Bacillus sp. R2: Promising Marine Bacterium with Chitinolytic/Agarovorant Activity and Multiple Enzymes Productivity

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Abstract. Seventy-two bacterial isolates from soil and aquatic sources were tested for their ability to produce chitinase. After successive rounds of primary and secondary screening, an agar-degrading marine bacterium from the Red Sea (Hurghada- Egypt), designated R2 attracted our attention not by its higher chitinolytic agarovorant activity but also by its multiple enzyme production (protease, gelatinase, lipase, esterase, amylase, cellulase, pectinase, dextranase, alginase, arabinase, peroxidase, manganese peroxidase ….) and its moderately halophilic/halotolerant physiology. This bacterial isolate was selected and identified using conventional methods as well as the 16S rRNA technique. The morphological, physiological, and biochemical characterization showed that the strain was Gram-positive or Gram- variable, endospore-forming rods, motile by a single polar flagellum, positive for oxidase, catalase, peroxidase, and urease, negative for nitrate reductase, indole, MR, and VP., therefore, it was assigned to the genus *Bacillus*. Moreover, the isolate was identified molecularly and submitted in the Gen Bank sequence database as Bacillus sp. R2 with a given accession number DQ 923,161. the higher chitinolytic agarovorant activity and multi enzymatic productivity of *Bacillus sp.* R2 makes it a suitable candidate for marine enzymes production in addition to its ecological role in marine biogeocycles.

Keywords: *Bacillus sp.* R2 · Identification · Chitinase · Hydrolytic enzymes · Marine biotechnology

1 Introduction

Enzymes are highly efficient biocatalysts produced by living organisms, they accelerate biochemical reactions rates faster by millions of times faster than any chemical reaction [1]. Enzymes from microbial origin were the most preferable source due to their availability, diversity, and stability than plant and animal enzymes, in addition to lower cost, high production rate in a short time and space [2, 3]. moreover, microbial enzymes have gained more attention globally for their widespread applications in various industrial

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sectors ranging from chemicals, food, feed, fuel, textile, detergent, paper, cosmetics, and pharmaceuticals to agricultural, environmental, and medical fields [4–7].

Many hydrolytic enzymes from fungal and bacterial sources were already produced and used in various commercial processes. chitinases and agarases are glycosyl hydrolases that attracted the attention of many researchers due to their versatile biotechnological uses and produced from various sources [8, 9]. The present work was designed to isolate chitinase and agarase potent bacterium and screen its ability for multiple enzyme productivity, moreover, identifying the isolate conventionally and molecularly for further biotechnological valorizations.

2 Material and Methods

2.1 Sampling and Chitinolytic and/or Agarolytic Bacteria Isolation

Different samples of soils, water, sands, and sediments samples from Mediterranean and red seas and Mariout lake, Nile River (Egypt) were collected, and 0.1 ml were spread directly, whereas soil, sewage, and wastewaters samples were diluted in sterilized seawater and plated on chitinase detection agar (CHDA) plates that contained seawater, 1% (v/v) colloidal chitin, 1.5% (w/v) agar, pH 7. Whereas agarolytic bacteria were screened on agarase detection agar (ADA) plates containing 1.5% (w/v) Agar, 0.02% (w/v) yeast extract dissolved in seawater pH: 7 and incubated at 30OC for few days (48−120 h).

2.2 Chitinolytic and Agarolytic Bacteria Detection

Chitinolytic bacteria can be detected by visualizing the clear zone formed on (CHDA) plates. while the agarolytic activity was assessed by liquefaction or shallow depression appearing around the colonies or with Gran's test [10] when the (ADA) plates were flooded with Lughole's iodine and kept at 4 °C for 1 h. Agarolysis was seen as clear yellow haloes formed around the colonies in contrast to the purple-brown background. This indicated that agarase diffused out from the colonies and reducing compounds were released during agar degradation. All colonies showing clear zone or formed depression, liquefaction, or pits on (CHDA) or (ADA) plates were picked up and purified by successive streaking on the same screening media.

2.3 Potent Strain Conventional Identification

Screenings resulted in potent isolate were subjected to conventional and molecular identifications. The conventional one was carried out according to the morphological, physiological, and biochemical tests described in Bergey's manual of systematic Bacteriology [11, 12]. Morphological characteristics, such as colonial characteristics, pigmentation, luminescence, and swarming were performed on plates of nutrient agar, marine LB agar, seawater agar, and chitin agar. Cell morphology, spore formation was studied by staining (simple and differential) and observed microscopically. Physiological tests were performed at different temperatures and different pHs and different sodium chloride concentrations as well as Biochemical tests were performed as described before. [13, 14,

15] : These tests included oxidation-fermentation (O-F) test, oxidase, catalase, nitrate reduction, methyl red (MR), Vogues-Proskauer (VP), indole, urease, citrate, carbohydrates or sugars fermentation, gelatinase, hemolysin, and utilization of some substrates. Moreover, plasmid miniprep of the potent strain was done according to the method of Zaghloul et al. (1985) [16] with minor modification.

2.4 Potent Strain Molecular Identification

DNA Extraction, Purification, and Amplification. The DNA was extracted according to Sambrook et al. 1989 [17] their purity and concentration were measured at 260 and 280 nm. The conserved 16S rDNA gene was amplified by polymerase chain reaction (PCR) using universal primers designed to amplify the full length (1,500 bp) of the 16S rDNA gene according to the Escherichia coli genomic DNA sequence. The forward primer was 5'- AGAGTTTGATCMTGGCTCAG-3' and the reverse primer was 5'-TACGYTACCTTGTTACGACTT-3'. Amplification of the entire 16S rDNA gene was performed by PCR using the thermocycler (Progene – Techne "Cambridge" LTD – UK). Hundred ng of purified genomic DNA was used in 50 μl reaction mixtures containing 30 p moles of each primer and 2 units of Taq DNA polymerase. The thermocycler was programmed as follow: an initial denaturation of 5 min at 94 ºC followed by 30 cycles of 1 min at 94 ºC (denaturation), 1 min at 55 ºC (primer annealing), and 1.5 min at 72 ºC (extension) plus one additional cycle for final elongation at 72 ºC for 5 min. The single DNA band of approximately 1.5 Kb (PCR product) was detected by agarose gel electrophoresis.

PCR Product Purification, DNA Sequencing, and Phylogenetic Analysis. The PCR product was purified to remove incorporated nucleotides and excess primers using (QIA gen PCR Purification Kit). The pure PCR product (1.5 Kb) was subjected to automated sequencing by ABI PRISM sequencer based on the dideoxy chain termination method developed by Sanger et al. (1977) [18]. The obtained nucleotide sequence of the 16S rDNA of the isolate R2 was aligned with various sequences of Bacillus members using the Blast search database [19] The sequence has been deposited in the GenBank sequence database and the phylogenetic analysis of the isolate R2 16S rDNA sequence and their relationship with Bacillus group was evaluated using the distant matrix method [20] followed by neighbor-joining analysis [21].

2.5 Potent Strain Multiple Enzymes Production And Assays

After growing the strain in seawater + 0.05% yeast extract supplemented separately with 0.5% of tested substrate (colloidal chitin, agar, cellulose, xylan, starch, blue dextran, pectin, alginate, Arabic gum, lactose, casein, and Tween 80), the flasks incubated at 37 0 C with 180 rpm agitation rate. The quantitative assays of cellulase, pectinase, amylase, dextranase, and alginase were carried out with the DNSA method as described below for agarase and chitinase. While xylanase activity was assayed according to Bailey et al. (1992) [22] using(sigma) oat spelt xylan. Proteolytic activity (neutral and alkaline) was measured according to the method of Cliffe and Law (1982) [23] using Hide Powder

Azure (HPA). One unit of proteolytic activity was defined as the amount of enzyme that developed a change of absorbance (0.1) against the control at 595 nm at 37 °C. Lipolytic or esterolytic activity was determined according to the method described earlier using P- nitro phenyl palmitate as substrate [24]. One unit of lipolytic or esterolytic activity was defined as the amount of enzyme that released one micromole of P- nitro phenyl from the substrate P- nitro phenyl palmitate per min. Furthermore, Peroxidase activity was assayed according to [25] using 2,20 -azino-bis (3-ethyl benzthiazoline-6-sulfonic acid) (ABTS) as a substrate, whereas manganese peroxidase was assessed according to [26, 27].

2.6 Analytical Methods

Electron Microscopy
Potent isolate cell and spore morphologies, size, and flagellation were studied by using JEOL scanning and transmission electron microscopy (scanning electron microscopy – SEM –and transmission electron microscopy –TEM), the true dimensions of the cell were calculated according to the following equation: True length (μm) = L (mm) × 103/M. Where L (mm) = Length on the photograph in millimeter. M = Magnification.

Chitinase, Agarase, and Protein Assays
Chitinase and agarase activities were analyzed according to the Miller method [28] by estimating the released reducing ends of sugar using N-acetyl - D-glucosamine (NAG) and galactose as standards, respectively. One unit of chitinase and agarase activities were defined as the amount of enzyme required to release 1 μ mol of NAG and galactose per minute respectively during these conditions. as described in the Bradford method (1976) [29] Soluble proteins were assessed using bovine serum albumin as a reference for standard curve preparation.

3 Results and Discussion

3.1 Potent Strain Conventional Identification

Seventy-two bacterial isolates from soil and aquatic sources were tested for their ability to produce chitinase. After successive rounds of primary and secondary screening, a marine bacterium from the Red Sea (Hurghada- Egypt), designated R2 attracted our attention by its higher chitinolytic agarovorant activity (Fig. 1). Consequently, it was selected for further identification. The morphological, cultural, as well as physiological, and biochemical characteristics summarized in Table 1: showed that the strain R2 was Gram-positive or variable, strictly aerobic, endospore-forming Bacilli, motile by a single polar unsheathed flagellum (Fig. 2). Cells were straight rods approximately 1 μm in diameter and 3–4 μm in height (Fig. 2). The endospores are oval or ellipsoidal, (1.25–1.66 × 2.95–3.95 μm), lie centrally or sub terminally in slightly swollen sporangia (Fig. 3). The isolate colonies were smooth, circular to slightly irregular, slightly raised, opaque, cream to pale yellow and 2–4 mm in diameter after one day of growth at 30 °C on MLB agar medium.

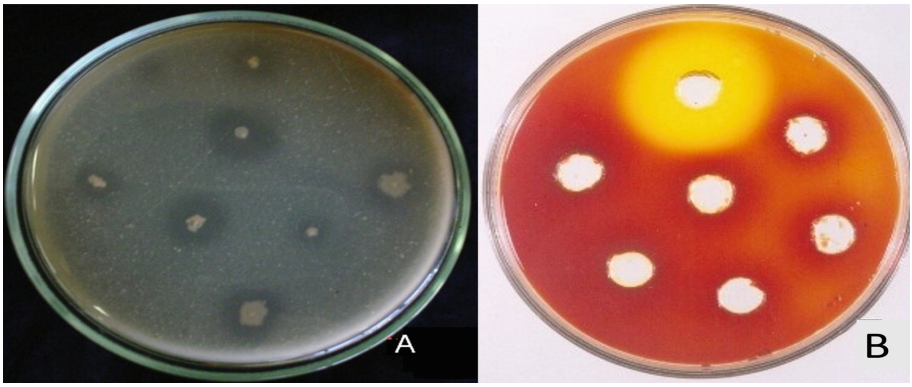

Fig. 1. A: Chitinase screening on (CHDA) plates, B: *Bacillus sp.* R2 agarase activity detection through well cut diffusion assay on agar plates.

Fig. 2. A: Transmission electron photomicrographs of the R2 isolate (negative form) cells have single polar flagellum (15000X), B: Ultra-thin section showing the rod-shaped morphology of the cells during division (40000X).

The growth of isolate R2 was between 4 and 42 °C while the optimal growth lied between 30–37 °C Table 1. Good growth occurred at pH 5–10 and was limited at pH 4 and 11. No growth was observed in the absence of NaCl, while good growth was noted in the presence of NaCl 2–18%. The strain tolerated weakly NaCl concentration 18–30%. A similar range was tolerated by closer Gram-positive or variable marine bacilli strains, such as *B. aquimaris* isolated from the yellow sea [30] which tolerated up to 18% NaCl.

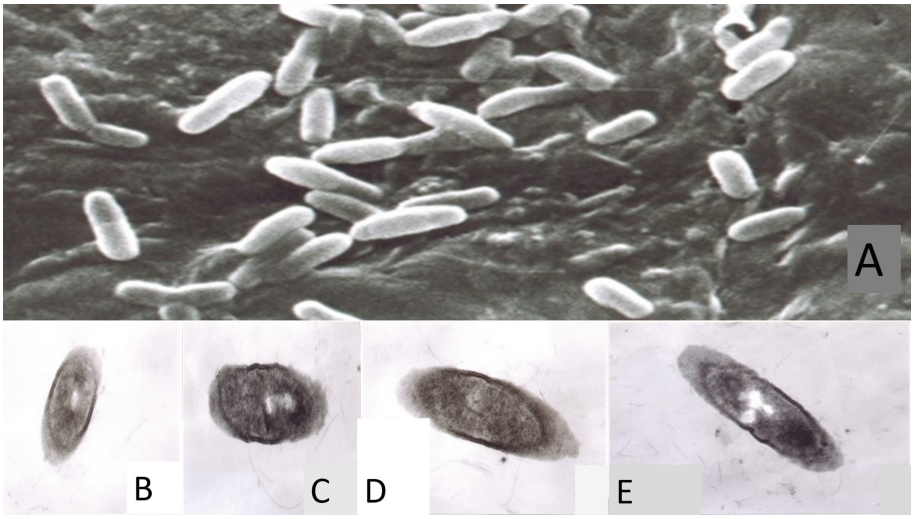

Fig. 3. Electron photomicrographs of the isolate R2 (sporulated form). Panel A; SEM micrograph showing the paracentral/subterminal spore position. Panels B, C and D and E; ultra-thin Sects. (50,000 X) showing the oval and ellipsoidal shapes of the spore, respectively.

Moreover, *Jeotgalibacillus alimentarius* grew at 19% NaCl [31]. The isolate R2 was aerobic and gave a positive reaction for oxidase, catalase, peroxidase, and urease. The isolate was negative for nitrate reductase, indole, MR, and VP. The isolate produced several enzymes such as agarase, chitinase, protease, lipase, amylase, gelatinase, dextranase, and β-galactosidase (Table 2). The isolate R2 fermented the following sugars to produce acid from glucose, galactose, mannose, xylose, glycerol, lactose and maltose, sucrose, arabinose, sorbose; however, fructose was not fermented (Table 1). Additionally, the plasmid profile of the isolate revealed the absence of any plasmids. The conventional identification, based on the criteria described in Bergey's manual, of systematic bacteriology [11, 12] assigned the isolate R2 to the genus Bacillus..

Table 1. Bacillus *sp. R2* morphological, physiological, and biochemical characteristics.

Characteristics	Results	Characteristics	Results
Gram-staining	V or +	(18–30)%	(vw)
Cell morphology	Straight rods	Growth in MacConkey	+
Cell size	(1.65 × 4) μm	Hemolysin	+
Cell flagellation	Single polar	Protease	+
Cell arrangement	Mono	Gelatinase	+
Sporangia:		Lipase	+
Spore shape	Ellipsoidal/oval	Amylase	+
Spore size	(1.45 × 3.35) μm	Dextranase	+
Spore position	Central/subterminal	Agarase	+
Sporangia swollen	Slightly	Chitinase	+
Motility	+	Hydrolysis of:	
Swarming	+	Casein	+
Colony color	Cream to pale yellow	Gelatin	+
Pigmentation	−	Tween 20	+
Luminescence	−	Tween 80	+
Salt requirement	+	Starch	+
Aerobe/anaerobe	Aerobe	Agar	+
O/F test	±	Agarose	+
Oxidase	+	Filter paper	−
Catalase	+	Cellulose	±
Peroxidase	+	CMC	+
Nitrate reductase $N^{O_3^-} \rightarrow N^{O_2^-}$	−	Chitosan	+
Methyl red (MR)	−	Alginate	+
Voges-Proskauer (VP)	−	Pectin	±
Indole from tryptophan	−	Arabic gum	+
Urease	+	Dextran blue	+
Citrate	+	Acid from:	
Growth at:		Glucose	+
(4, 20, 30, 37, 42) °C	+	Galactose	(w)
(50, 55, 60) °C	−	Fructose	(Alk)
Growth at pH:		Mannose	+

(*continued*)

Table 1. (*continued*)

Characteristics	Results	Characteristics	Results
4	(vw)	Sorbose	−
(5, 6, 7, 8, 9, 10)	+	Arabinose	−
11	W	Xylose	+
Growth in NaCl:		Lactose	+
0%	−	Maltose	+
(2–3)%	+	Sucrose	−
(3–12)%	+	Glycerol	+
(12–18)%	±	Presence of plasmids	−

(+) Positive, (−) Negative: (w) Weak positive, (v) Variable, (vw) very weak positive, Alk; alkaline reaction.

3.2 Potent Strain Molecular Identification

The resulting data indicated that the isolate R2 understudy belonged to Bacillus. sp (probability 97%). This confirmed the conventional identification and as evident from the taxonomy report that the isolate R2 exhibited 98% identity with Bacterium JL-74 [32] and 97% similarity with *Bacillus sp.* CNJ9O4 PLO4, Bacillus. sp. CNJ815 PLO4, Bacillus. sp. T5-12, *Bacillus. sp.* PO1, Bacillus holothurians, Marine *Bacillus* NRRLB–14851, and *B. barbaricus*. It was noticed that all the above strains were Gram-positive or variable moderately halophilic, halotolerant marine bacilli. Part of the nucleotide sequence (561 bp) was submitted to the GenBank database, with the accession number (DQ923161). A phylogenetic tree (Dendrogram) was generated using the Bio-Edit program and neighbor-joining algorithm. Figure 4 showed that the isolate R2 was phylogenetically related to members of the Bacillus group and formed a coherent cluster with *B. halodurans* and *B. clausi*. We must point out that the taxonomic position of our strain was very far from the terrestrial Bacilli such *as B.subtilus, B.cereus*…and very closer to the gram variable marine Bacilli with moderately halophilic or halotolerant properties, Interestingly, many recently isolated positive or gram variable bacilli with halophilic or halotolerant properties have been isolated from marine sources (Yellow sea of China and Korea, Red sea (this study), Died sea (2) and from salt lakes of China [33] and Mongolia [34] and have been identified as members of related new genera such as *Virgibacillus, Lentibacillus, Tenuibacillus, Cerasibacillus, Halobacillus* [35] *and Salinibacillus* [36] rather than members of the genus Bacillus. All these facts indicate the importance of the polyphasic approach for the determination of the exact taxonomic position of this special group of Bacilli which our strain R2 was a newly isolated strain from the red sea belongs to this group. Accordingly, the future study aimed to determine the exact taxonomic status of the strain R2 using a combination of phenotypic properties, chemotaxonomy since the strain was gram variable, and phylogenetic analysis based on full 16S rDNA gene (1500 bp) not partial sequence and genomic DNA-DNA relatedness and based on these data collectively the novel strain R2 may be placed at least in novel distinct specie.

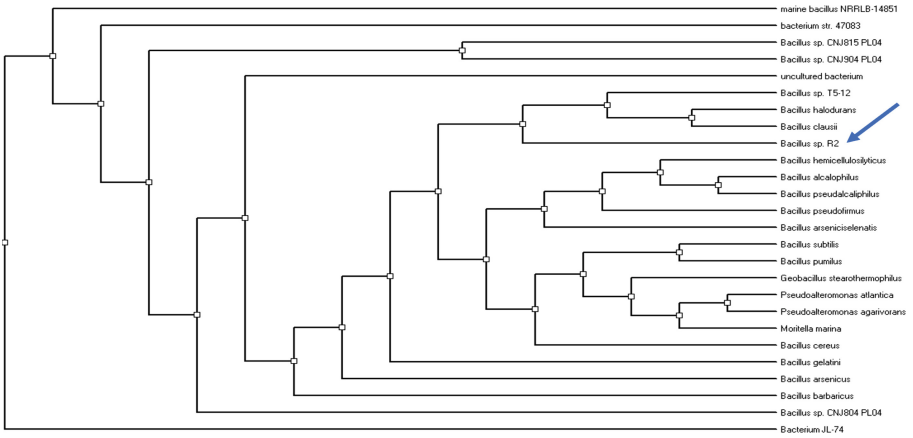

Fig. 4. Dendrogram (based on 16S DNA sequence comparisons) showing the taxonomic position and the phylogenetic relationship of Bacillus sp. R2 with several Bacillus species. Scale bar: 0.1 substitution pair nucleotide position.

3.3 Potent Strain Multiple Enzymes Productivity

Besides the agarovorant and chitinolytic activities, the results presented in Table 2 revealed that Bacillus sp. R2 produces multiple enzymes include cellulase, xylanase, pectinase, amylase, dextranase, arabinase, alginase, and peroxidase, in addition to other hydrolytic enzymes such as protease, gelatinase, lipase, and lactase. Bacillus sp. R2 multiple enzymes productivity was not surprising for the Bacillus genus that secretes numerous enzymes degrading various substrates, enabling them to survive in a continuously changing environment. Furthermore, Bacillus has become the major microbial cell factory for many industrial products [37, 38], including enzymes [39], heterologous proteins, amino acids, vitamins, and antibiotics [40].

Table 2. The enzymes produced by Bacillus sp. R2.

Enzyme	Production	Enzyme activity (U/ml)
Agarase	+ + +	32
Chitinase	+ + +	34.5
Cellulase	+	11.32
Xylanase	+	0.36
Dextranase	+	29
Amylase	+ +	22
Lipase	+ + +	198.86
Protease (Neutral)	+ +	10.2
Protease (Alkaline)	+	6.25

(*continued*)

Table 2. (*continued*)

Enzyme	Production	Enzyme activity (U/ml)
Gelatinase	+ +	ND
Urease	+ +	ND
Pectinase	+	19.24
Alginase or alginolytic act	+ +	21.12
Arabinase	+	12.5
Lactase (B-galactosidase)	+	45.6
Peroxidase	+	16 [(a)]
Manganese peroxidase	+	3.8 [(b)]

ND: not determined, (a) and (b) enzymes activities were estimated by Khelil et al. 2015, 2016 [41, 42]

4 Conclusion

Bacillus genus and its enzymes continue to show great potential for practical applications in various biotechnological fields. In these concepts *Bacillus sp.* R2 multiple enzymes productivity enables it to become a potential microbial cell factory for many hydrolytic enzymes which will be exploitable in food, feed, fuel, and fertilizers biotechnologies. in addition, the various substrates degradability by *Bacillus sp.* R2 explained its ecological role in marine organic matter mineralization.

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