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ORIGINAL ARTICLE

Investigating the Ability of Diverse Native *Bacillus* Species to Produce Hydrolytic Enzymes Using Agar Plate Assay

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Hydrolytic enzymes of Bacillus attracting a great attention due to their various applications in industrial bioprocesses. In this work, out of 525 *Bacillus* isolates, 40 were screened for their potential capacity to produce hydrolytic enzymes including xylanase, lipase, amylase, pectinase, protease and carboxymethyl cellulose (CMC) using agar plate assay. A pure culture of each isolate was streaked on the surface of agar media containing suitable substrate specific for every enzyme activity, and the diameter of hydrolytic zones were measured. Data showed clear zones around the colonies which were interpreted as evidence of the enzymes activities. The isolates were able to generate at least one of these enzymes among which CMC and protease were the most common enzymes detected in 40 isolates; amylase in 39, xylanase in 36, lipase in 32 and pectinase in 26. Based on 16S rRNA gene sequencing the isolates were classified as *B. atrophaeus*, *B. amyloliquefaciens*, *B. subtilis*, *Paenibacillus polymyxa*, *B. simplex* and *B. tequilensis*. The native *B. tequilensis* isolates formed the largest zone clearance and had high abilities to produce five hydrolases. The isolates which had the largest enzymatic activity zones and the largest diameter of the clear hydrolysis zones on agar plates were submitted for further research work and enzyme-based industrials.

Key words: Xylanase, lipase, amylase, pectinase, protease, carboxymethyl cellulose, Bacillus species, agar assay

Microbial hydrolytic enzymes are of great importance in view of their applications in different industrial bioprocesses (Hossain *et al.,* 2020). *Bacillus* has been considered as the bacterium of the choice due to its metabolic diversity to produce a high number of these enzymes with useful and/or novel characteristics (Danilova *et al.,* 2020). Therefore, improvement of new strains for high enzyme production is needed for reducing the cost of the industrial process and might also have some specialized attractive characteristics.

Bacillus species having extracellular xylanolytic, amylolytic, proteolytic, lipolytic, pectinolytic, cellulolytic, chitinolytic activities, which have great commercial value due to their important applications in many industrial bioprocesses (Alrumman *et al.,* 2018). These enzymes meet industrial demand since they can excrete extracellularly, facilitating extraction from fermentation media (Raveendran *et al.,* 2018; Harba *et al.,* 2020).

Application of modern techniques to improve the hydrolytic enzyme production does not invalidate the search for wild organisms producing useful enzymes (Baweja *et al.,* 2016). However, identification of naturally occurring bacteria might be the best way to find new strains for producing hydrolytic enzymes for industrial purposes. Furthermore, due to the demand to obtain these enzymes with specific processing characteristics, particularly in developing countries with low technological capabilities, screening isolates using simple *in vitro* assay will be quite cheap.

Despite the biotechnological prospects of *Bacillus sp.* in addition to its importance to the host, essential information on its hydrolytic enzyme potential has remained unknown. Furthermore, one of the main problems in screening large number of microbial strains for their enzymatic hydrolases producing ability is the lack of simple and fast reliable screening technique (Tseng *et al.,* 2000), therefore, agar plate screening method was tested here. Hence, in the present work, production of six hydrolytic enzymes from a set of *Bacillus* sp, i.e. *B. atrophaeus, B. amyloliquefaciens, B. subtilis, B. simplex, Paenibacillus polymyxa* and *B. tequilensis* was compared under agar media containing specific substrate for each enzyme to determine their potential as sources of industrial enzymes.

MATERIALS AND METHODS

Bacterial isolates

Soil samples (at 4 cm depth) were randomly taken from different geographical regions of Syria. One gram of each sample was mixed with 10 mL of sterile distilled water. Serial dilution was made from 10^{-3} to 10^{-} (Ammouneh et al., 2011), and 1 mL of each dilution was transferred onto sterilized Nutrient Agar (NA) medium and incubated overnight, the colonies of prospective *Bacillus* sp. were identified according to *Wulff et al.* (2002).

Identification of the selected isolates

The 16S rRNA genes were amplified by PCR using forward and reverse universal primers: BacF (5' GTGCCTAATACATGCAAGTC-3') and BcaR (5'-CTTTACGCCCAATAATTCC-3') (Nair et al., 2002). The PCR reaction mixture contained 2 µl genomic DNA, 1x reaction buffer (TrisKCl-$MgCl_2$), 0.2 mM dNTP, 2 mM $MgCl_2$, 1 µM of each primer, and 5U/µl Taq polymerase (Fermentas). PCR steps were as follows: A denaturation step at 95 °C for 5 min followed by a second denaturation step at 95 °C for 1 min, annealing for 1 min at 54 °C, an extension at 72 °C for 90 s, and a final extension step of 72 °C for 10 min. A total of 30 serial cycles of PCR amplification was performed. PCR products were run on a 1.5% agarose gel, stained with ethidium bromide and then visualized by UV light (302 nm). Before sequencing, PCR products were purified with QIAgen gel extraction kit. Sequencing was done using a ABI 310 Analyzer (Perklin-elmer, Applied Biosystems, USA), and the sequences were compared using the NCBI database.

Hydrolytic enzymes production

All the identified isolates were examined for the production of hydrolytic enzymes (xylanase, lipase, amylase, pectinase, protease and CMC) using typical methodologies as illustrated below:

Xylanolytic activity

Xylanolytic activity of the isolates was tested in agar medium supplemented with 05% oat spelts xylan. After

the incubation period, xylanolytic activity of the isolates was detected by clear zones around the colonies using remazol brilliant blue dyed xylan (Ellis and Magnuson, 2012).

Proteolytic activity

Proteolytic activity was tested by incubating *Bacillus* isolates on gelatin-agar media (10 g/L gelatin, 5 g/L tryptone, 1 g/L glucose, 2.5 g/L yeast extract, 20 g/L agar under pH 7) and incubated for 48 h at 30°C. Transparent circles around the colonies were observed after staining with mercuric chloride solution (Fry *et al.,* 1994).

lipolytic activity

For lipolytic activity, the isolates were inoculated on agar media (15 mL/L Tween 80, 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl, 20 g/L agar under pH 7) and incubated for 48 h at 30°C. The appearance of clear zones after staining with methyl red solution indicated the presence of lipolytic activity (Samad *et al.,* 1989).

Pectinolytic activity

For pectinolytic activity, isolates grown on pectic agar medium after it was stained by Cetyl trimethyl ammonium bromide. and examined for the appearance of clear zones to confirm pectinase production (Beg *et al.,* 2000).

Cellulolytic activity

For the determination of cellulolytic activity, the isolates were inoculated onto 0.5% CMC-agar plates for 48 h at 30°C. After incubation, the plates were stained for 10 min with Congo red solution (2 g/L) and next destained with NaCl (1 M) for 15 min; clear zones surrounding the colonies indicated cellulase production (Meddeb-Mouelhi *et al.,* 2014).

Amylolytic activity

For amylolytic activity, bacteria grown on starch-agar media (10 g/L soluble starch, 5 g/L tryptone, 3 g/L yeast extract, 20 g/L agar under pH 7) solution; transparent zones surrounding the colonies indicated amylase production after staining by remazol dye (Amoozegar *et al.,* 2003).

Data analysis

Bacterial identification and clear zone observations of hydrolytic enzyme-producing bacillus were analyzed descriptively and presented in figures and tables. Qualitative testing of the hydrolytic enzyme-producing bacillus spp. was done by observing the clear zones around the bacterial colonies and then dividing the diameter of the clear zone with the bacterial colony diameter (Ashok *et al.,* 2019). The results for the diameter were expressed as relative enzymatic activities.

RESULTS AND DISCUSSION

Based on colony morphology, each distinct morphological character was considered as different bacterial species, and by using 16S rRNA gene sequencing method these species were identified as *B. atrophaeus, B. amyloliquefaciens, B. subtilis, B. simplex, Paenibacillus polymyxa* and *B. tequilensis* as their sequences confirmed similarities ≤ 98% to their closely related type isolates (Table 1; Fig. 1).

A collection of 40 isolates of the *Bacillus* spp. were screened for their abilities to produce hydrolytic enzymes such as xylanase, lipase, amylase, pectinase, protease and CMC enzymes in agar plate tests. The results of the observations of the enzymatic activity revealed clearance zones around the inoculated sites as a result of hydrolytic enzymes activity for 3 days on agar media containing specific substrate for each enzyme (Fig. 2).

Comparative analysis of enzymes activity of *Bacillus* spp. demonstrated that the tested *Bacillus* spp. isolates had significantly different levels of enzyme activity through formation of clear zones around the colonies (Fig. 3). It was found that the isolates had the abilities to produce at least one of these enzymes among which CMC and protease were the most common enzymes detected in 40 isolates; amylase in 39, xylanase in 36, lipase in 32 and pectinase in 26 (Table 2). However, *Bacillus* sp. had the highest CMC hydrolysis zone (3.8 cm) followed by protease hydrolysis zone (2.3 cm). In addition, pectinolytic activity that degrades pectin, a plant-based biopolymer, was detected in rather higher hydrolytic zones (1.87 cm) as shown in Figure 3. The diameter of the hydrolysis zone showed the concentration and enzymatic activity produced (Palmer, 1995).

The results showed formation of a clear zone around the colonies on agar medium containing xylan, which indicated the ability of colonies to produce xylanase. This is in line with findings of Varghese *et al.* (2017). In addition, the proteolytic clear zone produced by *Bacillus* sp. occurs due to protease activity which catalyzes the hydrolysis of the protein molecules into large fragments and peptidases the hydrolyze polypeptide fragments into amino acids (Razzaq *et al.,* 2019).

The data showed that Bacillus sp. had the ability to produce the amylase enzyme since 39 (97.5%) of the isolates produced detectable quantities of this enzyme in the agar media (Fig. 2) which gives a direct visual indication of starch hydrolysis that appeared as clear zones around the bacterial colonies (Dhawale *et al.,* 1982). Additionally, *Bacillus* sp. could hydrolyze the Carboxymethyl cellulose contained in the media and produced a large CMC hydrolysis zone (3.8 cm) which reflect that the polysaccharides have been degraded into saccharides with shorter chains (Zhou *et al.,* 2016).

The present study was focused on screening *Bacillus* sp. for their abilities to produce hydrolytic enzymes including xylanase, lipase, amylase, pectinase, protease and CMC using agar plate assay. Data showed that *Bacillus* sp. could generate at least one of these enzymes among which CMC and protease were the most common enzymes detected in 40 isolates, and the largest hydrolysis zones were observed for CMC (3.8 cm) and protease (2.3 cm). The isolates which had the largest enzymatic activity zones and the largest diameter of the clear hydrolysis zone will be tested to characterize these enzymes and determine their biochemical properties.

Table 1: *Bacillus* species used in the study.

Bacillus Species	Colony morphology
Atrophaeus	Brown-black, opaque, smooth, circular
Amyloliquefaciens	Creamy white with irregular margins
Paenibacillus	Milky white, thin often with amoeboid spreading
Subtilis	Fuzzy white, opaque, rough, with jagged edges
Simplex	Cream, gloss, with irregular margins slightly raised
Tequilensis	Yellowish, opaque, smooth, circular

Table 2: Enzymatic activity of *Bacillus* species used in the study.

Characteristics	*B. atrophaeus*	*B. subtilis*	*B. polymyxa*	*B. amyloliquefaciens*	*B. tequilensis*	*B. simplex*	Positive isolate numbers	Percentage of positive isolates
Isolate numbers	3	20	2	10	4	1		
Enzymatic activities	0	20	2	10	4	0		
Xylanase	-	+	+	+	+	-	36	90
Amylase	+	+	+	+	+	-	39	97.5
Protease	+	+	+	+	+	+	40	100
CMC	+	+	+	+	+	+	40	100
Lipase	+	+	+	+	+	-	32	80
Pectinase	-	+	+	+	+	-	26	65

+: Presence of enzyme activity and -: Absent of enzyme activity

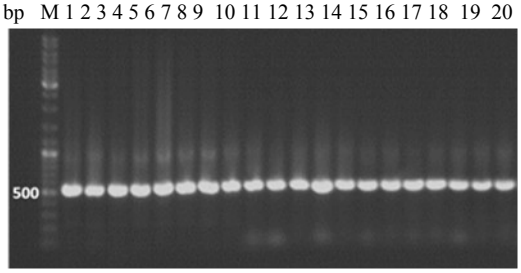

Figure 1: Agarose gel electrophoresis of 16S rRNA of some Bacillus sp. isolates used in the study. M represents the 100-bp DNA marker (HinfI; MBI Fermentas, York, UK).

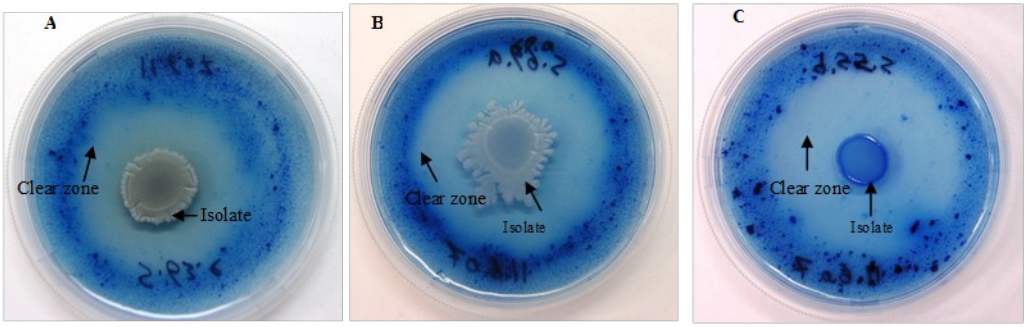

Figure 2: The clear zone of amylase activity by B. atrophaeus (F), B. tequilensis (B) and enbacillus polymyxa (C) on using agar plate assay.

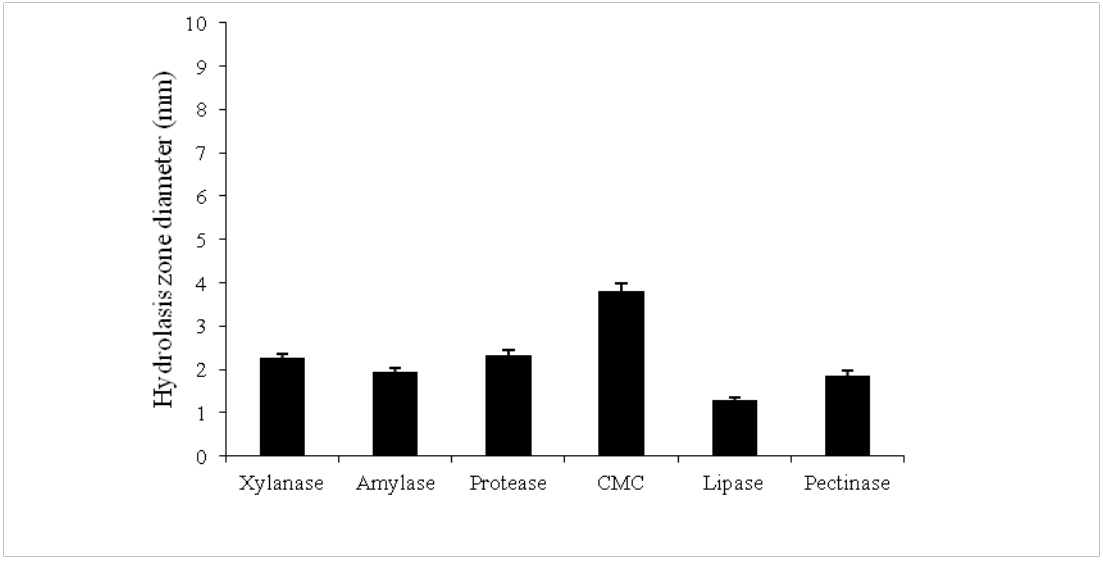

Figure 3: Formation of hydrolysis zones (cm) around the colonies of *Bacillus sp*. using agar plate method. Error bars display the standard deviation among two biological replicates.

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CONFLICTS OF INTEREST

The authors declare that they have no potential conflicts of interest.

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