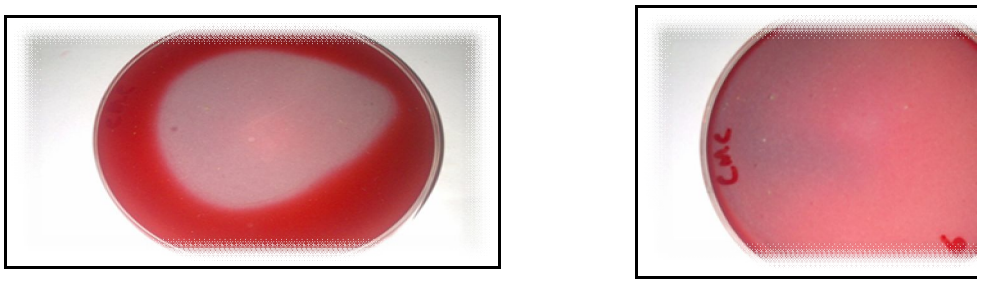
Table 2. Biochemical characteristics of Actinobacteria isolates.

Culture	Growth characteristics							
	S1	S2	S3	S4	S5	N6	M7	M8
Enzyme production								
Gelatinase	+	+	+	+	+	+	+	+
Urease	−	+	−	−	−	+	−	−
Nitrate reductase	+	+	+	+	+	+	+	+
Milk casein	−	−	−	+	−	−	−	+
Citrate	−	−	−	−	−	−	−	−
Esculine	+	+	+	+	+	+	+	+
Carbon sources								
Amidon	+	+	+	+	+	+	+	+
Amylose	−	−	−	−	−	−	−	−
Arabinose	+	+	−	+	+	−	−	+
Celebiose	−	+	+	+	−	+	+	+
Erythritol	−	−	−	−	−	−	−	−
Fructose	−	−	−	−	−	−	−	−
Galactose	−	+	−	+	−	+	−	+
Glucose	+	+	+	+	+	+	+	+
Indole	+	+	+	+	+	+	+	+
Inositol	+	+	−	−	+	−	+	−
Lactose	−	−	−	−	−	−	−	−
Maltose	+	−	−	+	+	+	−	−
Mannose	−	−	+	+	−	−	+	+
Mannitol	+	+	+	+	+	+	+	+
Melezitose	+	+	−	+	−	+	−	+
Melibiose	+	+	+	−	+	+	+	−
Palatinose	−	−	−	−	−	−	−	−
Raffinose	−	+	−	+	−	+	−	−
Rhamnose	−	−	−	+	−	−	−	+
Ribose	+	+	+	+	+	+	+	+
Saccharose	−	−	−	−	−	−	−	−
Sorbitol	−	−	−	−	−	−	−	−
Sorbose	−	−	−	−	−	−	−	−
Trehalose	−	−	−	−	−	−	−	−
Xylose	−	−	−	−	−	−	−	−

After measured the transparent halos diameter around cultivated colonies S5, N6, and M7 strains revealed high ability to produce cellulase in vitro with degradation efficiency above 90% (figure 2, table 3) **(**13**,** 28**)**, these strains were subjected later for CMCase production, and as a result both S5 and M7 strains showed high activity in CMCase production with maximum CMCase activity 0.43IU/mg and maximum value to degradation 97.5 % for M7 , 0.40IU/mg and 91.1% for S5. So S5 and M7 were selected for further studies to determine the optimal conditions for production (table 3).Initial pH of the media strongly influences many enzymatic systems and transport of several species of enzymes across the cell membrane, in addition each organism has its own pH range for growth and activity with an optimum value in this range(29, 30), so the optimal pH of CMCase production by selected strain was determined and the results revealed that maximum CMCase production was achieved in the range of pH 6.0-8.0,whilethe production of CMCase was increased as pH of the medium increases, it was maximum in pH=8 for M7 (0.46 IU/mg) and S5 (0.42 IU/mg), but after pH 8 there was a decrease in enzyme production (table 4, fig.3) (31). Furthermore, S7 was capable of producing CMCase in the range of 25 - 40°C with maximum production at 40°C for M7 (0.47 IU/mg) and S5 (0.43 IU/mg) (14). However, increase in temperature beyond 40°C led to decline in production of enzyme (table 4, fig. 5), proving that pH and temperature play a major role in CMCase production.

Table 3. Cellulose-degrading potential of the positive isolates.

Strain	Colony diameter	Halo diameter	hydrolysis capacity (HC)	CMCase activity (IU/mg)
S1	1	6.5	63,2%	0.25
S2	0.8	5.5	54%	0.21
S3	1.1	8.7	87.3%	0.28
S4	0.9	8.7	89.6%	0.35
S5	0.5	8.5	91.1%	0.40
N6	0.8	8.7	90.8%	0.38
M7	0.6	8.5	97.8%	0.43
M8	1.3	6.5	59.7%	0.20



S1 **S5**

Figure 2. Cellulose-degrading potential of stains

Table 4.Optimization of CMCase production conditions

Different parameters	Different values	M7	S5
Incubation period	24	0.26	0.18
	48	0.30	0.28
	72	0.43	0.40
	96	0.42	0.41
	120	0.35	0.32
pH	5	0.29	0.21
	6	0.33	0.28
	7	0.44	0.39
	8	0.46	0.42
	9	0.35	0.30
Temperature	25	0.25	0.19
	30	0.43	0.28
	35	0.44	0.40
	40	0.47	0.43
	45	0.35	0.31
Carbon sources	Starch	0.47	0.45
	Glucose	0.16	0.24
	Galactose	0.29	0.30
	Fructose	0.15	0.10
Nitrogen sources	Urea	0.21	0.15
	Peptone	0.31	0.38
	Casein	0.23	0.31
	Ammonium phosphate	0.34	0.36

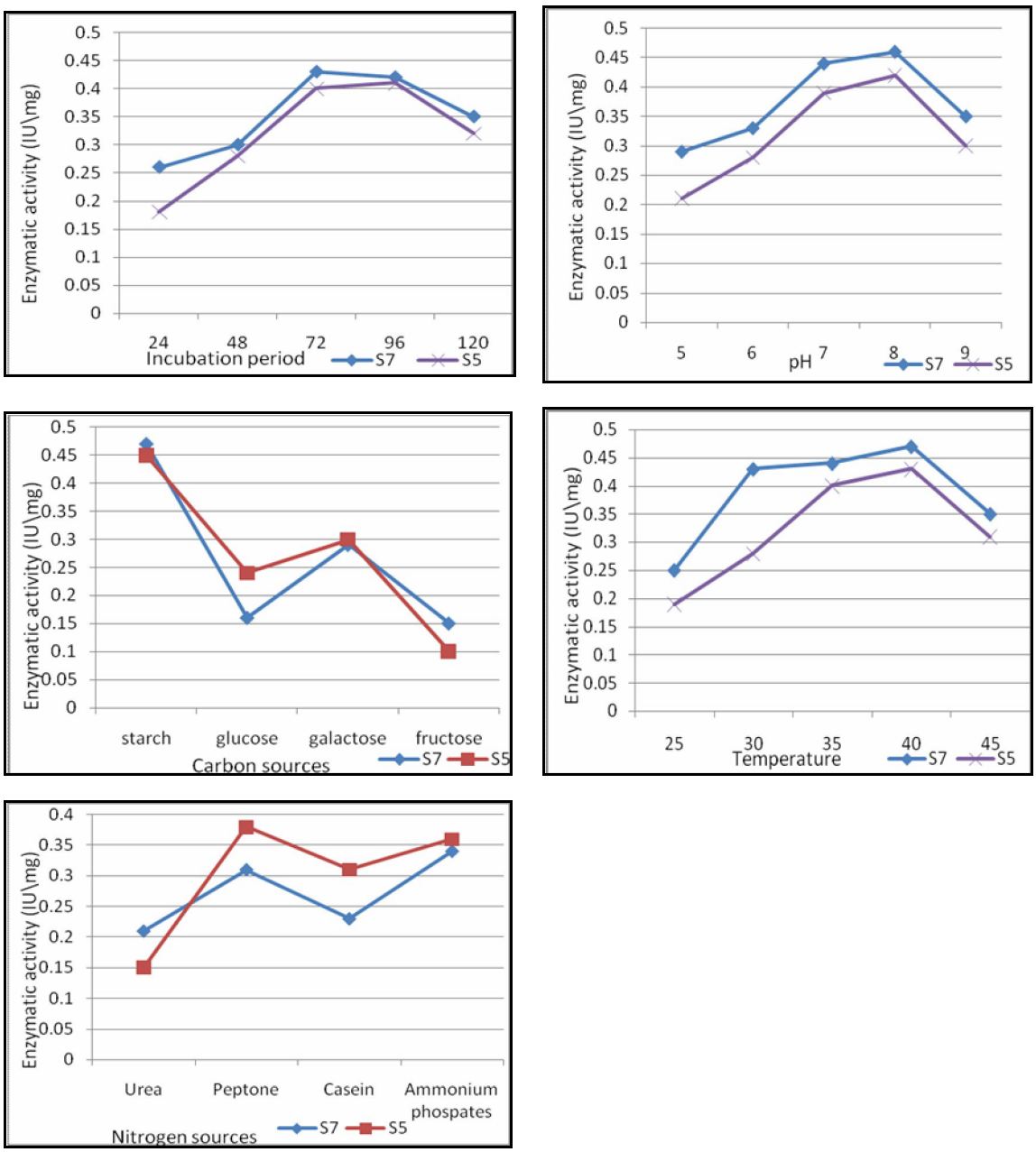


Figure 3. CMCase production in different parameters.

For incubation period results showed that maximal production of CMCase were detected between 72-96 hours of incubation, with maximal production after 72 hours of incubation for M7 (0.43 IU/mg), and after 96 hours for S5 (0.41 IU/mg), morever there was a decrease in production when the incubation period beyond 96 hours for two detected strains (table 4, fig. 4) (27, 32).

Finally, the ability of different carbon and nitrogen sources to stimulate maximum CMCase production was studied, and it has revealed that maximum activity for M7(0.47IU/mg) and S5(0.45 IU/mg) was detected in cultures contained starch as the growth carbon source, whereas cultures containing fructose as the growth carbon source presented the minimum CMCase activity for M7(0.15IU/mg) and S5 (0.10 IU/mg). About nitrogen sources, ammonium phosphate leads to maximum activity values for M7 (0.34 IU/mg), whereas peptone was the best nitrogen source for S5 (0.38 IU/mg), in spite of that urea leads to the minimum values of CMCase production for two strains (5, 27, 33), perhaps because thepeptone and ammonium phosphate were acomplex organic nitrogen source which might be stimulating growth by releasing$NH4^+$ and improving the expression of nitrogen assimilating enzymes (21).

The results of this investigation eventually demonstrated that actinobacteria strains is a potential source of CMCase production, with optimal production: pH=8, temperature=40C, incubation period= 72-96, carbon source= starch, and nitrogen source= peptone or ammonium phosphate, so the process of CMCase production from actinobacteria strains can be commercialized after further optimization for enhanced enzyme production. In addition, the extracellular CMCase can be further purified and used in different industrial applications.

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