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RESEARCH ARTICLE

Sugar beet is a suitable source for cellulases-producing bacteria and actinomycetes

Fahmy H. Fahmy[1], Abd El-Naser A. Zohri[2]*, Gamal E. Mohamed[3], Elsayed Hafez[4]

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Abstract

Sugar beet is considered the second source of sugar production in the world, but it becomes the first source in Egypt and several other countries all over the world. The present study aimed to convert the agro-industrial beet pulp that consists of cellulosic material into fermentable sugars as a friendly source of energy. The cellulases producing bacteria and actinomycetes are associated with beets' pulps and roots. The study also aimed to optimize the conditions of cellulases production, e.g., incubation time, temperature and pH. One hundred and two isolates of bacteria and actinomycetes were isolated from these samples and then screened to determine their potency to produce cellulases. Seven isolates were recorded as high producers (two from rhizospheres, one from endophytes, and four from the beet pulp). These seven isolates were classified according to morphological and biochemical tests as S11 (Streptomyces), S31 (Streptomyces), S45 (Bacillus), and S72 (Bacillus), S73 (Streptomyces), S85 (Streptococcus) and S88 (Bacillus). Optimization for the incubation period, temperature, and pH showed that activities of the highest three tested isolates S11, S45, and S88 were 0.73, 0.17, and 0.54 U/ml after two days of the incubation period. These levels increased to 1.33, 0.24 and 0.76 U/ml on the fourth incubation day at different temperatures and pH degrees. According to the results, it is recommended to use bacteria (Streptomyces), which is sample No. S11 isolated from the rhizosphere soil of beetroots was the high producer of cellulases at 50°C and pH 7.

Keywords: *Bacteria; Rhizosphere; Endophytes; Cellulase; Beet pulp.*

[1]Faculty of Sugar and Integrated Industries Technology, Assiut University, Egypt
[2]* Botany and Microbiology Department, Faculty of Science, Assiut University, Assiut, Egypt
[3] Microbiology Department, Faculty of Science, Zagazig University, Egypt.
[4] Plant Protection and Biomolecular diagnosis Department, Arid Lands Cultivation Research Institute (ALCRI), City of Scientific Research and Technology Applications, Alexandria, Egypt.
***Corresponding author:** Zohriassiut@yahoo.com

Introduction

Increasing environmental pollution and global warming encourage bioengineers to find sources of energy which are friendly to environment (Zhang et al. 2016). Nearly 60 countries are cultivating sugar beet in the world (Kumar and Pathak 2013). One ton of sugar beet yields 160 kg of sugar and 500 kg of wet pulp (about 88% moisture) (FAO 2009). More than one million tons of dry beet pulp (about 10% moisture) were produced in the US and nearly five million tons were produced in EU countries (Ziemínski and Kowalska-Wentel 2017). Egypt is one of the sugar beet producers in the world. Egyptian production of sugar beet increased from 500 tons year 1961 and 2.890360 tons in the year 2000 up to 13.043.612 tons harvested in 2020 (FAO 2022). Conversion of agro-industrial beet pulp consisting of cellulosic material into different products and energy is the best promising scheme. On the other hand, the high costs of forage production from beet pulp through mechanical squeezing, natural gas consumption during dehydration, mechanical pelleting and the high maintenance expenses during the beet pulp forage production.

The cellulolytic enzyme system is a complex system of enzymes composed of endoglucanase, exoglucanase, and β- glucosidase that acts synergistically to degrade cellulosic substrate (Zhang et al. 2006; Michelin et al. 2013). Cellulose with the complex structure having a hydrolytic enzyme system for the cleavage of bonds and its conversion into simple sugar like D-glucose units (Hussain et al. 2009). Due to the industrial importance of cellulases, its biosynthesis from microbes has been gaining interest (liming et al. 2004; Abdulhadi et al. 2021). Bacteria with a higher growth rate than fungi, has much potential to be employed in the production of cellulases (Thakkar et al. 2014). Cellulases are produced by several microorganisms commonly bacteria, actinomycetes, and fungi to hydrolyze cellulose to its monomer glucose. Based on their chemical composition, beetroots and beet pulps as well as soil attached to the beetroots might be

rich sources of actinomycetes were isolated from inside the sugar beet root tissues during the sugar beet growing campaign in the 2019 season from different 12 sites in two governorates (Sharkia and Dakahlia) in Egypt. Endophytic microbes enter tissues via germinating radicles (Gagne et al. 1987). The third source of isolation was beet pulp, which was collected during the sugar production campaign 2019 from Dakahlia Sugar Company; Egypt. The main reason for different sources of isolation is making diversity to have a wide range of bacteria and actinomycetes that might have economic feasibility in the production of cellulases for bioconversion of beet pulp to fermentable sugar in this investigation.

a lot of bacteria and actinomycetes that possess the capability to produce cellulases.

Beet pulp is the by-product of the beet sugar industry. It consists of cellulose (21-30%), hemicellulose (26-36%), pectin (20-22%), protein (10-15%), and lignin (1-2%) on a dry weight basis (Zheng et al. 2013; Kamzon et al. 2016). Cellulases are highly specific, and the enzymatic hydrolysis of cellulose usually was carried out under mild conditions of pressure, temperature, and pH. Unlike most the chemical and physicochemical pretreatment methods, biological pretreatment was preferred and offered advantages of low energy consumption and no chemical requirement, in addition to mild operational conditions and likely ease of integration into a consolidated bioprocessing setup. Bacteria are emerging as hotspots of versatility and variety genetically and functionally. They can degrade lignocellulosic biomass involving complex system of lignocellulolytic enzymes (López-Mondéjar et al. 2019). There has been increasing interest in obtaining new, stable and more specific enzymes using low-cost carbon sources, such as the beet pulp, and in searching for new and suitable microbial strains for large-scale cultivation for biotechnological processes (Parameswaran 2009). Therefore, the current study was designed to explore the bacteria and actinomycetes associated with beetroots, beet pulp, and the soil attached to beetroots to determine their efficiency in the production of cellulases.

Materials and Methods

Isolation of cellulolytic bacteria and actinomycetes from different sources.

Isolation of bacteria and actinomycetes from different sources understudy considered as possible sources for collection of target isolates. Sugar beets' pulp and roots as buried living organisms in soil considered as trap for catching the desired microbes to hydrolyze their contents. Helal (2005) used isolation trap technique for catching the desired microbes by burring grinded rice straw as a substrate in different cultivated soils this technique caught 47 species belonging to 24 genera of fungi. The rhizosphere bacteria and actinomycetes were isolated from sugar beet root-free soil. In comparison to the root-free soil, the rhizosphere forms a nutrient-rich niche for microorganisms because of the exudation of organic compounds by plants (Sorensen 1997). Additionally, this microenvironment is described as a microbial hot spot where various interactions between organisms, beneficial as well as pathogenic take place (Whipps 2001). In addition, the endophyte bacteria and

Rhizosphere and endophytic cellulolytic bacteria and actinomycetes

Rhizosphere and endophytic populations were enumerated as average colony-forming units (CFU/g soil) and (CFU/g tissue), respectively. The results are recorded in Tables (1,2). Seventy-one isolates were collected from the two examined governorates. in the SHARKIA governorate, the total number of isolates was 43 (20 rhizospheres and 23 endophytes). The mean count/plates of rhizosphere and endophytic microbes ranged from 6 to 30 and 6 to 24 CFU, respectively. While, the total microbial counts of rhizospheres and endophytes fluctuated between 6 x 104 to 30 x 104CFU/ g soil and 6 x 103 to 24 x 103CFU / g tissues, respectively Tables (1,2). On the other hand, 28 isolates 16 rhizospheres and 12 endophytes) were isolated from the DAKAHLIA governorate. The mean count/plates of rhizosphere and endophytic microbes in this governorate ranged from 5 to 28 and 3 to 30 CFU, respectively. While, the total microbial counts of rhizospheres and endophytes have fluctuated between 5 x 104 to 28 x 104CFU/ g soil and 3 x 103 to 30 x 103CFU/ g tissues, respectively Tables (1,2). Irfan et al. (2017) isolates thermophilic strain Bacillus subtilis from soil and used for cellulase production in submerged fermentation using potato peel as sole carbon source. Bai et al. (2017) isolated cellulolytic bacterium from soil and was identified as Cellulomonas sp. Microbial endophytes can be isolated from surface-disinfected plant tissue or extracted from internal plant tissue as cited in the extensive review by Kobayashi and Palumbo (2000).

Table 1 Cellulolytic both bacteria and actinomycetes isolated from rhizospheres (CFU/g soil) from 12 sites in Sharkia and Dakahlia governorates.

Governorate	Site of sample	Rhizosphere soil		
		Isolate No	Mean count /plates	CFU/g soil
Sharkia	Ebrahimia (Site 1)	1	18	18 x10^4
		2	6	6 x10^4
		3	15	15 x10^4
		4	12	12 x10^4
		5	8	8 x10^4
		6	8	8 x10^4
	Zagazig (Site 2)	7	15	15 x10^4
		8	30	30 x10^4
		9	17	17 x10^4
		10	15	15 x10^4
		11	12	12 x10^4
	Aboukbir (Site 3)	12	9	9 x10^4
		13	16	16 x10^4
		14	23	23 x10^4
	Kofornegm (Site 4)	15	25	25 x10^4
		16	9	9 x10^4
	Diarbnegm (Site 5)	17	23	23 x10^4
		18	30	30 x10^4
	Kafrsagr (Site 6)	19	27	27 x10^4
		20	26	26 x10^4
Dakahlia	Mansoura (Site 7)	21	28	28 x10^4
		22	20	20 x10^4
	Belkas (Site 8)	23	27	27 x10^4
		24	20	20 x10^4
	Sherbin (Site 9)	25	16	16 x10^4
		26	28	28 x10^4
	Satamony (Site 10)	27	19	19 x10^4
		28	15	15 x10^4
		29	6	6 x10^4
	Miitghamr (Site 11)	30	14	14 x10^4
		31	18	18 x10^4
	inbillawain (Site 12)	32	12	12 x10^4
		33	13	13 x10^4
		34	9	9 x 10^4
		35	5	5 x 10^4
		36	9	9 x 10^4

Table 2 Cellulolytic both bacteria and actinomycetes isolated from endophytes (CFU/g tissues) from 12 sites in Sharkia and Dakahlia governorates.

Governorate	Site of sample	Endophyte in beet tissues		
		Isolate No.	Mean count /plates	CFU/g tissues
Sharkia	Ebrahimia (Site 1)	37	10	10x10^3
		38	8	8 x10^3
		39	6	6 x10^3
		40	6	6 x10^3
		41	9	9 x10^3
		42	10	10 x10^3
	Zagazig (Site 2)	43	7	7 x10^3
		44	12	12 x10^3
		45	15	15 x10^3
		46	17	17 x10^3
		47	12	12 x10^3
	Aboukbir (Site 3)	48	14	14 x10^3
		49	18	18 x10^3
		50	9	9 x10^3
	Kofornegm (Site 4)	51	8	8 x10^3
		52	9	9 x10^3
		53	6	6 x10^3
	Diarbnegm (Site 5)	54	13	13 x10^3
		55	24	24 x10^3
		56	20	20 x10^3
	Kafrsagr (Site 6)	57	9	9 x10^3
		58	16	16 x10^3
		59	10	10 x10^3
Dakahlia	Mansoura (Site 7)	60	18	18 x10^3
	Belkas (Site 8)	61	30	30x10^3
	Sherbin (Site 9)	62	7	7 x10^3
		63	15	15 x10^3
		64	5	5 x10^3
		65	3	3 x10^3
		66	10	10 x10^3
		67	5	5 x10^3
	Satamony (Site 10)	68	16	16 x10^3
		69	25	25 x10^3
	Miitghamr (Site 11)	70	22	22 x10^3
	Sinbillawain (Site 12)	71	28	28 x10^3

Cellulolytic bacteria and actinomycetes from beet pulp samples.

Five samples of beet pulp were obtained during the sugar beet production campaign of 2019 from Dakahlia Sugar Company, Egypt. The samples were collected from the wet sugar beet pulp that comes out from the extraction stage after cutting beets into stripes (cossets) to make the extraction of juice easy. The moisture content of this wet pulp is about to 86 % and the dry matter is about 14%. This water content in the wet pulp makes it a good medium for the isolation of different types of microbes that may be hydrolyzed by the component of the pulp. The results are recorded in Table 3. Thirty-one isolates were collected from both bacteria and actinomycetes in this experiment. The mean count/plates of microbes ranged from one to 6 CFU, respectively. While their total counts fluctuated between one x 10^4 and 6 x 10^4 CFU/ g pulp Table 3. Schmidt and Walter (1978) isolated 400 pure cultures of microorganisms from 8 g bagasse during 6.5 days of storage. These organisms consist of bacteria (74%), yeasts (13%), actinomycetes (6%), and filamentous fungi (7%). Accordingly, Lara et al. (2014) isolated 198 and 160 yeast strains from decayed wood and bagasse, respectively.

Medium for isolation and screening of cellulases producing bacteria and actinomycetes.

This medium was composed of (g/l): carboxymethylcellulose (CMC), 10; tryptone, 2; KH_2PO_4, 4; Na_2HPO_4, 4; $MgSO_4.7H_2O$, 0.2; $CaCl_2$.$2H_2O$, 0.001; $FeSO_4.7H_2O$, 0.004; agar, 15, supplemented to 1-liter distilled water and pH adjusted to 7. This medium is considered a selective medium for cellulolytic bacteria and actinomycetes (Ray et al. 2007). The same medium without agar is used as broth medium for cellulases production.

Nutrient agar medium : This medium was used for morphological characterization of different isolates and it was composed of (g/l): Beef extract, 3; Peptone, 5; Glucose, 5; NaCl, 5; agar, 20 supplemented with1-liter distilled water. (Difco 1994).

Lauri-Bertani agar: This medium was used for the preservation of collected isolates, which were composed of (g/l): (tryptone,10; yeast extract,5; sodium chloride,10; agar,15. supplemented to 1-liter distilled water. The same medium without agar was used as broth medium for culture conditions optimization of cellulases production.(Bertani 1951).

Table 3 Cellulolytic both bacteria and actinomycetes (CFU/g pulp) isolated from beet pulp collected from Dakahlia sugar and refining factory, Egypt.

Samples	Isolate No.	Mean count /plates	CFU/g pulp
Sample 1	72	5	5 x 10^4
	73	5	5 x 10^4
	74	1	1 x 10^4
	75	3	3 x 10^4
	76	2	2 x 10^4
	77	1	1 x 10^4
Sample 2	78	3	3 x 10^4
	79	3	3 x 10^4
	80	3	3 x 10^4
	81	1	1 x 10^4
	82	1	1 x 10^4
	83	2	2 x 10^4
	84	6	6 x 10^4
	85	1	1 x 10^4
Sample 3	86	3	3 x 10^4
	87	2	2 x 10^4
	88	2	2 x 10^4
	89	3	3 x 10^4
Sample 4	90	2	2 x 10^4
	91	3	3 x 10^4
	92	5	5 x 10^4
	93	1	1 x 10^4
	94	3	3 x 10^4
Sample 5	95	2	2 x 10^4
	96	4	4 x 10^4
	97	1	1 x 10^4
	98	1	1 x 10^4
	99	4	4 x 10^4
	100	3	3 x 10^4
	101	4	4 x 10^4
	102	2	2 x 10^4

Isolation of cellulolytic bacteria and actinomycetes.

A selective medium specific for isolation of cellulolytic bacteria and actinomycetes was used as described by Ray et al. (2007). Bacteria and actinomycetes were isolated from rhizosphere soil and sugar beet pulp using the serial dilution method as described by Shaikh et al. (2013). Endophytic microbes were isolated by the method described by Shi et al. (2009). Spore suspension of each sample was prepared in sterilized distilled water and 0.1 ml of the diluted sample was spread on a CMC medium. Plates (3 for each sample) were incubated at 40°C for 2-4 days until the visible colonies formed. The purified colonies were preserved at

4ºC for further screening of cellulases production.

Screening of cellulases producing bacteria and actinomycetes.

Microorganisms capable of growing in the cellulose medium were isolated and checked for cellulolytic activity. Spore suspension of each isolate was prepared in sterilized distilled water and 0.1 ml of the suspension was injected inside an 8 mm diameter pipe centered and suspended on a CMC medium as described by Tagg and McGiven (1971). Plates (3 for each isolate) were incubated at 40°Cfor 2-4 days until clear zones of cellulose hydrolysis (enzymatic hydrolysis) can be visualized with 1% Congo red dye (15 min), followed by destining with 1 M NaCl solution for 15 min. Clear zones could be observed only around colonies of the active cellulolytic isolates (Wood and Bhat 1988).

Morphological and biochemical characterization of selected isolates.

Some morphological (shape & motility) and biochemical (Gram staining, starch hydrolysis, catalase & oxidase secretion) characteristics of the highly seven cellulases producing isolates were examined. Morphological characters were observed on the Nutrient agar medium (Difco 1994). After 24 h of incubation at 30°C cell morphology was determined using light microscope observations of Gram-stained smear preparations. Motility and shape were tested by the hanging drop method (Priest et al. 1988). Gram stain test was performed by the method of Potter and Beth (2008). Starch hydrolysis test achieved as the method described by Hemraj et al. (2013). Catalase and oxidase secretion were tested by methods described by Facklam and Elliott (1995) and Winn et al. (2006), respectively.

Optimization of culture conditions for enzyme production.

Sterilized **Lauri-Bertani** broth inoculated with 1.3 x 10^4 CFU/ml of pure colonies of each selected isolate (S11, S45, and S88), then incubated statically at 40°C for 2-4 days. In addition, the effect of different incubation temperatures at 30 - 60°C and pH at 5 – 7 on the enzyme activity was examined. At the end of the incubation period, the cultures were filtrated using Whatman No.1 filter paper. The culture filtrate was used directly for enzyme activity determination. Cellulase (CMCase) activity was determined by mixing 0.5 g of CMC in 50 mM acetate buffer pH 5 with 0.5 ml of suitably diluted enzyme and incubating at 50°C for 20 min (Ghose 1987). The reaction was terminated by the addition of 2 ml of 3,5dinitro salicylic acid (DNS) and the contents were boiled for 10 min. The color developed read 520 nm (UV spectrophotometer). The amount of reducing sugar liberated was quantified using glucose as standard. One unit of cellulases is defined as the amount of enzyme that liberates 1 μ mole of glucose equivalents per minute under the assay conditions. A blank was also prepared that contains 1 ml of distilled water instead of the enzyme.The proper statistical analyses of variance according to Gomez and Gomez, (1984). Mean comparison between treatments and their interactions was determined using Duncan's multiple range tests at a 0.05 probability significance level .

Results and Discussion.

Sugar beet factories face high maintenance cost in the beet pulp stations. Another high cost in the forage production from beet pulp during the industrial processes. So, our study aimed to convert the high amount of agro-industrial beet pulp into fermentable sugars as a friendly source of energy. Cellulases can be produced by many microbes, such as bacterial and fungal species of various ecological backgrounds (Santhi et al 2014). Cellulases are very important enzymes for the bioconversion of beet pulp into fermentable sugars suitable for ethanol production as clean biofuel as well as several other fermentation products. The cellulose content in sugar beet acts as trap for calling and catching the cellulolytic bacteria and actinomycetes from the surrounded rhizosphere soil during the sugar beet growth campaign.

Screening for cellulases production by collected bacteria and actinomycetes.

One hundred and two isolates of bacteria and actinomycetes were collected and screened for their abilities to produce cellulases in this study. Thirty– six isolates (No. 1 – 36 in Table 1 obtained from the rhizosphere soil (soil samples), 35 isolates (No. 37 – 71 in Table 2 obtained from the endophytic samples and 31

isolates (No. 72 – 102 in Table 3 obtained from the beet pulp samples. The results were recorded in Table 4 showed that the isolates under examination were classified into five categories. The first category (VH= very high cellulases producers) formed a clear zone of more than 3 cm and was represented by 7 isolates (two from rhizospheres, numbers 11 & 31; one from endophytes, number 45; and four isolates from the beet pulp samples, numbers 72, 73, 85 & 88). The second category (H= high cellulases producers) formed a clear zone ranging from 2 to 3 cm and represented by 3 isolates.The third category (M= moderate producers, formed a clear zone between 1 and less than 2 cm. This group is represented by 30 isolates. Nineteen isolates showed a low production level of cellulases (L= clear zone less than one cm) representing the fourth category. The rest 43 isolates could not produce cellulases and represented the fifth group.

Table 4 Screening of bacterial and actinomycete isolates obtained from rhizosphere soil, endophytes of sugar beetroots, and sugar beet pulp samples for their abilities to produce cellulases.

Isolate No.	Clear zone (cm)	activity	Isolate No.	Clear zone (cm)	activity	Isolate No.	Clear zone (cm)	activity
1	0.00	VE	35	0.00	VE	69	0.00	VE
2	0.90	L	36	0.80	L	70	0.85	L
3	0.00	VE	37	0.00	VE	71	0.00	VE
4	0.00	VE	38	0.00	VE	72	3.05	VH
5	1.35	M	39	1.20	M	73	3.15	VH
6	1.60	M	40	0.00	VE	74	0.00	VE
7	1.15	M	41	1.75	M	75	0.00	VE
8	2.10	H	42	0.00	VE	76	1.85	M
9	1.35	M	43	1.10	M	77	0.00	VE
10	1.25	M	44	0.00	VE	78	1.25	M
11	3.75	VH	45	3.20	VH	79	0.00	VE
12	0.00	VE	46	1.60	M	80	0.00	VE
13	0.00	VE	47	0.85	L	81	1.70	M
14	0.00	VE	48	2.05	H	82	0.85	L
15	0.75	L	49	0.00	VE	83	0.85	L
16	0.00	VE	50	0.00	VE	84	1.85	M
17	0.75	L	51	0.00	VE	85	3.05	VH
18	1.25	M	52	0.00	VE	86	0.85	L
19	0.00	VE	53	1.25	M	87	1.25	M
20	0.00	VE	54	0.00	VE	88	3.25	VH
21	1.10	M	55	0.00	VE	89	1.25	M
22	1.10	M	56	0.00	VE	90	1.45	M
23	1.25	M	57	0.00	VE	91	0.90	L
24	0.00	VE	58	0.00	VE	92	0.80	L
25	0.00	VE	59	0.85	L	93	0.00	VE
26	0.00	VE	60	1.35	M	94	1.25	M
27	0.00	VE	61	1.45	M	95	1.25	M
28	0.00	VE	62	0.00	VE	96	0.95	L
29	1.40	M	63	1.60	M	97	0.00	VE
30	0.00	VE	64	0.85	L	98	0.50	L
31	3.05	VH	65	0.00	VE	99	1.40	M
32	1.60	M	66	0.00	VE	100	0.80	L
33	0.90	L	67	0.85	L	101	0.85	L
34	0.00	VE	68	1.75	M	102	2.05	H

CZ: clear zone diameter measured by cm.Activity: very high= VH (CZ:more than 3 cm), high=H (CZ:2-3cm), moderate=M (CZ: 1-2cm), low=L (CZ: less than 1cm) and Negative= VE (CZ = zero cm).

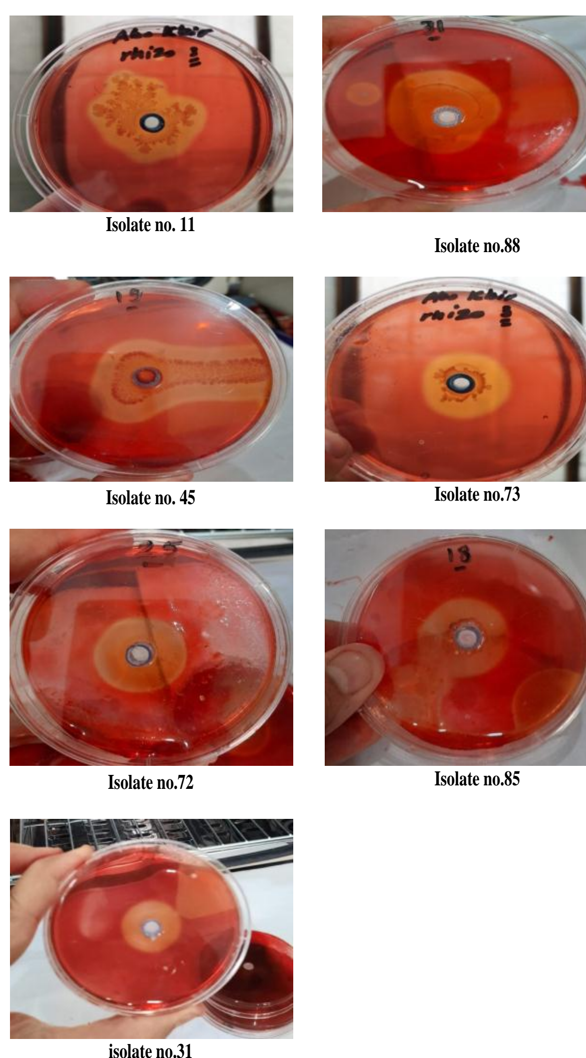

Figure 1 The highly seven-cellulases producer isolates during the study.

The highest seven isolates for cellulases enzyme production on CMC agar medium were obtained during the study. Figure 1 shows clear zones as evidence of cellulases enzyme production**.**

Characterization of the highly seven cellulases producing isolates.

The highly seven cellulases producing isolates (11, 31, 45, 72, 73, 85 & 88) were characterized through their morphological appearance, motility, Gram staining, in addition to catalase, starch hydrolysis, and oxidase tests. Data was obtained from the cultural, morphological, and biochemical studies compared with the standard description given in *Bergey's Manual* of Determinative Bacteriology and the cellulolytic actinomycetes and bacterial isolates identified in Table 5 . From morphological and biochemical tests**,** the 7 isolates were Gram-positive and show no motility except isolate No. 45, isolate No. 72 and isolate No. 88. All isolates are positive in catalase except isolate No. 45 and isolate No. 85. All isolates show positive results in starch hydrolysis activity except isolate No. 31and isolate No. 85. All isolates show negative oxidase test except isolate No. 31, isolate No. 45 and isolate No. 85. After staining isolate No. 45, isolate No. 72 and isolate No. 88 appears in rod shape and isolate No. 85 appears cocci in pairs and few ones' cocci in single and isolate No. 11, isolate No. 31 and isolate No. 73 appears in the filamentous shape.

Table 5 Some morphological and biochemical characteristics of the seven highly cellulases producer isolates.

Characterization Isolates	Shape	Motile	Gram stain	Catalase	Starch hydrolysis	Oxidase
S11, Streptomyces	Filamentous	Non	+	+	+	-
S31, Streptomyces	Filamentous	Non	+	+	-	+
S45, Bacillus	Rod	+	+	-	+	+
S72, Bacillus	Rod	+	+	+	+	-
S73, Streptomyces	Filamentous	Non	+	+	+	-
S85, Streptococcus	Cocci in pairs	Non	+	-	-	+
S88, Bacillus	Rod	+	+	+	+	-

Bacteria and actinomycetes have a high growth rate as compared to fungi because of their fast growth rates. Therefore, they have good potential to be used in cellulases production. Celluloytic property of some bacterial genera such as *Bacillus*, *Cellulomonas*, *Cellovibrio*, *Micrococcus, Pseudomonas,* and *Sporosphytophaga* spp., previously, recorded by several researchers (Immanuel et al. 2006). Cellulases yields appear to depend on a complex relationship involving a variety of factors like inoculum size, pH value, temperature and growth time, etc. (Immanuel et al. 2006).

Optimization of culture conditions for enzyme production.

Effect of incubation period on cellulases activity.

Isolates S11, S45, and S85, which were recorded in the screening experiment as the highest cellulases producers and isolated from rhizosphere soil, as endophytes from beetroot and beet pulp, respectively, were selected for quantitative determination for their cellulases activities. The results in Table 6 indicated that the activities of cellulases produced by the tested isolates S11, S45, and S88 were 0.73, 0.17, and 0.54 U/ml after two days of incubation period, respectively. These levels of cellulases activity were increased to 1.33, 0.24, and 0.76 U/ml on the fourth incubation day, respectively. Based on the obtained results, it was found that isolate S11 is the highest CMCase producer and the second one is isolate S88.Sethi et al. (2013) found that the cellulases activity of E. *coli* and *Bacillus* sp. were 0.6 and 0.5 U/ml, respectively when grown on agro-based waste sources. Somkidet al. (2015) found that the enzyme activities of both Bacteria PD6-1 and Actinomyces PD2A2 were 0.001 U/ml.

Table 6 Cellulases activity by the promising isolates after 2 and 4 days of incubation.

Isolates	Cellulases activity (U/ml)	
	2 days	4 days
S11	0.73 ± 0.1	1.33 ± 0.1
S45	0.17 ± 0.1	0.24 ± 0.1
S88	0.54 ± 0.2	0.76 ± 0.2

The experiments were done on the tested isolates to measure the effect of different temperature and pH degrees on the rate of cellulases activity produced after two and four days.

The effect of temperature and pH degrees on the rate of cellulases activity produced after two days.

Table 7 shows the significant differences between isolates and temperature degrees except for pH degrees where there were no significant differences in studying the isolates, where the isolate S11 exceeded the other isolates where it recorded 0.662, during the studying of temperature we found that the temperature 40°C exceeded the other temperatures than the temperature 50°C and the last one was 30°C, and there are no significant differences among the different pH degrees where the highest degrees were 5,6,7 pH.

Table 7 Cellulases activity produced was affected by the main factors after two days.

Characters Treatments	Cellulases activity (U/ml) 2 days
A- Isolates	
S11	0.662^{A}
S45	0.163^{C}
S88	0.455^{B}
F-test	**
B- Temperature	
30°C	0.352^{C}
40°C	0.462^{A}
50°C	0.461^{A}
60°C	0.431^{B}
F-test	*
C- PH	
5 pH	0.443^{A}
6 pH	0.421^{A}
7 pH	0.411^{A}
F-test	n.s

*, **, ns: significant at 5%, 1% level، and not significant, respectively. Sharing a letter in common within a column does not differ significantly ($p<0.05$).

Table 8 shows the bilateral interaction between isolates and temperature degrees where the differences were highly significant and the highest value of cellulases activity produced was at 50°C in isolate S11, and the lowest value was in isolate S45 at 30°C.

Table 8 Effect of temperature on cellulases activity produced by the three tested isolates after two days.

A- Isolates	Temperature			
	30°C	40°C	50°C	60°C
S11	0.527^{d}	0.650^{c}	0.760^{a}	0.710^{b}
S45	0.130^{i}	0.200^{h}	0.160^{h}	0.163^{h}
S88	0.400^{g}	0.537^{d}	0.463^{e}	0.420^{f}
F-test	**	**	**	**

Table 9 shows the binary interaction between the isolates and the pH degrees where there were highly significant differences and the highest value of cellulases activity produced was at pH7 in the first isolate S11, and the lowest one was in S45 at the pH degree pH7.

Table 9 Effect of pH on cellulases activity produced by the three tested isolates after two days.

A- Isolates	pH		
	5	6	7
S11	0.625^{c}	0.663^{b}	0.698^{a}
S45	0.200^{g}	0.158^{h}	0.133^{i}
S88	0.518^{d}	0.448^{e}	0.400^{f}
F-test	**	**	**

Table 10 shows the bilateral relationship between temperature and pH degrees where the relationship was highly significant and the highest ratio was at 40°C, and pH 5, and the temperature was 50°C, and pH 5 where the lowest value of cellulases activity produced was at the temperature 30°C, and pH7.

Table 10 Cellulases activity produced is affected by the interaction between temperature and pH after two days.

Temperature	pH		
	5	6	7
30°C	0.367^{f}	0.347^{g}	0.343^{g}
40°C	0.500^{a}	0.447^{c}	0.440^{de}
50°C	0.480^{b}	0.460^{c}	0.443^{c}
60°C	0.443^{c}	0.437^{dc}	0.413^{e}
F-test	**	**	**

Table 11 shows the triple interaction among the isolates' temperature and pH degrees and the difference was highly significant, the isolate S11 exceeded the other isolates and the highest value of cellulases activity produced was at 50°Cand pH degree pH 7 where it was recorded 0.800 according to El-Naggar and Abdelwahed (2012); Alam et al. (2011)., the lowest isolates of cellulases activity produced S45 which recorded 0.100 at 30°C and pH degree pH 7 where was found that the isolates S45 and S88 preferred the temperature 40°C and the pH 5 according to Tiwari et al. (2015). Irfan et al. (2017) obtained CMCase enzyme 3.5 U/ml from *Bacillus subtilis* K-18 at pH 7.0 and incubation temperature of 50 °C under submerged fermentation. Bai et al. (2017) found the optimum CMCase enzyme activity at pH 7.0 and incubation temperature 50 °C.

Table 11 Cellulases activity produced was affected by the interaction between different isolates, temperature, and pH after two days.

Isolates	Temperature	pH		
		5	6	7
S11	30°C	0.480^{e}	0.530^{de}	0.570^{d}
	40°C	0.610^{c}	0.640^{c}	0.700^{bc}
	50°C	0.730^{b}	0.750^{b}	0.800^{a}
	60°C	0.680^{bc}	0.730^{b}	0.720^{b}
S45	30°C	0.170^{h}	0.120^{i}	0.100^{i}
	40°C	0.260^{g}	0.180^{h}	0.160^{h}
	50°C	0.190^{h}	0.160^{h}	0.130^{i}
	60°C	0.180^{h}	0.170^{h}	0.140^{i}
S88	30°C	0.450^{c}	0.390^{f}	0.360^{fg}
	40°C	0.630^{c}	0.520^{de}	0.460^{e}
	50°C	0.520^{de}	0.470^{e}	0.400^{f}
	60°C	0.470^{e}	0.410^{f}	0.380^{f}
F-test =	**	**	**	**

The effect of temperature and pH degrees on the rate of cellulases activity produced after four days.

Table 12 shows the differences were significant between isolates and temperature degrees except for the pH degrees where there were no significant differences in studying the differences between the isolates, where isolate S11 exceeded the other isolates where it recorded 1.202 in the studying of temperature degrees, the 50°C exceeded, then 40°C, and in the last rank was 30°C, and there were no significant differences between the different pH degrees and the highest were 5, then 6, then pH 7.

Table 12 Cellulases activity produced was affected by the main factors after four days.

Characters Treatments	Cellulase activity (U/ml) 4 days
A- Isolates	
S11	1.202^{A}
S45	0.230^{C}
S88	0.640^{B}
F-test	**
B- Temperature	
30°C	0.564^{C}
40°C	0.742^{A}
50°C	0.756^{A}
60°C	0.701^{B}
F-test	*
C- pH	
5 pH	0.714^{A}
6 pH	0.689^{B}
7 pH	0.680^{B}
F-test	n. s

sharing a letter in common within a column does not differ significantly ($p<0.05$).

Table 13 shows the bilateral interaction between the isolates and temperature degrees and the differences were highly significant the highest value of cellulases activity produced was at 50°C in isolate S11 and the lowest value was in isolate S45 at 30°C.

Table 13 Effect of temperature on cellulases activity produced by the three tested isolates after four days.

A- Isolates	Temperature			
	30°C	40°C	50°C	60°C
S11	0.944^{c}	1.192^{c}	1.392^{a}	1.281^{b}
S45	0.185^{h}	0.280^{g}	0.224^{f}	0.230^{f}
S88	0.562^{f}	0.755^{d}	0.652^{e}	0.591^{f}
F-test	**	**	**	**

Table 14 shows the bilateral interaction between the isolates and the pH degrees. There was a highly significant and the highest value of cellulases activity produced at pH7 in the isolate S11 and the lowest at S45 at pH7.

Table 14 Effect of pH on cellulases activity produced by the three tested isolates after four days.

A- Isolates	pH		
	5	6	7
S11	1.135c	1.205b	1.267a
S45	0.281g	0.222h	0.187i
S88	0.727d	0.630e	0.563f
F-test	**	**	**

Table 15 shows the bilateral relation between temperature and pH degrees where the relationship was highly significant and the highest ratio was at 40°C and pH 5, then 50°C, and pH 5, and the lowest value of cellulases activity produced was at 30°C, and pH 6.

Table 15 Cellulases activity produced is affected by the interaction between temperature and pH after four days.

Temperature	pH		
	5	6	7
30°C	0.579^{c}	0.554^{c}	0.557^{c}
40°C	0.790^{a}	0.718^{c}	0.719^{c}
50°C	0.773a	0.756b	0.739b
60°C	0.716c	0.713c	0.673d
F-test	**	**	**

Table 16 shows the triple interaction between the isolates, temperature, and pH degrees, and the differences were highly significant, where the isolate S11 exceeded the other isolates, and the highest value of cellulases activity produced was at 50°C, and pH 7 and recorded 1.472 according to Alam et al. (2011) and the lowest one was S45 which recorded 0.142 at 30°C and pH 7 where the isolates S45 And S88 preferred the temperature 40°C and pH degree 5 according to Tiwari et al. (2015).

Table 16 Cellulases activity produced was affected by the interaction between different isolates, temperature, and pH after four days.

Isolates	Temperature	pH		
		5	6	7
S11	30°C	0.863[h]	0.945[gh]	.023[g]
	40°C	1.123[fg]	1.171[f]	1.283[d]
	50°C	1.321[c]	1.382[b]	1.472[a]
	60°C	1.234[e]	1.320[c]	1.290[d]
S45	30°C	0.241[m]	0.171[n]	0.142[o]
	40°C	0.362[l]	0.251[m]	0.227[n]
	50°C	0.268[m]	0.223[n]	0.182[n]
	60°C	0.253[m]	0.241[m]	0.196[n]
S88	30°C	0.632[j]	0.547[k]	0.507[kl]
	40°C	0.885[h]	0.732[i]	0.648[j]
	50°C	0.730[i]	0.663[j]	0.563[k]
	60°C	0.662[j]	0.577[k]	0.534[k]
F-test	**	**	**	**

Conclusion

The results which were achieved in this investigation demonstrated the suitable habitats for cellulolytic bacteria and actinomycetes, the optimum conditions (time, temperature, and pH) for cellulases enzymes production, and interaction among these conditions. In this study, we recommended with the use of bacteria *(Streptomyces)* sample No. S11 that was isolated from the rhizosphere soil of sugar beet. This isolate recorded the maximum value of cellulases production at 50°C and pH 7.

References

Abdulhadi Y, Ashish V (2021) Production, optimization and deinking capacity of alkaline cellulose produced from Mucor circinelloides WSSDBS2F1. CELLULOSE CHEMISTRY AND TECHNOLOGY, 55(5-6): 605-618.

Alam MZ, Sultana M, Anwar MN (2011) Isolation, identification and characterization of four cellulolytic actinomycetes and their cellulases. Chittagong University Journal of Biological Sciences, 6(1-2): 159-173.

Bai H, Irfan M, Wang Y, Wang H, Han X (2017) Purification and charachterization of cellulose degrading enzyme from newly isolated cellulomonas sp. Cellulose chemistry and technology. 51 (3-4), 283-290.

Bertani G (1951) Studies on lysogenesis I: the mode of phage liberation by lysogenic Escherichia coli. Journal of bacteriology. 62(3): 293-300.

Difco (1994) Manual of dehydrated culture media reagents for microbiology. DIFCO Laboratories. Detroit Michgan (Tenth Edition), 48232 U.S.A.

El-Naggar NA, Abdelwahed NAM (2012) Optimization of process parameters for the production of alkali-tolerant carboxymethyl cellulases by newly isolated Streptomyces sp. strain NEAE-D. Afr. J. Biotechnol. 11(5): 1185-1196.

Facklam R, Elliott JA (1995) Identification, classification, and clinical relevance of catalase-negative, Gram-positive cocci, excluding the streptococci and enterococci. Clinical Microbiology Reviews. 8(4):479-495.

FAO (2022) Food and Agriculture Organization of the United Nations, https://www.fao.org/faostat/en/#data/QCL.

FAO Sugar Beet/White Sugar (2009) FAOSTAT. Food and Agriculture Organization of the United Nations.

Gagne S, Richard C, Rousseau H, Antoun H (1987) Xylem-residing bacteria in alfalfa roots. Canadian Journal of Microbiology. 33:996–1000.

Ghose TK (1987) Measurement of cellulases activities. Pure Appl. Chem. 59:257–268.

Gomez KA, Gomez AA (1984) Statistical Procedure for Agricultural Research, 2nd ed. John Wiley and Sons Inc. Croda.

Helal GA (2005) Bioconversion of straw into improved fodder: mycoprotein production and cellulolytic acivity of rice straw decomposing fungi. Mycobiology, 33(2): 90-96.

Hemraj V, Diksha S, Avneet G (2013) A review on commonly used biochemical test for bacteria, Innovare Journal of Life Sciences, 1(1): 1-7.

Hussain S, Siddique T, Saleem M, Arshad M, Khalid A (2009) Impact of pesticides on soil microbial diversity, enzymes, and biochemical reactions. Advances in agronomy. 102:159-200.

Immanuel G, Dhanusha R, Prema P, Palavesam A (2006) Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. International Journal of Environmental Science & Technology. 3(1): 25-34.

Irfan M, Mushtaq Q, Tabssum F, Shakir HA, Qazi JI (2017) Carboxymethyl cellulase production optimization from newly isolated thermophilic Bacillus subtilis K-18 for saccharification using response surface methodology. AMB Express. 7(1):1-9.

Kamzon MA, Abderafi S, Bounahmidi T (2016) Promising bioethanol processes for developing a biorefinery in the Moroccan sugar industry. international journal of hydrogen energy. 41: 20880–20896.

Kobayashi DY, Palumbo JD (2000) Bacterial Endophytes and Their Effects on Plants and Uses in Agriculture. In Microbial Endophytes (pp: 213-250). CRC Press.

Kumar R, Pathak AD (2013) Recent trend of sugarbeet in world. Souvenir, IISR-Industry interface on Research and Development Initiatives for Sugar beet in India. May 28–29, 2013 Sugar beet Breeding Outpost, Mukteswar: 46–47.

Lara CA, Santos RO, Cadete RM, Ferreira C, Marques S,Girio F, Fonseca C (2014) Identification and characterisation of xylanolytic yeasts isolated from decaying wood and sugarcane bagasse in Brazil. Antonie Van Leeuwenhoek, 105(6): 1107-1119.

Liming X, Xueliang S (2004) High-yield cellulase production by Trichoderma reesei ZU-02 on corn cob residue. Bioresource technology. 91(3):259-262.

López-Mondéjar R, Algora C, Baldrian P (2019) Lignocellulolytic systems of soil bacteria: a vast and diverse toolbox for biotechnological conversion processes. Biotechnology advances. 37(6): 107374-107374.

Michelin M, Polizeli ML TM, Ruzene DS, Silva DP, Teixeira JA (2013) Application of lignocelulosic residues in the production of cellulases and hemicellulases from fungi. Fungal enzymes. CRC Press/Taylor & Francis, Boca Raton, pp: 31–64.

Parameswaran B (2009) Sugarcane bagasse. In: P.S.N.Nigam and P. A. Pandey (eds.). Biotechnology for agro-industrial residues utilization - Utilization of agro- residues. Springer, pp: 239-252.

Potter D, beth B (2008) "Biochemical Tests." Microbiology 202. Penn State Erie, The Behrend College. 30 Oct. 2008 – 11 Nov.

Priest F, Good M, Todd C (1988) A numerical classification of the genus Bacillus. Journal of general microbiology 134: 1847–1882.

Ray AK, Bairagi A, Ghosh KS, Sen SK (2007) Optimization of fermentation conditions for cellulases production by Bacillus subtilis CY5 and Bacillus circulans TP3 isolated from fish gut. Acta Ichthyologica ET Piscatoria, 37(1): 47-53.

Santhi VS, Gupta A, Saranya S, Jebakumar SRD (2014) A novel marine bacterium Isoptericola sp. JS-C42 with the ability to saccharifying the plant biomasses for the aid in cellulosic ethanol production. Biotechnology Reports. 1: 8-14.

Schmidt O, Walter K (1978) Succession and activity of microorganisms in stored bagasse. European journal of applied microbiology and biotechnology.5: 69 - 77.

Sethi S, Datta A, Gupta BL, Gupta S (2013) Optimization of Cellulase Production from Bacteria Isolated from Soil. International Scholarly Research Notices Biotechnology.3 :1–7

Shaikh NM, Patel AA, Mehta SA, Patel ND (2013) Isolation and screening of cellulolytic bacteria inhabiting different environment and optimization of cellulases production. Universal Journal of

Environmental Research & Technology.3(1): 39 – 49.

Shi Y, Lou K, Li C (2009) Isolation, quantity distribution and characterization of endophytic microorganisms within sugar beet. African Journal of Biotechnology. 8 (5): 835-840.

Somkid D, Duangpen D (2015) Isolation and screening of cellulose producing microorganisms and the study of some characteristics of enzymes. Biology Education and Research, 978 - 981, 287-524-2_16.

Sorensen J (1997) The rhizosphere as a habitat for soil microorganisms, In: Van Elsas, J.D., Trevors J. T and Wellington E. M. H. (eds.) Modern Soil Microbiology. Marcel Dekker, New York/ Basel/ Hongkong. Pp:21-45.

Tagg JR, McGiven AR (1971) Assay system for bacteriocins. Applied Microbiology. 21: 943-943.

Thakkar A, Saraf M (2014) Application of Statistically Based Experimental Designs to Optimize Cellulase Production and Identification of Gene. Natural Products and Bioprospecting, 4(6):341–351.

Tiwari S, Jadhav SK, Tiwari KL (2015)Bioethanol production from rice bran with optimization of parameters by Bacillus cereus strain McR-3. International journal of environmental science and technology. 12(12): 3819-3826.

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany. 52: 487-511.

Winn W, Allen S, Janda W, Koneman E, Procop G, Schreckenberger P, Woods G (2006) Color atlas and textbook of diagnostic microbiology, 6th ed. Lippincott Williams & Wilkins, Philadelphia, PA.

Wood TM, Bhat KM (1988) Methods for measuring cellulase activities. In Methods in enzymology Academic Press. Vol. 160pp: 87-112.

Zhang W, Sathitsuksanoh N, Barone JR, Renneckar S (2016) Enhanced enzymatic saccharification of pretreated biomass using glycerol thermal processing (GTP). Bioresource technology.199:148-154.

Zhang YHP, Himmel ME, Mielenz JR (2006) Outlook for cellulases improvement: screening and selection strategies. Biotechnology advances. 24(5): 452-481.

Zheng Y, Lee C, Yu C, Cheng YS, Zhang R, Jenkins BM, Vander-Gheynst JS (2013) Dilute acid pretreatment and fermentation of sugar beet pulp to ethanol. Applied Energy 105: 1-7.

Ziemiński K, Kowalska-Wentel M (2017) Effect of different sugar beet pulp pre-treatments on biogas production efficiency. Applied Biochemistry and Biotechnology. 181: 1211–1227.