INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print), 2222-5234 (Online)
http://www.innspub.net
Vol. 17, No. 3, p. 34-48, 2020

RESEARCH PAPER OPEN ACCESS

Direct Screening and Isolation of Carboxymethyl Cellulase producing Bacilli from Environmental Sources of Khyber Pakhtunkhwa

Sahar Nigar[1*], Bashir Ahmad[1], Sumera Afzal Khan[1], Ibadullah Jan[2], Amin Ullah[1]

[1]*Centre of Biotechnology and Microbiology (COBAM), University of Peshawar, Khyber Pakhtunkhwa, Pakistan*

[2]*College of Veterinary Sciences, The University of Agriculture, Peshawar, Khyber Pakhtunkhwa, Pakistan*

Key words: Carboxymethyl cellulase, *Bacillus* bacteria, Heat shock method, Congo red assay, Grams iodine assay.

http://dx.doi.org/10.12692/ijb/17.3.34-48 Article published on September 14, 2020

Abstract

The bacterial species, which belong to the genus *Bacillus,* can be economically exploited for the efficient industrial production of carboxymethyl cellulases (CMCases/endo-β-1,4-glucanases) by utilizing the cheap cellulose-based medium. The present study was undertaken to directly screen and isolate CMCase producing bacilli from cellulose associated environmental sources of Khyber Pakhtunkhwa. The screening and isolation of 150 samples were performed by heat shock, serial dilution, and spread plate techniques using Czapek-mineral-salt (CMS) agar medium, Congo red and Gram's iodine clearing zone assays. The isolates were purified on CMS agar medium and were identified. The CMCase positive efficient isolates were selected by using Congo red and Gram's iodine assays. The overall population of CMCase positive bacilli varied from $3.2\times10^5\pm1$ to $2.9\times10^6\pm1$ CFU/g in paper-pulp/paper waste (PPW) and organic-fertilizer/manure/animal waste (OMA) respectively. A total of 609 isolates were obtained with an average distribution of 20% in the central districts of the province. The maximum diversity of CMCase positive *Bacillus* isolates was observed in OMA samples. Out of the 609 isolates, 16 isolates displayed a significant CMCase activity, whereas a high, moderate and lower CMCase activity was exhibited by 100, 325, and 168 isolates, respectively. This study concludes that direct screening and isolation is a good primary screening technique for isolation of industrially important CMCase producing bacilli. Further, it may provide the basis for the utilization of inexpensive cellulose-based medium for the economical production of CMCases and other bio-products on an industrial scale to meet the local and national/international needs of such products.

* **Corresponding Author:** Mrs. Sahar Nigar ✉ cobam09@gmail.com

Introduction

The microbial cellulases are a major group of industrial enzymes. They are employed in animal feed, food, paper, textiles, detergent, bioconversion and fermentation industries (Nigam, 2013; Liu and and Kokare, 2017; Sahoo *et al.*, 2019). Carboxymethyl cellulase (CMCase) is a member of cellulase enzyme system and is an endoglucanase enzyme that acts on soluble cellulose such as carboxy methyl cellulose (CMC) and thereby cleaving the β- 1,4 glucosidic bonds randomly within the cellulose polymer (Nigam, 2013; Sadhu and Maiti, 2013).

Cellulose is a major component of lignocellulosic biomass which is an abundantly available and mainly unexploited resource. Cellulose has a great potential for its conversion into bio-based products and bioenergy. However, a huge amount of cellulose in the form of agro/ industrial waste is accumulating in the environment or utilized inefficiently, mainly because of expansive utilization processes (Maki *et al.*, 2009; Limayem and Ricke, 2012; Nigam, 2013; Sadhu and Maiti, 2013; Behera *et al.*, 2017; Sharma *et al.,* 2019). An effective cellulase enzyme system could utilize the full potential of cellulose for its economical bioconversion. Microorganisms produce diverse cellulase systems (Nigam, 2013; Sadhu and Maiti, 2013; Cragg, 2015; Gupta, 2015; Behera, 2017; Srivastava, 2019). The bacterial species, which belong to the genus *Bacillus*, primarily produce and secrete carboxymethyl cellulase to breakdown cellulose and consume it as carbon and energy source (Maki, 2009; Shweta, 2012; Walsh, 2015; Cragg *et al.*, 2015; Gupta and Verma, 2015; Srivastava *et al.*, 2019).

Bacillus species are Gram's positive endospore-forming bacteria. Such bacteria can be isolated from almost every habitat in the environment. Bacillus bacteria are heavily exploited in the bioconversion and fermentation industry due to its ability to produce a wide variety of metabolites, and ease in its handling. Therefore, an efficient cellulase producing *Bacillus* species can have the potential to be economically exploited by utilizing the cheap cellulose-based medium for the production of various metabolites (Lyngwi and Joshi, 2014).

The previous studies isolated and screened the cellulase producing bacilli in two rounds (Kim *et al.*, 2012; Amore *et al.*, 2013; Chantarasiri *et al.*, 2015). The current study directly screened and isolated CMCase positive *Bacillus* bacteria from environmental sources in a single round.

Materials and methods

Sample collection

A total of 150 cellulose associated environmental samples, including leaf/grass litter, manure/animal waste, compost, paper waste, organic and rhizosphere soil samples, were collected from central districts i.e. Peshawar, Nowshera, Charsadda, Mardan, and Swabi of Khyber Pakhtunkhwa. Approximately, 1 kg of each sample was aseptically collected and zipped locked in commercially available sterile polyethylene bags. Each site was characterized in terms of temperature, pH, texture (soil texture by field method (McDonald *et al.*, 1998) and moisture content (Hausenbuiller, 1978), and geographical location. All the samples were labeled with sample number, date, and location and were placed in a cool box. Samples were sent to the laboratory where they were kept at 4°C until further use.

Screening of CMCase producing Bacillus species

The CMCase producing *Bacillus* species were directly screened in a single round by heat shock (modified from: Vos, 2011; Manzum and Al Mamun, 2018), serial dilution, spread plate, and standard plate count techniques using Czapek-mineral salt (CMS) agar medium (Aneja, 2007), Congo red (Ponnambalam,*et al.*, 2011; Ahmad *et al.*, 2013a,b) and Gram's iodine assay (Kasana *et al.*, 2008; Gopinath *et al.*, 2014) methods.The inoculated CMS agar plates were incubated at 37 °C for 2- 3 days. After incubation, the agar plates were flooded with 0.1% (w/v) Congo red dye solution for 15- 20 min. The plates were then rinsed with distilled water and kept at 1 M NaCl solution for 15- 20 min. The NaCl solution was discarded, and the plates were analyzed for clear yellow halo zone around the bacterial colonies in the

red or pink background. The colonies surrounded by a clear yellow halo zone were selected as CMCase positive bacteria. In the case of Gram's iodine assay, the incubated plates were directly flooded with Gram's iodine for 3-5 minutes. Colonies with a clear yellow halo zone in the brown background were selected as CMCase producing bacteria. The morphology of each colony was observed and Gram's staining procedure was performed as a preliminary identification criterion for recognizing CMCase positive isolates as belonging to the genus *Bacillus*.

Heat shock and serial dilution

A 10% sample suspension, prepared in saline solution of 0.85% was incubated at 80°C in water bath for 10 min and then was cooled under tap water for 2-3 min. This suspension was then serially diluted further to obtain 10^{-2} to 10^{-7} dilutions.

Isolation and culturing

The morphologically different forms of screened bacterial colonies on CMS agar plates were selected to obtain pure isolates. The selected bacterial colonies were sub cultured and re- sub cultured on the CMS agar medium plates, until a pure culture was obtained. The plates were then incubated at 37 °C for 24 h. The whole process was carried out aseptically.

Storage of isolates

The pure isolates were stored and maintained for a month on CMS agar slant at 4 °C. The long term storage of these isolates was done in 15% glycerol containing CMS broth at -20 °C.

Identification of efficient isolates

Identification of pure, efficient bacterial isolates was performed according to "Bergey's Manual of Determinative Bacteriology" (Robert *et al.*, 1957). The procedure for morphological and biochemical identification of isolates was followed from the standard Laboratory manual (Aneja, 2007).

Semi-quantitative analysis for determination of efficient Isolates

The CMCase positive isolates were semi quantitatively analyzed. The efficient isolates were selected on CMS agar medium by using two independent methods, i.e., Congo red and Gram's iodine assays. Bacterial isolates were spot inoculated on CMS agar medium plates, followed by incubation at 37 °C for 24 h. After incubation, the Congo red assay was proceeded by flooding the plates with 0.1 % (w/v) Congo red dye for 20 min. The dye was then rinsed with distilled water and 1M NaCl was applied for 20 min. In Gram's iodine assay, plates were flooded with Gram's iodine for 3-5 min. The diameters of the clear yellow halo zone (formed as a result of either Congo red or Gram's iodine assay) and colony were measured and halo zone to colony ratio was determined.

The halo zone to colony ratio was designated as Hydrolysis Capacity (HC value). Two measurements were taken from each isolate and the isolates with the largest halo zone to colony ratio were recognized as efficient isolates.

Statistical analysis

All experiments were performed in duplicates. They were statistically evaluated by MS Excel and results were presented as mean ±SEM (standard error of the mean).

Results

Sample collection

An investigation into the properties of the samples that were collected indicated that most samples showed moderate conditions of temperature, pH, and moisture content. The nature, source and properties of the collected samples are enlisted in Table 1.

Screening, isolation, and purification

Our preliminary investigations showed that all the isolates belonged to the genus *Bacillus* and were positive for the CMCase detection assay (Table 2 and Fig.1). The purification of the collected samples led to the identification of 609 CMCase producing *Bacillus* isolates. Of these, 131, 128, 124, 122, and 104 isolates were obtained from the samples collected from Peshawar, Charsadda, Nowshera, Mardan and Swabi districts, respectively.

Table 1. Nature/source and properties of collected samples from central districts of Khyber Pakhtunkhwa.

S.No.	Sample Nature/source	Texture	Temperature °C	pH	Moisture %
1	Leaf /Grass litter/Straw/sugarcane Bagasse (LGSB)	Coarse to fine	27-30	8.38-8.6	41.8-60
2	Organic fertilizer/Manure/ Animal waste (OMA)	Coarse to fine	20-25	8.2-9	36.2-60
3	Compost	Fine to slurry	27-60	5.3-8.4	32-70
4	Paper pulp/Paper wastes (PPW)	Slurry		7.97-8.38	30-55
5	Organic/Greenish/Black soil in water stream/ dam/river side soil (OGBDRS)	Soft solid to Slurry	21-23	7.45-7.8	41.8-60
6	Crop/Harvested crop Rhizosphere Soil (CHRS)	Clay loam	20-23	7-8	18-34%

Table 2. Preliminary identification of *Bacillus* bacteria.

Characteristics		Observations
Growth		Aerobic
Colony Morphology	Colony size and shape	Large size with round to irregular shape
	Margin	Entire or undulate (wavy)
	Elevation	Flat, convex or umbonate
	Color/Light transmission	White to off white, matte and creamy opaque
	Texture/Appearance	Smooth to wrinkled, pasty, dull dry rough, wet, shiny or blistery
Cell Morphology	Gram's staining	Gram's positive
	Shape	Round or square-ended rods
	Endospore	Present

The overall population of CMCase positive *Bacillus species* varied from $3.2 \times 10^6 \pm 1$ to $2.9 \times 10^6 \pm 1$ CFU/g in paper pulp/paper waste (PPW) and organic fertilizer/manure/animal waste (OMA) respectively (Tables 3 & 4). The average distribution of CMCase producing *Bacillus* species in the central districts of Khyber Pakhtunkhwa was determined to be 20 % (Fig.2). The diversity of CMCase positive *Bacillus* species, in terms of the number of isolates found in different samples, is represented in Fig. 3.

Table 1. Colony Forming Unit of CMCase producing Bacilli per gram in samples of Peshawar, Charsadda and Nowshera.

S.N.	Peshawar		Charsadda		Nowshera	
	Source	CFU/g (10^5)	Source	CFU/g (10^5)	Source	CFU/g (10^5)
1	Leaf litter	11±1	Animal waste	15±1	Black soil	16 ±1
2	Organic greenish B.S	10±1	Wheat straw	4.0 ±1	River side soil	11±1
3	Wheat R.S	5.2±1	Black soil	17 ±1	Leaf litter	8.7±1
4	Organic F/manure	22±1	Wheat R.S	9.0 ±1	Animal waste	19±1
5	Paper pulp	3.2±1	Animal manure	11 ±1	Compost	22±1
6	Wheat R.S	6.0 ±1	Leaf litter	3.5 ±1	Organic Black soil	24 ±1
7	Animal manure	20±1	Sugarcane R.S	11±1	Maize R.S	21 ±1
8	Leaf litter	10.1±0.5	Sugarcane bagasse	8.1 ±1	Maize R.S	23±1
9	Organic manure	25±1	Sugarcane compost	12 ±1	Animal waste	14±1
10	Wheat R.S	10±1	Harvested crop soil	3.7±0.5	Vegetable R.S	17±1
11	Wheat R.S	15.2±0.5	Animal waste	20± 1	Citrus R.S	19±1
12	Wheat R.S	11±1	Organic soil	10 ±1	Sugarcane bagasse	11±0.5
13	Compost	18±1	Wheat straw	15±1	Sugarcane compost	24±1
14	Grass/leaf litter	9.0±1	Tomato R.S	21±1	Tobacco R.S	20±1
15	Animal waste	17.4±0.5	Peach R.S	19±0.5	Tobacco R.S	21±0.5
16	Organic B.S	9.0 ±1	Organic soil	22±1	Paper and pulp	10±0.5
17	Leaf litter	11±1	River side soil	3.8 ±1	Paper waste	7.4 ±1
18	Straw	8.5 ±1	River side soil	3.3 ±1	Wheat R.S	23±1
19	Animal waste	20 ±1	Paper waste	3.9±1	Wheat R.S	24±1
20	Vegetable R.S	7.55 ±0.5	Tomato R.S	20 ±1	Wheat straw	11 ±1
21	Fruit R.S	8.0 ±1	Wheat straw	5.8± 0.5	Animal waste	26±0.5
22	Wheat R.S	12 ±1	Harvested crop soil	4.8 ±1	Harvested crop soil	12 ±1
23	Animal waste	22 ±1	Animal waste	25 ±1	Vegetable R.S	15 ±0.5
24	Sugarcane bagasse	16±1	Harvested crop soil	4.0 ±1	Vegetable R.S	8.0±1
25	Sugarcane compost U.L	28±1	Organic manure	19±1	Organic B.S	21 ±1
26	Sugarcane compost M.L	20±1	Leaf litter	7.1 ±1	Harvested crop soil	8.4 ±1
27	Sugarcane compost L.L	22±1	Animal waste	20 ±5	Organic waste soil	27±1
28	Vegetable R.S	4.6±0.5	Maize R.S	22 ±1	Mustard R.S	19±1
29	Animal waste	18±1	Maize R.S	20 ±1	Mustard R.S	21±1
30	Wheat R.S	5.5±1	Maize R.S	21 ±1	Mustard R.S	21±1

Note. CFU/g = Colony Forming unit per gram, B.S= Black soil, R.S = Rhizosphere soil, F= Fertilizer, U.L= upper layer, M.L= middle layer, and L.L= Lower Layer.

The maximum diversity of CMCase positive *Bacillus* isolates was observed in OMA samples. CMCase positive *Bacillus* bacteria screened and isolated on CMS agar medium are displayed in Fig. 4 & Fig. 5.

Identification of CMCase producing Bacillus species

The identification revealed that the initially screened bacterial isolates belonged to the *Bacillus* species. Biochemical tests demonstrating CMCase activity among *Bacillus* isolates are detailed in Table 5.

Semi-quantitative Analysis for determination of Efficient Isolates

The results of the Congo red and Gram's iodine assays indicated that a total 609 isolates exhibited CMCase activity. The degree of activity of the isolates ranged between 1 and 20 in terms of hydrolysis capacity (HC) (Table 6 and Fig. 6). Out of total isolates only 16 were found to express significant CMCase activity in the range of 7- 20 HC (Table 7). Of these 16 isolates, the isolate 35A exhibited the highest HC value of 20.

Table 2. Colony Forming Unit of CMCase producing Bacilli per gram in samples of Mardan and Swabi.

S.No.	Mardan		Swabi	
	Source	CFU/g (10^5)	Source	CFU/g (10^5)
1	Wheat R.S	16±1	Wheat R.S	17±1
2	Wheat R.S	14±0.5	Wheat R.S	13±1
3	Wheat R.S	15 ±1	Wheat straw	10±1
4	Compost	28 ±1	Barley R.S	21±1
5	Organic soil	26 ±1	Barley R.S	22 ±1
6	Animal waste	22±1	Gram R.S	18±1
7	Sugarcane R.S	21 ±1	Gram R.S	16 ±1
8	Sugarcane R.S	20 ±0.5	Gram leaves	10 ±1
9	Sugarcane bagasse	19 ±0.5	Tobacco R.S	20 ±1
10	Tobacco R.S	23 ±1	Tobacco R.S	21 ±1
11	Tobacco R.S	22±1	Tobacco leaves	16 ±1
12	Tobacco leaf	17±0.5	Sugarcane R.S	27±0.5
13	Compost	27 ±1	Sugarcane R.S	25 ±1
14	Barley R.S	28±1	Sugarcane bagasse	20±1
15	Barley R.S	23 ±1	Maize R.S	25±1
16	Barley R.S	23 ±1	Maize R.S	22 ±1
17	Barley straw	4.1 ±1	Maize R.S	17 ±1
18	Maize R.S	17 ±1	Maize straw/bagasse	14 ±1
19	Maize R.S	15 ±1	Jowar R.S	16 ±1
20	Maize straw/bagasse	12±0.5	Jowar R.S	20 ±1
21	Organic waste	27 ±1	Jowar bagasse	10±0.5
22	Greenish black soil of stream	18 ±1	Harvested crop soil	5.0 ±1
23	Organic manure	22 ±1	Harvested crop soil	8.0 ±1
24	Wheat R.S	9.0 ±1	Compost	28 ±1
25	Wheat R.S	11±1	Organic soil	26 ±1
26	Wheat R.S	1.2 ±1	Leaf litter	9.7 ±1
27	Animal waste	25 ±1	Animal organic waste	29±1
28	Harvested crop soil	5.0 ±1	Animal waste	27±1
29	Harvested crop soil	3.8 ±0.5	Guava R.S	11±1
30	Harvested crop soil	4.7 ±0.5	Damside soil	20 ±1

Note. CFU/g = Colony Forming unit per gram, R.S = Rhizosphere soil.

The CMCase activity of a few *Bacillus* isolates in the Congo red and Gram's iodine assay is shown in Figs., 7 and 8. The HC values of CMCase/cellulase positive *Bacillus* isolates from different sources/geographical origins are compared in Table 8. The isolates of the present study have shown high HC value in comparison of the previous studies.

Discussion

The current study uses a direct method for screening

and isolation of CMCase producing *Bacillus* species. In our experience, this direct method has proved to be extremely efficacious for the purpose. The selection of nature and source of a sample is important for successful screening and isolation of any type of microbe.

Table 3. Biochemical tests for identification of prominent CMCase activity showing *Bacillus* species isolates.

S.N.	Isolate code	G.S	E.S	Motility	Catalase	Starch hydrolysis	Aerobic	Remarks
1.	5D	G +	+	+	+	+	+	*Bacillus* Spp.
2.	12K	G +	+	+	+	+	+	*Bacillus* Spp.
3.	16b	G +	+	+	+	+	+	*Bacillus* Spp.
4.	19D	G +	+	+	+	+	+	*Bacillus* Spp.
5.	24A	G +	+	+	+	+	+	*Bacillus* Spp.
6.	26B	G +	+	+	+	+	+	*Bacillus* Spp.
7.	35A	G +	+	+	+	+	+	*Bacillus* Spp.
8.	43A	G +	+	+	+	+	+	*Bacillus* Spp.
9.	58A	G +	+	+	+	+	+	*Bacillus* Spp.
10.	60B	G +	+	+	+	+	+	*Bacillus* Spp.
11	70B	G +	+	+	+	+	+	*Bacillus* Spp.
12	81A	G +	+	+	+	+	+	*Bacillus* Spp.
13	89A	G +	+	+	+	+	+	*Bacillus* Spp.
14	107C	G +	+	+	+	+	+	*Bacillus* Spp.
15	120B	G +	+	+	+	+	+	*Bacillus* Spp.
16	136A	G +	+	+	+	+	+	*Bacillus* Spp.

Note. G.S= Gram's staining, E.S= Endospore staining.

Table 6. The degree of CMCase activity of *Bacillus* species isolates.

Degree of CMCase Activity	No. of Isolates
CMCase activity of total isolates (*HC value 1 - 20)	609
Low CMCase activity (HC value 1 - 1.9)	168
Moderate CMCase activity (HC value 2 - 3.9)	325
High CMCase activity (HC value 4 - 6.9)	100
Significant CMCase activity (HC value 7 - 20)	16

Note. HC = Hydrolysis Capacity.

The samples included in the present investigation are mostly derived from habitats that are indogenous to bacilli. Saini *et al.,* 2012 concluded in their study that natural habitats, such as compost, vermicompost, farmyard manure and rhizospheres are among the best sources for procuring cellulose degrading bacteria. The present study is in line with previous studies (Ahmad *et al.*, 2013a, b; Amore *et al.*, 2013; Chantarasiri *et al.*, 2015; Gaur and Tiwari, 2015; Saini *et al.*, 2017) that have reported similar sampling for the purpose of isolating cellulolytic bacteria, including those belonging to the *Bacillus* species, from cellulose- associated environmental sources.

The method for screening and isolation of any microbe is also very important especially to speed up the process and to obtain specific result. Here in the current study the heat shock method along with testing of initial isolates directly for CMCase activity yielded both fast and specific results. In the present investigation the high population dynamics of CMCase positive *Bacillus* bacteria and the diversity in

terms of the number of CMCase positive *Bacillus* isolates in different samples clearly indicates the efficiency of the screening protocol used. However previously published studies (Kim *et al.*, 2012; Amore *et al.*, 2013; Chantarasiri *et al.*, 2015) isolated and screened bacterial samples indirectly within two rounds. A study published by Chantarasiri *et al.* (2015) reported the identification of 87 cellulolytic bacteria among 145 microbial isolates from 42 soil samples of Thai coastal wetland.

Table 4. Efficient CMCase producing *Bacillus* species isolates (HC value ≥ 7).

S.No.	CMCase +ve isolate code	Holozone diameter (cm)		Colony diameter (cm)		HC value = Zd/Cd	
		Congo red assay	Gram's iodine assay	Congo red assay	Gram's iodine assay	Congo red assay	Gram's iodine assay
1	5D	1.4	1.4	0.2	0.2	7	7
2	12K	1.4	1.4	0.1	0.1	14	14
3	16B	2.1	2.1	0.2	0.2	10.5	10.5
4	19D	1.4	1.4	0.1	0.1	14	14
5	24A	1.9	1.9	0.1	0.1	19	19
6	28B	1.5	1.5	0.1	0.1	15	15
7	35A	2	2	0.1	0.1	20	20
8	43A	1.5	1.5	0.2	0.2	7.5	7.5
9	58A	1.6	1.6	0.1	0.1	16	16
10	60B	2	2	0.2	0.2	10	10
11	70B	1.6	1.6	0.2	0.2	8	8
12	81A	2	2	0.2	0.2	10	10
13	89A	1.4	1.4	0.2	0.2	7	7
14	107C	2	2	0.2	0.2	10	10
15	120B	1.4	1.4	0.1	0.1	14	14
16	136A	1.7	1.7	0.1	0.1	17	17

Note. HC = Hydrolysis Capacity; Zd = Holozone diameter; Cd = Colony diameter. Each value is the mean (± 0.00 S.E) of two replicates.

Table 8. Comparison of HC values of CMCase/cellulase positive *Bacillus* isolates from different sources/geographical origins.

S.No.	*Bacillus* isolates	Source/Geographical origin	HC value for CMCase/cellulase	References
1	*Bacillus licheniformis*	wastes dumpsites in Lagos, southwest Nigeria	8.5 (cellulase)	(Ojo-Omoniyi *et. al.*, 2016)
2	*Bacillus sphericus*	wastes dumpsites in Lagos, southwest Nigeria	8 (cellulase)	(Ojo-Omoniyi *et. al.*, 2016)
3	*Bacillus subtilis*	wastes dumpsites in Lagos, southwest Nigeria	6.5 (cellulase)	(Ojo-Omoniyi *et. al.*, 2016)
4	*Bacillus* sp. MSL2	Rice paddy fields, Ayutthaya province, Thailand.	2.50 ± 0.11 (Average)	(Sriariyanun *et. al.*, 2016)
5	*Bacillus cereus* CDB F5	Woody forest soil, Pachmarhi, M.P.	4.44	(Behera *et. al.*, 2014)
6	*Bacillus brevis* CDB1	mangrove soil of Mahanadi river delta, Odisha, India	2	(Rathore, 2014)
7	*Bacillus* spp. CDB12	mangrove soil of Mahanadi river delta, Odisha, India	2.1	(Rathore, 2014)
8	*Bacillus* spp. 35A	Agricultural cellulosic waste, Charsadda, KP, Pakistan	20	(Present study)
9	*Bacillus* spp. 12K	Organic fertilizer/manure, Peshawar, KP, Pakistan	14	(Present study)
10	*Bacillus* spp. 16B	Organic fertilizer/manure, Peshawar, KP, Pakistan	10.5	(Present study)

The screening method included inoculation and incubation of microbes on CMC agar plates followed by Gram's iodine assay. The authors reported that among all such isolates identified in their study, *B. cereus* strain BR0302 demonstrated the highest CMCase activity. Amore *et al.* reported that industrial waste based compost is a source for novel cellulolytic strains and enzymes. A total of 90 bacterial isolates,

obtained from raw composting materials were screened for their cellulolytic activity using CMC based medium and the Congo red assay. The results revealed the presence of 31 cellulolytic microbes, including several belonging to the *Bacillus* species, with high levels of Azo-CMCase activity (Amore *et al.*, 2013).

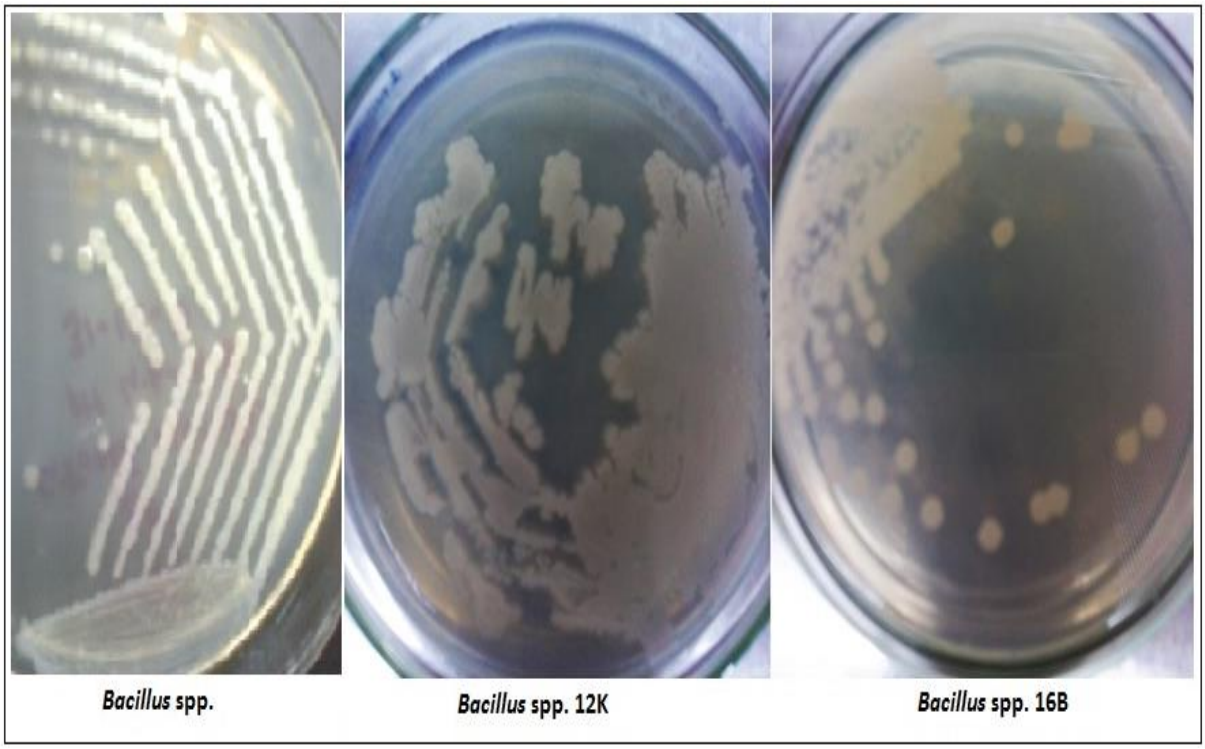

Fig. 1. Colony morphology of few CMCase positive *Bacillus* species isolates.

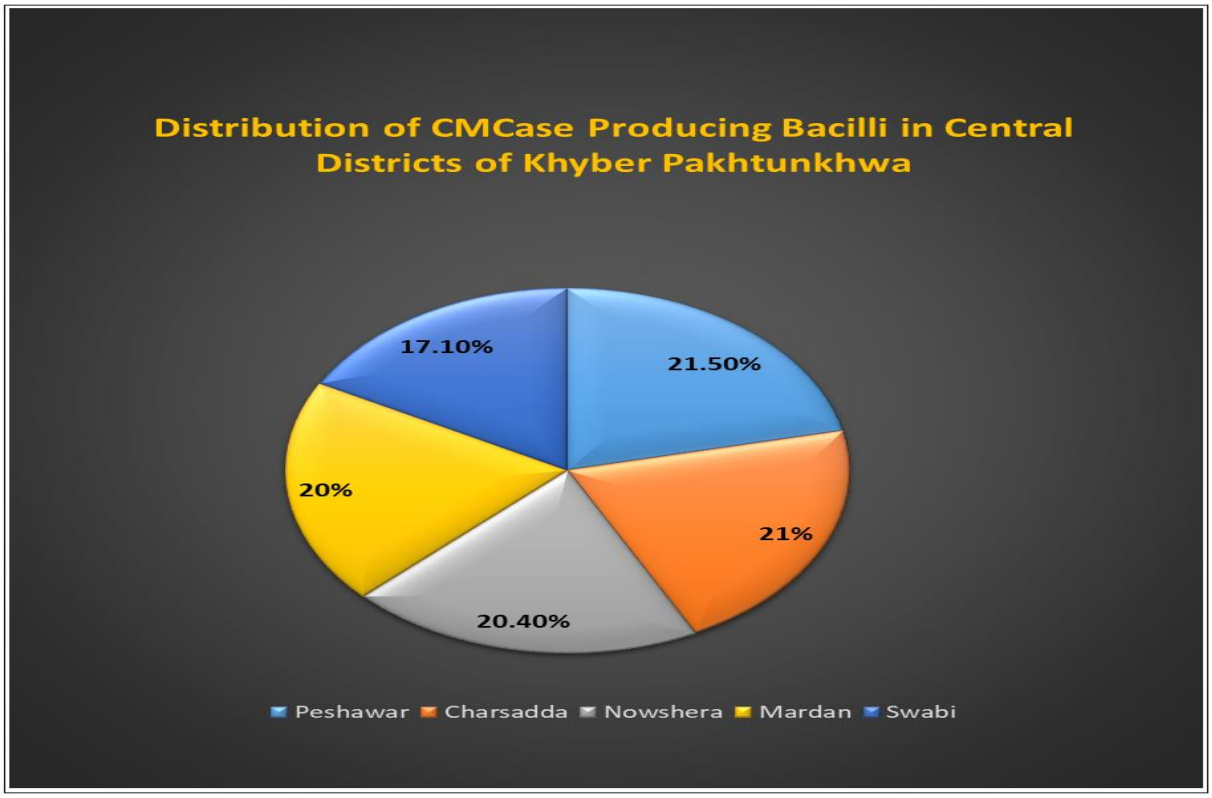

Fig. 2. Distribution of CMCase producing Bacilli in central districts of Khyber Pakhtunkhwa.

A study conducted by Kim *et al.*, (2012) analyzed 176 samples isolated from agricultural sources such as soil, compost, and animal waste slurry in Jeju Island, South Korea. The authors screened 309 CMCase positive clones on CMC agar medium containing trypan blue. Amongst the isolates, three strains that demonstrated the highest CMCase activity were identified as belonging to the *Bacillus* species.

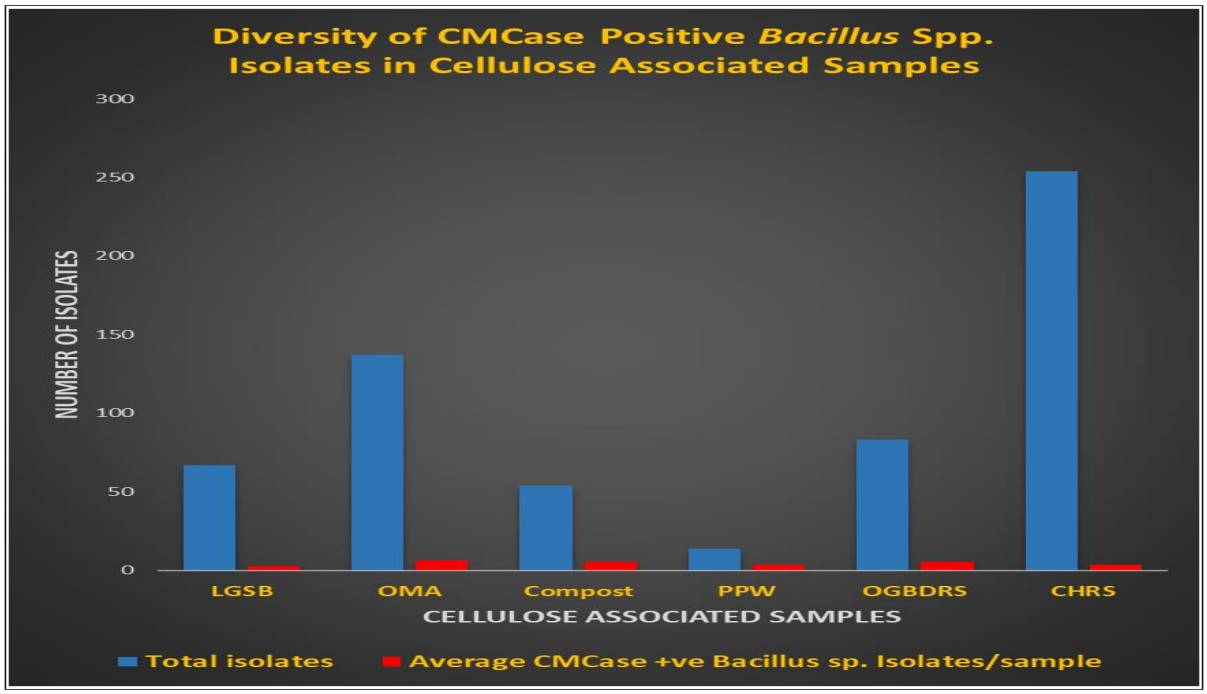

Fig. 3. Diversity of CMCase positive *Bacillus* spp. isolates in cellulose associated samples of Khyber Pakhtunkhwa.

Note. LGSB= Leaf/Grass litter/Straw/Bagasse, OMA= Organic fertilizer/Manure/Animal waste, PPW= Paper pulp/paper waste, OGBDRS= Organic Greenish/Black/Dam/River side Soil, CHRS= Crop/Harvested crop Rhizosphere soil.

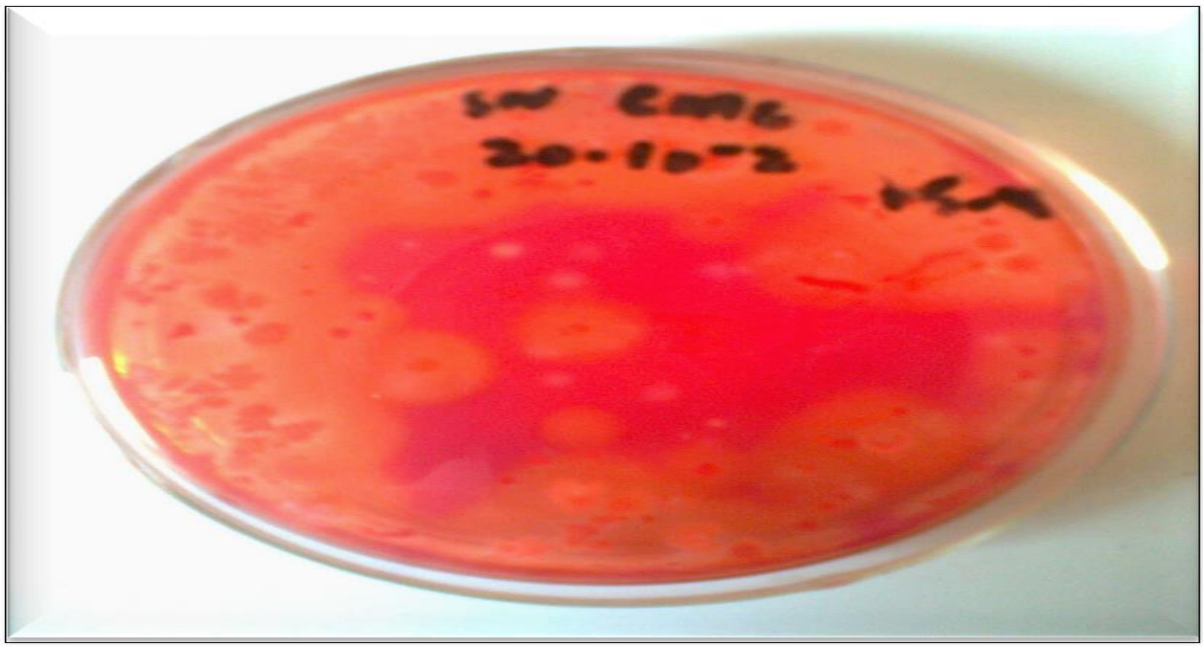

Fig. 4. CMCase positive *Bacillus* bacteria screened and isolated on CMS agar medium. Clear yellow holozone around colonies in red background is visible after Congo red assay.

Although Saini and Lakshmi (2012) reported a protocol, similar to the present study, involving simultaneous isolation of cellulolytic bacteria and selection of efficient producer. Their results were comparable to those obtained by us. Robson and Chambsliss (1884) previously used heat shocked samples from soil to isolate the Group I *Bacillus* strain DLG that has cellulolytic activity, their methodology differed from the current investigation as it involved an initial inoculation of the heat shocked samples onto nutrient agar plates followed by analyzing them for cellulase production.

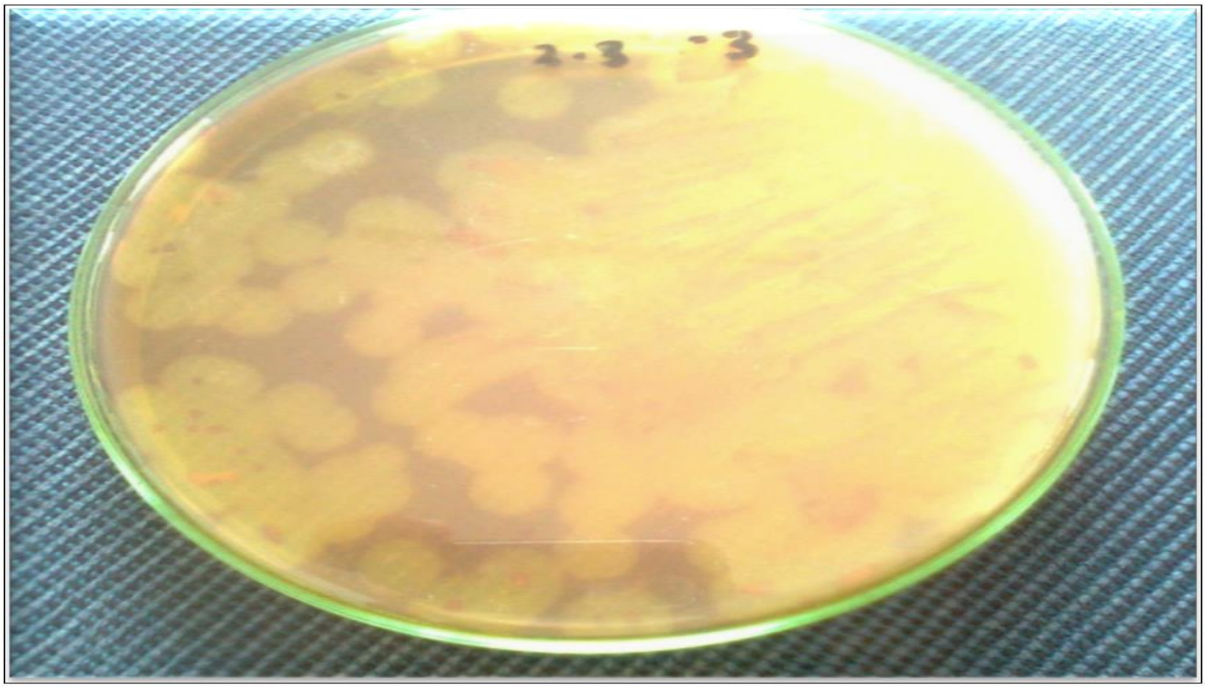

Fig. 5. CMCase positive *Bacillus* bacteria screened and isolated on CMS agar medium. Clear yellow holozone around colonies in brown background is visible after Gram's iodine assay.

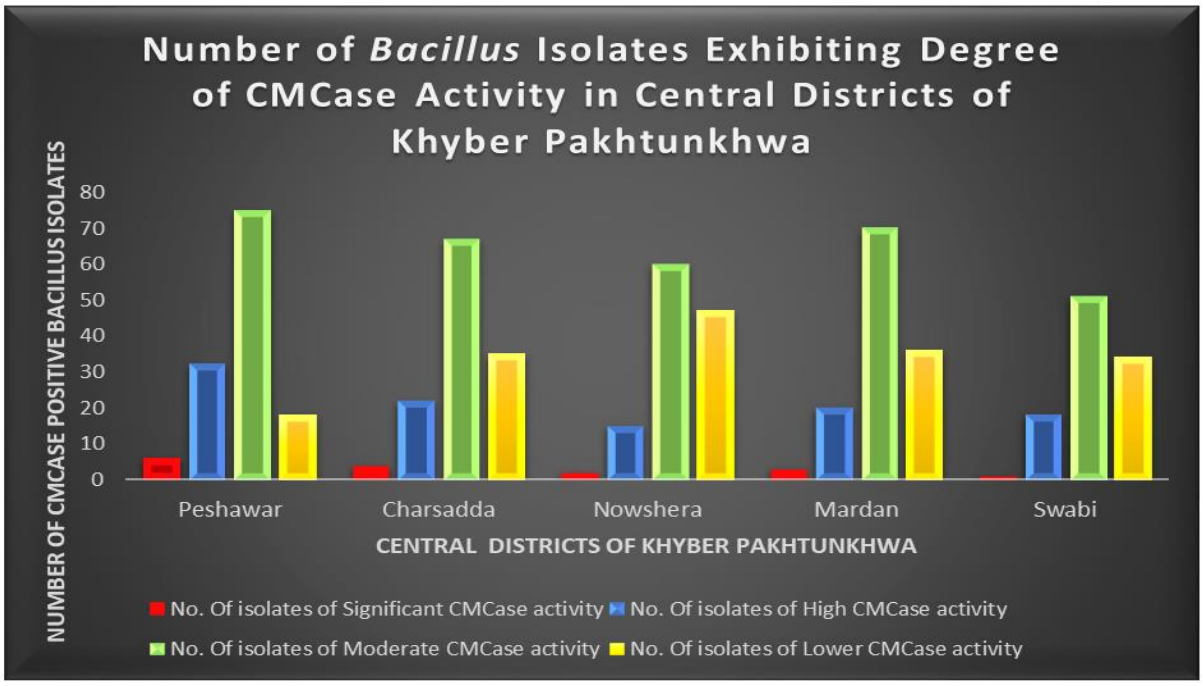

Fig. 6. Number of isolates of *Bacillus* species exhibiting degree of CMCase activity in central districts of Khyber Pakhtunkhwa.

Results of our study indicate that there is no significant difference between Congo red and Gram's iodine assay in terms of resolution. Both assays were equally capable of resolving the clear zones formed around CMCase positive colonies. However, we observed that both assays have distinctive advantages as well as disadvantages.

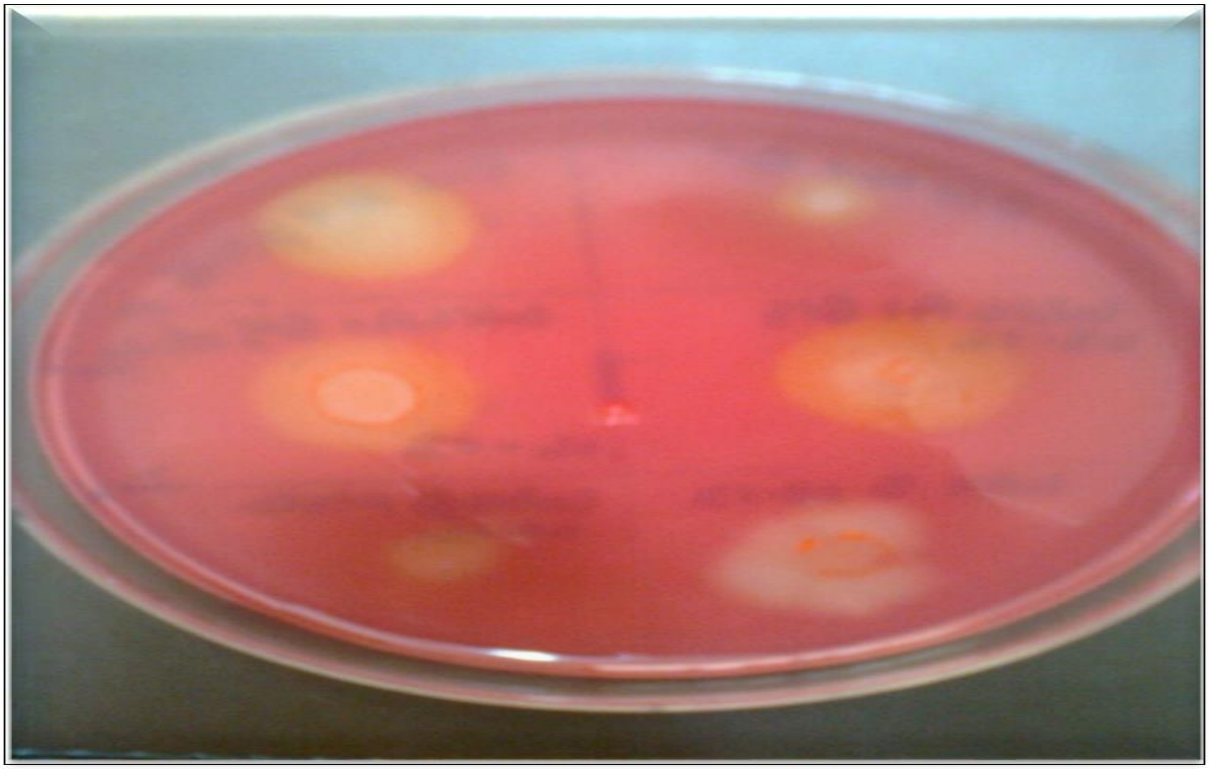

Fig. 7. CMCase activity of few *Bacillus* species isolates in Congo red assay (yellow zone surrounding each colony represents CMCase activity).

The Congo red assay was found to be slow as it has several steps and there is a risk that the colonies may wash out. But the clear zone formed by this method seen is to be very stable. In contrast, the Gram's iodine assay was rapid, single step process. There was no risk of colony wash out but the clear zone became fainted and disappeared after some time. A comparative study aimed at analyzing various staining techniques for determining extracellular cellulase activity on CMC agar plates reported that best results are obtained by using Gram's iodine assay with the Congo red assay coming in second place (Gohel *et al.*, 2014). Kakkar *et al.* (2016) also reported Gram's iodine as best stain to measure cellulolytic activity.

Several researchers have reported the isolation of cellulase producing *Bacillus* strains with high CMCase activities in the last decade (Reddy *et al.*, 2016; Patagundi, 2014; Singh *et al.*, 2013). Such strains were identified based on morphological, biochemical and Molecular characteristics. The present study used morphological and biochemical characteristics for identification purpose. All the isolates belong to *Bacillus* species. The results shows that the *Bacillus* species is widely distributed in Khyber Pakhtunkhwa.

In the present study HC value for CMCase activity of *Bacillus* species was determined independently through the Congo red as well as the Gram's iodine assay. The diameter of the clear zone formed for both these assays was observed to be identical. This is a sharp contrast to a previously published study that did a comparative analysis of Gram's iodine and Congo red as indicators of hydrolysis. The results indicated that the Gram's iodine test may lead to the identification of false positives in a typical screening procedure. This can be avoided by using the Congo red test instead, which also allows for the detection of

cellulase activity from live microbial colonies unlike Gram's iodine (Meddeb-Mouelhi *et al.*, 2014). The conclusion of such study is in contradiction to the findings of another comparative study, which concluded that the Congo red staining is less efficient as it deactivates the microbes (Gohel *et al.*, 2014).

Similar to our investigation, several previously published studies (Behera *et al.*, 2014; Rathore, 2014; Ojo-Omoniyi *et al.*, 2016; Sriariyanun *et al.*, 2016) have also used HC values to quantify the CMCase cellulase activity of *Bacillus* species/microbial isolates.

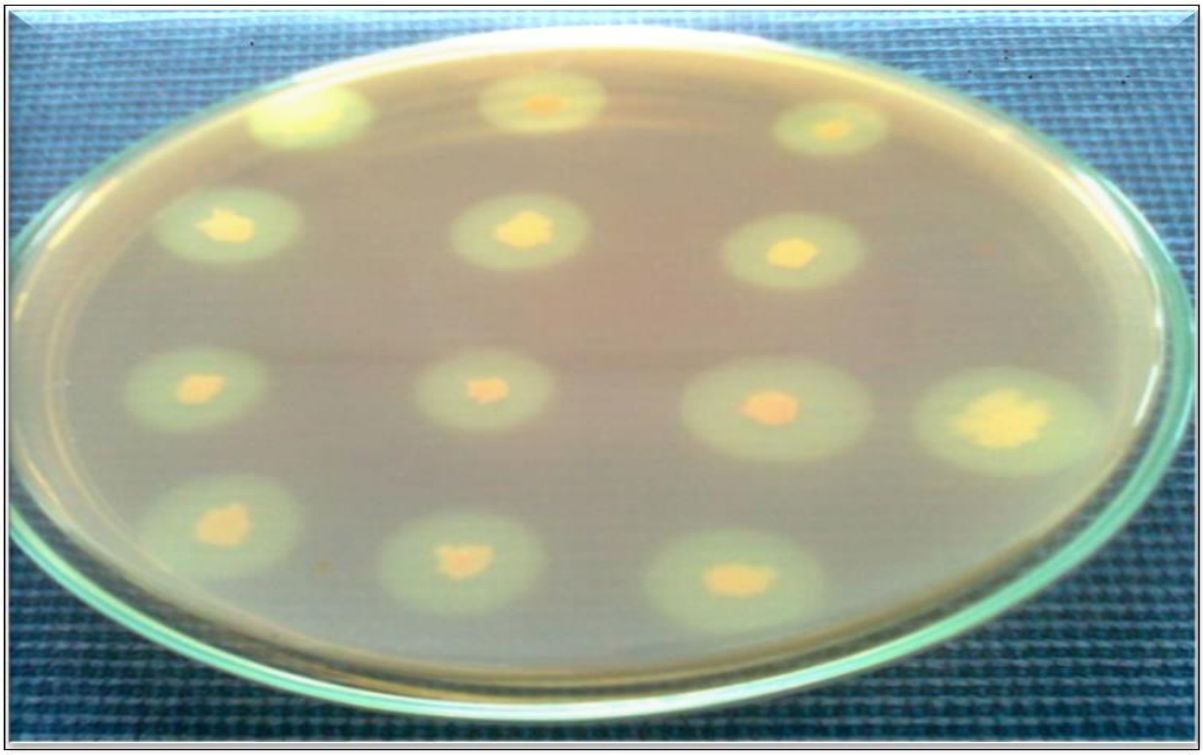

Fig. 8. CMCase activity of few *Bacillus* species isolates in Gram's iodine assay (yellow zone surrounding each colony represents CMCase activity).

In such studies, it was observed that the HC value for CMCase/cellulase activity of the isolates of *Bacillus* spp. did not exceed 8.5.

This was in contrast to our observations wherein HC values ranged from 1 to 20. Ojo-Omoniyi *et al.* (2016) also reported that *Bacillus* species, along *with Aspergillus* and *Trichoderma* spp., have exceptionally high cellulase activity as compared to other isolates.

The isolates of the present study have shown high HC value in comparison of the previous studies.

Conclusion

The direct approach used in the present study has proved to be very efficacious for the screening and isolation of CMCase producing bacilli. The results revealed that CMCase positive *Bacillus* species with varying degree of CMCase activity is widely distributed in Khyber Pakhtunkhwa. The efficient isolates of the present study have been further studied in part to achieve their benefits at industrial scale.

Acknowledgement

This study was funded by the Directorate of Science and Technology (DOST), Khyber Pakhtunkhwa and was conducted at Microbiology Research Laboratory, Centre of Biotechnology and Microbiology (COBAM), University of Peshawar.

Ethical approval

Ethical approval were obtained from the Ethics Committee of the Center of Biotechnology and Microbiology, University of Peshawar.

Conflict of interest

There were no potential conflicts of interest.

References

Ahmad B, Nigar S, Malik NA, Bashir S, Ali J, Yousaf S, Bangash JA, Jan I. 2013. Isolation and characterization of cellulolytic nitrogen fixing *Azotobacter* species from wheat rhizosphere of Khyber Pakhtunkhwa. World Applied Sciences Journal **27(1),** 51-60. http://dx.doi.org/10.5829/idosi.wasj.2013.27.01.81120

Ahmad B, Nigar S, Shah SS, Bashir S, Ali J, Yousaf S, Bangash JA. 2013. Isolation and identification of cellulose degrading bacteria from municipal waste and their screening for potential antimicrobial activity. World Applied Sciences Journal **27(11),** 1420-1426. http://dx.doi.org/10.5829/idosi.wasj.2013.27.11.81162

Amore A, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V. 2013. Industrial waste based compost as a source of novel cellulolytic strains and enzymes. FEMS Microbiology letters **339(2),** 93-101. http://dx.doi.org/10.1111/1574-6968.12057

Aneja KR. 2007. Experiments in microbiology, plant pathology and biotechnology. New Age International.

Behera BC, Parida S, Dutta SK, Thatoi HN. 2014. Isolation and identification of cellulose degrading bacteria from mangrove soil of Mahanadi River Delta and their cellulase production ability. American Journal of Microbiological Research **2(1),** 41-46. http://dx.doi.org/10.12691/ajmr-2-1-6

Behera BC, Sethi BK, Mishra RR, Dutta SK, Thatoi HN. 2017. Microbial cellulases–Diversity & biotechnology with reference to mangrove environment: A review. Genetic Engineering and Biotechnology **15(1),** 197-210. http://dx.doi.org/10.1016/j.jgeb.2016.12.001

Chantarasiri A, Boontanom P, Yensaysuk N, Ajwichai P. 2015. Isolation and identification of a cellulase-producing *Bacillus* sp. strain BR0302 from Thai coastal wetland soil. King Mongkut's University of Technology North Bangkok International Journal of Applied Science and Technology **8(3),** 197-203. http://dx.doi.org/10.14416/j.ijast.2015.07.002

Cragg SM, Beckham GT, Bruce NC, Bugg TD, Distel DL, Dupree P, Etxabe AG, Goodell BS, Jellison J, McGeehan JE, McQueen-Mason SJ. 2015. Lignocellulose degradation mechanisms across the Tree of Life. Current Opinion in Chemical Biology **29,** 108-119. http://dx.doi.org/10.1016/j.cbpa.2015.10.018

Gaur R, Tiwari S. 2015. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from *Bacillus vallismortis* RG-07. BMC biotechnology **15(1),** 19. http://dx.doi.org/10.1186/s12896-015-0129-9

Gohel HR, Contractor CN, Ghosh SK, Braganza VJ. 2014. A comparative study of various staining techniques for determination of extra cellular cellulase activity on Carboxy Methyl Cellulose (CMC) agar plates. International Journal of Current Microbiology and Applied Sciences **3,** 261-266.

Gopinath SM, Shareef I, Ranjit S. 2014. Isolation, screening and purification of cellulase from cellulase producing *Klebsiella variicola* RBEB3 (KF036184.1). International Journal of Science and Research **3(6),** 2319-7064.

Gupta A, Verma JP. 2015. Sustainable bio-ethanol production from agro-residues: a review. Renewable and sustainable energy reviews **41,** 550-567. http://dx.doi.org/10.1016/j.rser.2014.08.032

Hausenbuiller RL. 1978. Soil science; principles and practices. WMC brown company publishers, IOWA, Washington.

Kakkar N, Gupta SK, Saharan B. 2016. Comparison of different stains in determination of extracellular cellulose activity of *Odontotermes obesus* gut bacteria. International Journal of Entomology Research **1(5),** 54-56.

Kasana RC, Salwan R, Dhar H, Dutt S, Gulati A. 2008. A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Current Microbiology **57(5),** 503-507. http://dx.doi.org/10.1007/s00284-008-9276-8

Kim YK, Lee SC, Cho YY, Oh HJ, Ko YH. 2012. Isolation of cellulolytic *Bacillus subtilis* strains from agricultural environments. ISRN Microbiology,Article ID 650563, 9 pages. http://dx.doi.org/10.5402/2012/650563

Limayem A, Ricke SC. 2012. Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Progress in energy and combustion science **38,** 449-67. http://dx.doi.org/10.5402/2012/650563

Liu X, Kokare C. 2017. Microbial enzymes of use in industry. In: Biotechnology of Microbial Enzymes, 1st edition, pp. 267-298. Academic Press. http://dx.doi.org/10.1016/B978-0-12-803725-6.00011-X

Lyngwi, NA, Joshi SR. 2014. Economically important *Bacillus* and related genera: a mini review. In Biology of Useful Plants and Microbes, p. 33 -43, Ed. A.Sen (ed.). Narosa Publishing House, New Delhi.

Maki M, Leung KT, Qin W. 2009. The prospects of cellulase-producing bacteria for the bioconversion of lignocellulosic biomass. International journal of biological sciences **5(5),** 500-516. http://dx.doi.org/10.7150/ijbs.5.500

Manzum AA, Al Mamun MA. 2018. Isolation of *Bacillus* spp. Bacteria from Soil for Production of Cellulase. Nepal Journal of Biotechnology **6(1),** 57-61. http://dx.doi.org/10.3126/njb.v6i1.22338

McDonald RC, Isbell RF, Speight JG, Walker J, Hopkins MS.1998. Australian soil and land survey: field handbook. CSIRO publishing.

Meddeb-Mouelhi F, Moisan JK, Beauregard M. 2014. Comparison of plate assay methods for detecting extracellular cellulase and xylanase activity. Enzyme and Microbial Technology **66,** 16-9. http://dx.doi.org/10.1016/j.enzmictec.2014.07.004

Nigam P. 2013. Microbial enzymes with special characteristics for biotechnological applications. Biomoleculess **3(3),** 597-611. http://dx.doi.org/10.3390/biom3030597

Ojo-Omoniyi, Olusola A, Okwa, Omolade O, Junaid, Iswat OI, Abosede O. 2016. Sustainable Solid Waste Management: Isolation of Cellulolytic Microorganisms from Dumpsites in Lagos, Southwest Nigeria. International Journal of Current Microbiology and Applied Sciences **5(11),** 842-853. http://dx.doi.org/10.1136/bmjopen-2015-forum2015abstracts.105

Patagundi BI, Shivasharan CT, Kaliwal BB. 2014. Isolation and characterization of cellulase producing bacteria from soil. International Journal of Current Microbiology and Applied Sciences **3(5),** 59-69.

Ponnambalam AS, Deepthi RS, Ghosh AR. 2011. Qualitative display and measurement of enzyme activity of isolated cellulolytic bacteria. Journal of Biotechnology, Bioinformatics and Bioengineering **1(1),** 33-7.

Rathore P. 2014. Cellulase production by liquid state fermentation of *Bacillus* species isolated from woody forest soil. International Journal of Research Studies in Biosciences **2,** 8-13.

Reddy KV, Lakshmi TV, Reddy AV, Bindu VH, Narasu ML. 2016. Isolation, Screening, Identification and Optimized Production of Extracellular Cellulase from *Bacillus subtilis* Sub. sps using Cellulosic Waste as Carbon Source. International Journal of Current Microbiology and Applied Sciences **5(4),** 442-51.

Robert SB, Murray EGD, Nathan RS. 1957. Bergey's Manual of Determinative Bacteriology. 7th edition, Williums & Wilkins Company.

Robson LM, Chambliss GH. 1984. Characterization of the cellulolytic activity of a

Bacillus isolate. Applied and Environmental Microbiology **47(5),** 1039-46. http://dx.doi.org/10.1128/AEM.47.5.1039-1046.1984

Sadhu S, Maiti TK. 2013. Cellulase production by bacteria: a review. British Microbiology Research Journal **3(3),** 235-258. http://dx.doi.org/10.9734/BMRJ/2013/2367

Sahoo K, Sahoo RK, Gaur M, Subudhi E. 2019. Isolation of cellulase genes from Thermophilies: A Novel Approach toward new gene discovery. In: New and future developments in Microbial Biotechnology and Bioengineering, 1st edition, p 151-169. Elsevier. http://dx.doi.org/10.1016/B978-0-444-63503-7.00009-7

Saini A, Aggarwal NK, Yadav A. 2017. Isolation and Screening of Cellulose Hydrolyzing Bacteria from Different Ecological Niches. Bioengineering and Bioscience **5(1),** 7-13. http://dx.doi.org/10.13189/bb.2017.050102

Saini JK, Lakshmi T. 2012. Simultaneous isolation and screening of cellulolytic bacteria: selection of efficient medium. Journal of Pure and Applied Microbiology **6(3),** 1339-1344.

Sharma A, Choudhary J, Singh S, Singh B, Kuhad RC, Kumar A, Nain L. 2019. Cellulose as Potential Feedstock for Cellulase Enzyme Production: Versatility and Properties of Various Cellulosic Biomasses. In: New and Future Developments in Microbial Biotechnology and Bioengineering, 1st edition, p 11-27. Elsevier.

Shweta A. 2012. Cellulases of bacterial origin and their applications: A review. International Journal of Science and Research **3,** 1652-1655.

Singh S, Moholkar VS, Goyal A. 2013. Isolation, identification, and characterization of a cellulolytic *Bacillus amyloliquefaciens* strain SS35 from rhinoceros dung. ISRN microbiology, 2013, Article ID 728134, 7 pages. http://dx.doi.org/10.115/2013/728134.

Sriariyanun M, Tantayotai P, Yasurin P, Pornwongthong P, Cheenkachorn K. 2016. Production, purification and characterization of an ionic liquid tolerant cellulase from *Bacillus* sp. isolated from rice paddy field soil. Electronic Journal of Biotechnology **19,** 23-28. http://dx.doi.org/10.1016/j.ejbt.2015.11.002

Srivastava N, Srivastava M, Ramteke PW, Mishra PK. 2019. Synthetic Biology Strategy for Microbial Cellulases: An Overview. In New and Future Developments in Microbial Biotechnology and Bioengineering p 229-238. Elsevier. http://dx.doi.org/10.1016/B978-0-444-63503-7.00014-0

Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB. 2011. Bergey's manual of systematic bacteriology: 3, The Firmicutes. Springer Science & Business Media. http://dx.doi.org/10.1007/978-0-387-68489-5

Walsh G. 2015. Industrial enzymes: proteases and carbohydrases. In: Proteins; Biochemistry and Biotechnology, p 327-369, John Wiley and Sons. Ltd. http://dx.doi.org/10.1002/9781119117599.ch12